Nanomechanics of cell membrane and cellular contacts in control and failing hearts

Pamela Swiatlowska
Born: 3.04.1991

Faculty of Medicine
National Heart and Lung Institute
Imperial College London

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

London 2019
Abstract

In recent years, a growing number of studies have shown that mechanical properties play an important role in both structure and function of cells. Heart is an extremely dynamic organ; therefore, cardiac myocytes are constantly subjected to a mechanical stress. To date, titin protein and collagen fibers were considered to be the main regulators of tissue Young’s modulus, one of the standard measures of mechanical properties. Recently, studying mechanical properties at cellular, tissue and organ level, demonstrated contribution of mechanical cues to the development of different diseases, including heart failure. During the progression of this pathology, cells undergo several changes in physiology and mechanobiology, where a significant increase in Young’s modulus is observed.

Work presented in this thesis examines cardiomyocyte nanomechanical properties focusing specifically on measuring transverse cortical Young’s modulus by using high resolution Scanning Ion Conductance Microscopy in different mouse, rat and human disease models of heart failure. Further work investigates the role of different intracellular elements such as generic and cardiac-specific cytoskeleton, mitochondria and mechanical load that can affect cardiac mechanics. In order to determine their role RT-PCR, Western blot, Transmission Electron Microscopy and immunofluorescent staining techniques were used. To obtain a bigger picture on cardiac mechanics, co-cultures of myocytes alone and with fibroblasts were established where changes in Young’s modulus at the homo- and hetero-cellular cell-cell junction were studied. Using a novel Junctional Mapper software precise quantification of intercalated disc proteins population was attainable.

Scanning Ion Conductance Microscope was adapted to measure cell Young’s modulus at a nanoscale resolution and used in an extensive study of cardiomyocytes mechanics. In normal myocytes, the contribution of individual cellular elements to cell mechanical properties was assessed via inhibitor analysis. Consequently, actin, microtubules and caveolae were found to have the biggest contribution to cardiomyocyte mechanics. In a rat model of heart failure (16 weeks after myocardial infarction), cardiac myocytes show a markedly increased Young’s modulus with a significantly higher value in surface crest areas than Z-grooves. This could be related to mitochondria rearrangement, actin-myosin incomplete relaxation and increased microtubular network densification. In fact, microtubule post translational modifications (acetylation and detyrosination) were found to be increased in failing cells and that will correlates with an increased Young’s modulus. Moreover, a cross-talk has been revealed between these two populations of microtubules, as increased level of acetylation results in reduced detyrosination. Removal of load from both control and failing heart markedly reduces Young’s modulus of myocytes; in fact, after unloading the hearts failing cardiomyocytes present a similar Young’s modulus value to healthy cells. Other changes can be observed after the removal of load, for example
the level of acetylated and detyrosinated microtubules and the mitochondria numbers are also reduced.

Long term exposure to Angiotensin II (Ang II) is known to exert a hypertrophic effect on cardiac myocytes, whereas little is known about acute, short-term action of Ang II. This work suggests a novel, beneficial role of acute treatment with Ang II in regulating cardiomyocyte mechanics. Reduced Young’s modulus is observed in Ang II treated myocytes, which is driven by changes in microtubular network, including acetylation and detyrosination modifications. More importantly, Ang II acts equally upon failing myocytes bringing Young’s modulus value to the normal level. Therefore, it can be potentially used to treat diseased heart muscle cells.

Overall, this thesis describes a novel technique of measuring Young’s modulus in live cells. Using this method, a detailed study on changes in cardiomyocyte mechanics is presented. In line with other studies, we observe that understanding myocardial mechanobiology is imperative to fully disclose the mechanism of initiation and progression of heart failure.
Manuscripts accepted, in revision and preparation


4. Swiatowska P, Sanchez-Alonso J, Wright P, Novak P, Gorelik J. “Regulation of cardiac mechanics in the failing heart”


*Authors equally contributed to the performed work
Attended conferences, meetings and workshops

1. **Heart Failure Congress**, European Society of Cardiology, Athens 2019, Greece

2. **63rd Biophysical Annual Meeting**, Biophysical Society, Baltimore 2019, USA
   Swiatlowska P, Sanchez-Alonso J, Wright P, Novak P, Gorelik J., “**Role of microtubules in transversal Young’s modulus of normal and failing cardiac myocytes measured by a novel technique.**”

3. **Heart Failure Winter Meeting**, European Society of Cardiology, Les Diablerets 2019, Switzerland

4. **Additive Manufacturing and Biofabrication Summer School 2018**, Cellink-ETH Zurich, Switzerland.


6. **National Heart and Lung Institute Postgraduate Day** London 2018, UK;
   Swiatlowska P, Sanchez-Alonso J, Wright P, Novak P, Gorelik J., “**Regulation of cardiac mechanics in the failing heart**” oral presentation.


8. **National Heart and Lung Institute Postgraduate Day**, London 2017, UK;


11. **Cardiac Mechano-Electric Coupling and Arrhythmias Conference**; Freiburg 2016, Germany

Received grants and awards

1. The Company of Biologists, Conference Travel Grants 2019
   • “Single cell mechanics in failing heart: role of actin, microtubules and mitochondria rearrangement” poster presentation, Heart Failure Congress 2019, Greece

2. Physiological Society, Outreach Grant 2019
   • “Pint of Science 2019” event organization

3. British Society of Cell Biology, Travel Grant 2018
   • “Single cell mechanics in failing heart: role of actin, microtubules and mitochondria rearrangement” poster presentation, Heart Failure Winter Meeting, European Society of Cardiology, Les Diablerets 2019, Switzerland

4. Departmental NHLI Travel Award, 2018
   • “Role of microtubules in transversal Young’s modulus of normal and failing cardiac myocytes measured by a novel technique.” 63rd Biophysical Annual Meeting, Biophysical Society, Baltimore 2019, USA

5. Biochemical Society, Travel Grant 2018
   • Additive Manufacturing and Biofabrication Summer School 2018, Cellink-ETH Zurich, Switzerland

6. President’s Award for Excellence in Research for Outstanding Research Team, Imperial College London 2018

7. Physiological Society, Travel Grant 2018
   • “Regulation of cardiac mechanics in healthy and diseased hearts” poster presentation, ESRIC Super-Resolution Microscopy Summer School 2018, Royal Microscopical Society, Edinburgh, UK; Swiatlowska P, Sanchez-Alonso J, Wright P, Novak P, Gorelik J

8. Imperial College London General Trust Conference Grant 2017
   • “Mapping cell mechanosensation: new approach to study nanomechanical properties of normal and failing heart cells” poster session, International Society for Heart Research Meeting, Hamburg 2017, Germany

9. AMC Meeting “Nanomechanics of cell membrane in normal and failing hearts” poster Award, 2015

10. National Heart and Lung Institute, Imperial College London, 3-year PhD Studentship 2015
Declaration of Originality

I, Pamela Swiatlowska declare that this thesis and the work described in it is my own. Work described by others have been clearly referenced.

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Acknowledgements

First of all I would like to express my sincere gratitude to my two main supervisors Prof Julia Gorelik and Dr Vania Braga for continuous encouragement, constructive criticism and scientific guidance for the past 3 years.

Special gratitude goes to Dr Jose Sanchez Alonso-Mardones, my third supervisor that was granted the title of the best NHLI Post-Doc supervisor 2018/2019, following my application. Could not imagine a better candidate for this title. Patience, endless support, guidance, being available for assistance even in those really busy days, feedback on all prepared conference posters and oral presentations, immense sense of humor is a combination of characteristics for a role model supervisor and all of them can be assigned to Dr Sanchez. Every next student supervised will be extremely lucky to have Dr Sanchez as a supervisor.

I would like to thank Dr Pavel Novak, Dr Anita Alvarez-Laviada, Dr Ben Reilly-O’Donnell, Dr Peter Wright and Dr Ivan Diakonov, other Post-Docs in our lab group for being always willing to help.

I express my warm thanks to Peter O’Gara, Dr Catherine Mansfield, Dr Peter Wright, Sean Bello, Elisa Ferraro and Navneet Bhogal for animal models generation, cell isolation and kind cell portioning that I could use for my experimental studies. Special thanks go to Peter O’Gara for unbelievable great quality of isolated cardiomyocytes that I used in several of my experiments.

I would like to thank Stephen Rothery for his kind help with confocal imaging and Andrew Rogers for assistance with transmission electron microscopy.

I am very thankful to Cheryl Costello for her help with the administrative work related to several travel grants.

I am also grateful to Prof Sylwia Jafra, Prof Zygmunt Derewenda, Prof Gary Owens and Dr Olga Cherepanova, without their support I would not make that far in my scientific inter-country journey.

Thanks to the NHLI Department for the positive consideration of my PhD application and offering me a PhD Studentship, without which this project would not take place.
Special thanks go to all my friends scattered around the world but still willing to keep in touch with me, despite miles away. Also, thanks to those that I’ve met in London, especially my labmates. You’ve all contributed to the maintenance of my work-life balance.

Finally, I would like to thank my family for supporting me throughout my entire life and encouraging me to pursue my scientific career in different parts of the world. I hope they will never stop being proud of me.
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## Abbreviation and acronym list

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<th>Description</th>
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<tbody>
<tr>
<td>4PB</td>
<td>4-phenylbutyrate</td>
</tr>
<tr>
<td>A.U.</td>
<td>arbitrary units</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic Force Microscopy</td>
</tr>
<tr>
<td>AJ</td>
<td>adherens junctions</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>AT1</td>
<td>Angiotensin Type 1 receptor</td>
</tr>
<tr>
<td>Cav3</td>
<td>caveolin 3</td>
</tr>
<tr>
<td>CM</td>
<td>cardiomyocyte</td>
</tr>
<tr>
<td>Cx43</td>
<td>connexin 43</td>
</tr>
<tr>
<td>Da</td>
<td>daltons</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCM</td>
<td>dilated cardiomyopathy</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated kinase</td>
</tr>
<tr>
<td>FB</td>
<td>fibroblasts</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>FHL</td>
<td>half LIM domain</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GJ</td>
<td>gap junction</td>
</tr>
<tr>
<td>HOECHST</td>
<td>trihydrochloride trihydrate</td>
</tr>
<tr>
<td>IBZ</td>
<td>internal border zone</td>
</tr>
<tr>
<td>ICM</td>
<td>ischemic cardiomyopathy</td>
</tr>
<tr>
<td>KD</td>
<td>knock down</td>
</tr>
<tr>
<td>LINC</td>
<td>Linker of Nucleoskeleton and Cytoskeleton</td>
</tr>
<tr>
<td>LVAD</td>
<td>Left Ventricular Assist Device</td>
</tr>
<tr>
<td>MFB</td>
<td>myofibroblasts</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial Infarction</td>
</tr>
<tr>
<td>MTs</td>
<td>microtubules</td>
</tr>
<tr>
<td>OE</td>
<td>overexpression</td>
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<tr>
<td>OT</td>
<td>optical trap</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PTMs</td>
<td>post-translational modifications</td>
</tr>
<tr>
<td>RBM20</td>
<td>RNA binding motif 20</td>
</tr>
<tr>
<td>RAAS</td>
<td>renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SICM</td>
<td>Scanning Ion Conductance Microscopy</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>transforming growth factor-β1</td>
</tr>
<tr>
<td>TT</td>
<td>transverse tubule</td>
</tr>
<tr>
<td>YM</td>
<td>Young’s modulus</td>
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Chapter 1

General introduction
1. Introduction

1.1 Mechanical forces in biology
Every human is exposed to various mechanical forces originating from different sources. Gravity is the most commonly known force that is working on our bodies. Spine compression is where the gravity effect is the most noticeable in the human anatomy (Kane, Karl, and Swain 1985). Cartilage and bones are severely affected by compressive force especially during walking and exercising (Khalsa and Eisenberg 1997). During movement, bones are subjected to tension exerted by muscles (Firestein et al. 2017) whereas lung tissue is regularly stretched while breathing. Skin is the most abundantly exposed to the external environment tissue in the body and it is exceptionally durable to tensile forces. These examples show that mechanical force generation, maintenance and detection is an indispensable part of our daily life. Equally, heart muscle is affected by changing mechanical load due to continuous changes in blood volume and pressure.

It was recognized since a very long time that cells and tissues are able to sense mechanical cues from the surrounding environment. German 19th century anatomist, Julius Wolff, extensively explored the bone remodeling process and observed that if the bone is exposed to increased load this rigid structure remodels and adapts to it in the process known as Wolff’s law (Wolff 1986). Also, cell division along ‘long axis’ as opposed to random orientation is less prone to mechanical force disturbances according to Hertwig’s rule. This has its further implications in cell morphogenesis, fate and tissue architecture (Hertwig 1884). These studies prove that mechanical forces indeed play a significant role in cell and tissue construction. Moreover, in recent years further studies have uncovered the cellular and sub-cellular mechanisms showing the role of forces in cell, tissue and organ organization.

1.2 Mechanical regulation in the cell
Cells like any other non-biological material have their own mechanical properties that are equally relevant as structural and functional features. Taking advantage of their mechanical characteristic cells counteract the mechanical stress to carry out their physiological function. Likewise, mechanical force contribute to the physiological cell performance in control and diseased states (Petridou, Spiró and Heisenberg 2017; James, Wang and Thampatty 2006).
Additionally, force detection, maintenance, and generation at a single cell level is an important intermediate in the hierarchical body organization, being placed between mechanosensitive molecules and tissue-organ physiology. Therefore, studying cellular mechanics turns out to be a potent instrument to unravel intricate cell features in a more extensive way as opposed to individual morphological or biochemical traits. Cells sense mechanical stimuli, both from outside and from inside, in the process of mechanosensation and render them to biochemical information via mechanotransduction, controlling several physiological events such as cell proliferation, migration or differentiation (Chen, Tan, and Tien 2004; Leckband and de Rooij 2014). Mechanosensation and mechanotransduction have been proven to be important in embryogenesis, development and disease progression (Chanet and Martin 2014; Jaalouk and Lammerding 2009). Evidence shows mechanosensation is a reciprocal phenomenon and mechno-responsive elements can trigger a feedback loop. A given mechanical stimulus can initiate a specific signaling pathway, which can change mechanical characteristics of the cell such as Young’s modulus (YM) that in turn will impact on any further reaction of this cell to any mechanical cues.

![Image showing mechanosensation and mechanotransduction](image)

**Figure 1.** Schematic demonstrating sensing and transmission of the mechanical signal in a cell. First stage is known as mechanosensation when the stimuli reaches the cell. In the next step the mechanical information is transformed into a biochemical signaling pathway and reaching its final target in the process of mechanotransduction.

Depending on the type, function and location cells experience different kinds of mechanical stimuli that can act together or separately (Janmey and McCulloch 2007). The most common stimuli include:
1) mechanical stress, where the force is applied orthogonally to the surface of the cell. Tensile mechanical stress is when the force pulls the surface of the cell out, whereas, compression mechanical stress works to squash the cell.

\[ \sigma = \frac{F}{A} \]

where \(\sigma\) is the mechanical stress \([N/m^2]\), \(F\) is the applied force, \(A\) is the cell surface area

2) shear stress, where shear force acts parallel to the surface of the cell

\[ \tau = \frac{F}{A} \]

where \(\tau\) is the shear stress \([N/m^2]\), \(F\) is the applied stress, \(A\) is the cell surface area

Strain generated by deformation of the cell is a unitless parameter expressed as the ratio of the deformed cell length \([m]\) to the original length \([m]\),

\[ \varepsilon = \frac{\Delta L}{L} \]

where \(\varepsilon\) is the strain, \(\Delta L\) is the length after deformation and \(L\) is the original length

Despite being influenced by the mechanical environment, cells are still able to carry out their functions and maintain the structure. That is due to the unique cellular composition. Cell functions as a mechanical continuum composed of a number of structural elements that make up the individual cell mechanome. To date, different cell mechanical properties have been investigated; one of the most important is viscoelasticity. Cells represent both elastic solid material and viscous fluid, as structurally cells are composed of a filamentous protein structure embedded in a liquid cytoplasm. In that respect cells behave as structured liquid.

Another critical parameter is intracellular tension which develops by the cytoskeletal structure and it is used to keep all the intracellular components at place. A powerful model has been introduced by Ingber (model of tensegrity) suggesting that cell represents a continuum of forces and any change in tension at a particular part of the cell is quickly transferred to another, via cytoskeletal network (Ingber 1993).

Lastly, elastic property of a cell can be characterized by Youngs Modulus \([E]\), which is defined as follows:

\[ E = \frac{\sigma}{\varepsilon} = \frac{F/A}{\Delta L/L} \text{ [Pascal, Pa]} \]

where \(\sigma\) is mechanical stress, \(\varepsilon\) is strain (deformation)
This parameter has been investigated in a variety of cells, such as epithelial cells, fibroblasts isolated from pulmonary fibrosis patients, neural crest cells, cardiomyocytes, murine fibroblasts (NIH3T3), red blood cells, human mesenchymal stem cells and cancer cells, eventually used as a biomarker of the metastatic colony (Jaffar et al. 2018; Solon et al. 2007; Barns et al. 2017; Xu et al. 2012; Sarem et al. 2019).

1.3 Techniques used to measure cell YM
Currently, there is a wide range of accessible tools to investigate different cell mechanical properties in both space and time. In order to choose an appropriate technique to perform a reliable study, several factors need to be considered: sample size, mechanical resistance, time of analyzed process and within the sample, heterogeneity, rapid structural as well as mechanical changes (Moeendarbary and Harris 2014). Mechanical properties can differ in the same cell type as they may change according to the stage of the cell cycle, surrounding environment and cell activity. Studying YM of intracellular objects at different scale requires different methods (Fig. 2), equally, studying cells differs from studying mechanical properties at the scale of tissues and organs.

![Figure 2. Schematic representing most common techniques used for measuring cell Young’s modulus in relation to scale. Both, micropipette aspiration and magnetic twisting cytometry are applied to study object at a similar range. Optical tweezers can be used for objects smaller than 100nm. Whereas, atomic force microscopy is designed to examine objects as small as few nm up to µm range.](image)

In the next chapters I will describe the most commonly used techniques in more details.
1.3.1 Micropipette aspiration

Micropipette aspiration has been already known for several decades and has been widely used. This technique depends on the immobilizing a single cell on a micropipette. Next, a negative pressure is applied through the syringe to aspirate the cell. Deformation of the cell surface can be tracked using light microscopy with the accuracy of around 25 nm and the suction force as small as 0.1-0.2 pN/m² (Hochmuth 2000). Changes in cell distortion are plotted onto a graph that depends on the stiffness and viscoelastic properties, specific to each cell. Being an inexpensive technique, it has been used to study mechanics of different cell types both soft such as red blood cells, neutrophils and more rigid, like chondrocytes and endothelial cells (Barns et al. 2017; Lee, Patel and Park 2011; Hsieh et al. 2008; Chtcheglova et al. 2010). Micropipette aspiration uses the continuum model, which assumes that cell is either a liquid surrounded by a cortex or a uniform solid. The model type will then affect the cell behaviour during pressure aspiration. Stiffer cells will slowly extend in the pipette, whereas liquid cells will flow into the pipette with ease (Hochmuth 2000).

1.3.2 Optical tweezers

The development of optical tweezers (optical trapping, OT) for application in biological systems was recognised as great achievement by scientific community and the 2018 Nobel Prize in Physics was given to Arthur Ashkin at a remarkable age of 96. This technique uses a ray of light, usually infrared to avoid sample boiling, to control the position of a nanometer sized object. The light focuses on the sample that is steered by two types of forces. The scattering force is created when the ray of light is scattered on the sample plane and the light momentum is transferred to the sample simultaneously moving it in the direction of beam propagation. The second gradient force arises from the laser intensity that directs the sample to the light of the highest intensity. To measure mechanical properties of a cell, a plastic bead that is bound to a cell surface receptor is trapped in the ray of light. Then a defined light force in the order of pN is applied and the displacement from the laser centre is recorded. So far, mechanical properties of different cell types have been studied using this technique, such as red blood cells, chondrocytes, epithelial cells, fibroblasts, tumour cells or even DNA structure (Wang et al. 1997; Beutler et al. 1979; Resnick 2010; H. Zhang and Liu 2008). Major
advantage of OT is the opportunity to measure very small objects (atom-size) in a non-contact working mode. However, limitations are also present, such as the need to work in water solutions with relatively low throughput. Moreover, long exposure to a laser light of high intensity can affect sample viability as well as mechanical properties.

1.3.3 Atomic Force Microscopy
At present, the most commonly used technique to investigate mechanical properties of biological samples is Atomic Force Microscopy (AFM), a kind of Scanning Probe Microscopy, which was invented in 1986. The working principle of AFM is based on recording of the displacement of a probe (cantilever tip) which directly touches the sample. With the cantilever, similarly to previous method, the relationship between the force applied to it and the displacement of the cell membrane is recorded. The movement of the cantilever is detected optically in most cases. However, different detector systems exist; various tips geometries are also available, such as rectangular, circular, square or spike. To maintain the constant sample-tip interaction a feed-back loop system has been employed. A piezoelectric drive controls the cantilever movements, which allows accurate scanning. Vertical position of the cantilever is recorded and the sample deflection is calculated. AFM measurement can give information on sample active forces, viscoelastic response and YM, according to Hertz model. Advantages of AFM are in its nanometer resolution and in the lack of need to use an objective lens, which removes the aberration and diffraction issues. Also, there is no necessity to stain the cells (Chang et al. 2012). Mechanical properties of many cell types were analysed using AFM (Dague et al. 2014; Wagh et al. 2008; Haase and Pelling 2015; Y. J. Lee, Patel, and Park 2011). Commonly accepted disadvantages of AFM are the following: slow scanning rate, small scanning image size and direct cantilever-sample contact that can cause damage to the cell (Chang et al. 2012).

1.4 Mechanical properties in the cardiovascular system
Cardiovascular system is composed of three elements: the blood as a liquid, the blood vessels that deliver the blood to the heart, and the heart which resembles a pump. The blood flow has been recognized to generate enough pressure and shear stress to control the morphology and physiology of the vascular network as well as a four-chamber heart (Haga, Li, and Chien 2007; Li, Haga, and Chien 2005; Gimbrone et al. 2000). In vivo studies of zebrafish embryos
demonstrated that pronounced shear stress occurs during morphogenesis at the time of critical heart developmental stages. Introduced alterations to the shear flow patterns caused incorrect heart formation. Developmental deformations in the zebrafish heart, introduced by incorrect flow patterns, such as faulty valves, are identical to some inborn heart defects in humans (Hammerschmidt et al. 2007). In a different study also in zebrafish, by restricting hemodynamic pressure using microbeads, blood flow into the ventricle was inhibited and that eventually held back cardiac looping, one of the critical stages of cardiogenesis (McCain and Parker 2011). In the adult heart undisrupted blood flow is also of great importance as unstable flow has been shown to be the primary cause of atherosclerosis (Chiu and Chien 2011). These data are consistent with the dogma that force is a relevant factor for the organ origin and development.

Heart undergoes considerable changes during developmental stages until it reaches the mature state. But even after the heart is formed it maintains its kinetic features during the lifespan. Due to varying dynamics of cardiac blood flow, cardiomyocytes need to be resistant and highly adaptive to the variable mechanical load. To do that, myocardial structural organization is of great importance.

The heart is a four-chambered muscular pump in which left ventricle (LV) is discharging blood through the aortic valve into the aortic arch and further to the whole-body system of arteries and small blood vessels. Being the most muscular chamber LV is the one that generates the highest force. LV is also distinguished from the right ventricle (RV) by a longer isovolumetric contraction time at the baseline, a period when ventricles contract with no volume change while both heart valves are closed. Differences in contraction behavior between LV and RV show that chamber-specific properties have been developed in order to maintain an optimal cardiac function by adjusting contraction force and rate. The differences in electrophysiological parameters between LV and RV are summarized in an elegant review (Molina, Heijman, and Dobrev 2016). For example action potential of the plasma membrane displays different waveform patterns between LV and RV; systolic Ca2+ and cell shortening are higher in the LV than in the RV (Kondo et al. 2006); or sarcoplasmic reticulum calcium uptake leading to the calcium transient decay is slower in the RV than in the LV (Protsenko et al. 2018). Thus, physiological differences between LV and RV are well-documented; few studies have focused on the mechanical differences of both ventricles. Several research groups reported that changes in LV mechanical properties in accordance with load may
contribute to the chamber functionality (Elzinga and Piene 1980; Vonk Noordegraaf, Westerhof and Westerhof 2017; de Asua and Rosenberg 2017). On the other hand, mechanical properties of RV are less documented and more studies are needed.

1.5 Cell mechanics in a single-cell cardiomyocyte model
Adequate muscle organization is an obligatory requirement for the heart muscle contraction and uniaxial force transmission (Gautel and Djinović-Carugo 2016). During every heart beat each heart myocyte undergoes both length and load alterations. Frank-Starling law describes the direct proportional relation between LV volume and pressure (preload) and the contraction force (de Tombe et al. 2010; Allen and Kentish 1985). In the preload a certain relation between the length of the sarcomere (basic unit of muscle contractile mechanism) and the tension is set. This controls the rate of muscle shortening and further LV contraction and ejection of blood to the aorta. At the cellular level, increased LV volume results in cardiac myocyte elongation exerting the highest possible force, a calcium-dependent process. Therefore, the more Ca ions are available, the higher force is generated, depicting a direct relationship between sarcomere length and calcium concentration. However, in the systole (afterload) the cardiomyocytes shorten as the blood is pumped out against the arterial resistance (Chatterjee and Massie 2007; Kitzman et al. 2002). Cardiac myocytes are able to sense mechanical load of different magnitudes; they respond by converting these mechanical signals to biochemical pathways, which eventually exert structural and functional alterations in the cells. This serves as an adaptation of the heart to the applied load. It has been widely accepted that sarcomere, the main contractile unit of the muscle, is the substrate of mechano-transduction in cardiac myocytes (Lyon et al. 2015). However, there are also other confirmed and suggested mechanosensitive proteins at
different myocyte locations. The schematic below shows distribution of mechanosensors within cardiomyocyte that will be further discussed.

![Schematic depicting mechanosensitive elements within a cardiac myocyte. At the membrane, sarcolemma invaginations, caveolae are present, as well as integrins, transmembrane receptors, stretch-activated channels and intercalated discs, located at the cell edges (see detailed description in chapter 6). Inside the cell multiple mechanosensors are present: nucleus, mitochondria, generic cytoskeleton that includes microtubules, actin and intermediate filaments as well as cardiac-specific sarcomeric cytoskeleton containing Z-disc proteins, actin, myosin and titin.]

**Figure 3.** Schematic depicting mechanosensitive elements within a cardiac myocyte. At the membrane, sarcolemma invaginations, caveolae are present, as well as integrins, transmembrane receptors, stretch-activated channels and intercalated discs, located at the cell edges (see detailed description in chapter 6). Inside the cell multiple mechanosensors are present: nucleus, mitochondria, generic cytoskeleton that includes microtubules, actin and intermediate filaments as well as cardiac-specific sarcomeric cytoskeleton containing Z-disc proteins, actin, myosin and titin.

### 1.5.1 Cell membrane

Cell membrane is formed by a double layer of lipids and scattered cholesterol complexes (lipid rafts) with embedded proteins, some of them are ion channels or receptors. Functions carried out by the cell membrane are: protective barrier formation from the extracellular environment, bidirectional molecule and ion transport and sensing function. The plasma membrane also serves a role of anchor for the cytoskeleton; that helps to maintain the overall cell structure. Therefore, maintaining correct mechanical properties is imperative for the overall integrity of the cellular plasma membrane. The sarcolemma of the cardiac myocytes is
characterized by high degree of organization. Transverse tubules (T-tubules) are formed as deep membrane invaginations into the cytoplasm. These structures harbor many proteins involved in the excitation-contraction coupling, including receptors, ion channels and effector molecules. Interestingly, it has been shown recently that in rat cardiomyocytes treated with formamide, which removes T-tubules, passive mechanical properties do not change (Ferrantini et al. 2014). This finding suggests that T-tubules do not contribute to cell mechanics.

Another relevant membrane compartment are caveolae, small membrane pits made of specialized proteins and lipids. Caveolae are significantly flatter than T-tubules, but they similarly collect different ion channels, structural proteins and receptors. Numerous studies have proposed caveolae to significantly contribute to cell mechanics (Echarri and Del Pozo 2015; Nassoy and Lamaze 2012). Subjected to osmotic swelling and uniaxial stretch caveolae were able to flatten, but when the mechanical input was removed the flask-shaped invaginations regained their shape (Gervásio et al. 2011).

1.5.2 Membrane channels
Membrane-associated proteins perform different functions, such as ion transport, cell signaling or extracellular adhesion, as discussed later (Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter 2003). One particularly interesting class of proteins on the plasma membrane are the stretch-activated ion channels (SAC). Local mechanical stress provokes a change in the open/close state of the channel, triggering the signaling cascade. The activating mechanism is usually dependent of the lipid bilayer tension. Secondly, a bond can form between the channel and cytoskeleton and/or extra cellular matrix, which forces the channel to open (Markin and Sachs 2004; Markin and Martinac 1991). A wide range of SAC are present in archaea, bacteria and eukaryotes, such as TRP channels, DEG/NAC channels, MscS-like proteins and K(2P) channels (Yin and Kuebler 2010; Valle et al. 2012; Bianchi 2007; Booth and Blount 2012; Kloda and Martinac 2002). The level of selectivity and sensitivity of these channels may vary considerably. They are mostly found in sensory cells, for example hair or inner ear cells, but they also take part in the blood pressure regulation and heart electrophysiology (Árnadóttir and Chalfie 2010; Sidi, Friedrich, and Nicolson 2003; Corey 2006). Interestingly, it has been shown that not only SAC can be activated by cell stretch. The trans-membrane voltage gated channels, which regulate the plasma membrane potential, are
also affected. For example L-type calcium channels activity is increased by longitudinal mechanical stress through a polycystin-1-dependent mechanism, as shown in neonatal cardiac myocytes (Córdova-Casanova et al. 2018). Another group of ion channels on the plasma membrane are the ligand-gated channels. The main activation pathway is the binding of a specific ligand, however it has been shown that such channels can be induced by mechanical stretch. For example, Angiotensin II receptor 1 is a G protein-coupled receptor which is normally ligand-activated, however recent findings show that it can also be regulated by mechanical stretch (Schleifenbaum et al. 2014).

1.5.3 Integrins as the cell-ECM linker
Focal adhesions (FA) are specialized membrane-embedded dynamic multi-molecular protein complexes that anchor the cell to the surrounding extracellular matrix (ECM). FA consist of several components including transmembrane proteins, cytoskeleton and signaling molecules. FA are able to respond to mechanical stimulus, such as stretching, by engaging more FA proteins from the cytoplasm which results in strengthening of FA (Sawada and Sheetz 2002). Conversely, in the absence of external force, FA are maintained by the tension force provided within the cell via myosin; this force is then transmitted outwards (Bershadsky, Balaban, and Geiger 2003). FA proteins are cell specific but most likely include isoforms of integrins as the main adhesion complex constituents (Zamir and Geiger 2001; Mostafavi-Pour et al. 2003). Due to the FA connection with the ECM on one side and cytoskeleton on the other, the bidirectional communication is possible and very effective (Parsons, Horwitz, and Schwartz 2010). Tensional forces exerted by actin fibers within the cells can be matched by the ECM tension. When transmitted externally this tension regulates cell structure, movement and adhesion (Fletcher and Mullins 2010). Cells reside in a very dynamic ECM environment that along with the adhesion complexes and cytoskeleton balances the mechanical stress. The external milieu is a complex but well-organized structure of proteins that provide mechanical resistance, molecule adhering regions, scaffold and a storage of different proteins to supply cellular functions (Lukashev and Werb 1998; Raines 2000). This multi-functional intersecting network consists of collagen, proteoglycans and glycoproteins. The most abundant ECM constituent is collagen of several types (e.g type I, II, III and IV) that makes the bulk of ECM and exists in several forms of high mechanical strength, such as fibers and mesh scaffolding present in tendons, ligaments, cartilage, skin and basement membrane vascular structures.
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(Eyre 2004; Hulmes 1992; Brown and Timpl 1995). ECM is also characterized by the presence of different sulfated and non-sulfated proteoglycans that carry spatial organization and are associated with cell movement and migration. In addition, a large group of glycoproteins is present in the ECM, including fibronectin, laminin, vitronectin and tenasin (Lodish et al. 2000).

1.5.4 Intercalated discs
Intercalated discs (IDs) are cardiomyocyte-specific structures located at the lateral cell ends. Each cardiac myocyte generates force that is laterally transmitted to the IDs. End-to-end cell interactions make it possible for the mechanical force to be passed on to the adjacent cell to provide a synchronized contraction in the whole organ. IDs are composed of three different protein complexes: adherens junctions (AJ), desmosomes and gap junctions (GJ) (Sheikh, Ross, and Chen 2009). Undoubtedly, mechanical stimuli play crucial role in the maintenance and functioning of IDs. To date, maintaining ID’s structural integrity has been attributed mainly to AJ and desmosomes. However, emerging data from us and others show that GJ are not only responsible for the electrical coupling but also contribute to the mechanical properties at the junction (Salameh et al., 2010; Schultz, Swiatlowska, Alvarez-Laviada et al., 2019). A paragraph detailing our studies on cardiomyocyte IDs is present in chapter 6.

1.5.5 Mitochondria
Constant energy supply is one of the prerequisites of life; multitude of cell functions depends on energy consumption that is provided mainly by mitochondria. However, apart from renowned function of supplying energy in the form of ATP, these organelles are also responsible for apoptosis regulation and calcium buffering (Kluge, Fetterman, and Vita 2013). In the event of overstimulation, mitochondria can produce reactive oxygen species (ROS), molecules that readily release damaging free radicals. ROSs react with other molecules in the cells and may interrupt and damage ongoing physiological processes. In order to adapt to the everchanging cellular demand of energy mitochondria form a very mobile network which is constantly modified by fission (division) and fusion of individual organelles. Mitochondria are tightly connected to the cytoskeleton, (Boldogh and Pon 2007; Bereiter-Hahn and Vöth 1994; Palmer et al. 2011). With the help of motor proteins, dyneins and kinesins, mitochondria can move along the microtubular filaments within the cell (Ligon and Steward 2000). Also, these
small energy factories interact with mechano-responsive actin filaments. It has been proposed that since mitochondria are attached to the cell cytoskeleton that is able to transmit mechanical signals, they may sense and respond to mechanical cues; this leads to structural and/or functional alterations in the mitochondria. It is believed that interaction between mitochondria and actin filaments is a bidirectional relationship. Mitochondria affect several actin-dependent cellular functions, for example actin network provides cells with the resistance to deformation but achieving this requires energy stored in mitochondria in the form of ATP (Bartolák-Suki et al. 2017). Interestingly, it has been shown that mechanosensitive signals that come from stretching promote mitochondria function maintenance (Bartolák-Suki et al. 2015). Yet, others reported negative effects of stretching on the mitochondria life cycle. For example, transiently increasing the length of lung fibroblasts by 30\% causes mitochondria rupture and disrupting cell membrane using the glass micropipette brings the same effect of mitochondria fission (Imsirovic et al. 2015; Gonzalez-Rodriguez et al. 2015). These findings confirm that both internal and external mechanical stimulus can affect mitochondria structure. Emerging data show that several diseases that are associated with mitochondria malfunctioning are at the same time related to the mechanical factors (Bartolák-Suki et al. 2017). In fact, during fibrosis YM of the ECM is markedly increased, and this is transmitted to the cell cytoskeleton, also exerting an effect on mitochondria (Mora, Bueno, and Rojas 2017). In inflammatory lung airway disease, patients experience increased smooth muscle cells contraction that requires more ATP production, and it correlates with an increased YM in the airway wall ECM (Bartolák-Suki et al. 2017). Nevertheless, the role of mechanics in mitochondrial function is not well understood and warrants further studying.

### 1.5.6 Nucleus

So far, the majority of studies has focused on the mechano-transduction that occurs between ECM in the outside and cytoskeleton inside the cell. Recently, it has been shown that mechanical overload damages the nuclear envelope and disrupts the binding of the associated proteins. It has been shown, in an elegant microbead stretching experiment, that not only the plasma membrane elongates in the same direction as the pulling force, but also the nucleus. This was further shown to be an integrin-dependent process indicating a physical contact with the cell membrane (Maniotis, Chen, and Ingber 1997). At the nuclear membrane, a protein
complex known as LINC (Linker of Nucleoskeleton and Cytoskeleton) is located, which, similarly to the FA at the cell plasma membrane, is capable of recognizing a mechanical stimuli. With the aid of these mechanisms cell can directly sense mechanical disturbances in the cytoskeleton and transmit it to the nucleus (Luxton et al. 2010; Starr and Han 2002; Starr and Fridolfsson 2010). LINC interacts with the nuclear matrix composed primarily of lamin A that has also been confirmed to respond to mechanical cues, for example increased lamin A expression is associated with stiffer cells (Lee et al. 2007). Generally, the nucleus is stiffer and more viscous than the cytoplasm, with nuclear YM being from 3 to 10 times higher in the nucleus (Guilak, Tedrow, and Burgkart 2000; Chen et al. 2012). Moreover, distorting the shape of the nucleus has shown to affect DNA arrangement which further projects onto the gene transcription (Lammerding 2011).

1.5.7 Cardiac-specific cytoskeleton
Sarcomere is a high-order arrangement of contractile proteins that is present in muscle cells. The border between two sarcomeres is formed by the Z-disc. It is known that the Z-disc is one of the most molecule-abundant structures being composed of different proteins and phospholipids. Sarcomere is responsible for the force generation, serving as a mechano-sensing hot spot, as many of the sarcomere components play a role in mechanical stress detection and response. Muscle LIM protein (MLP) is one of the many tension sensors in the z-disc complex, reported by other groups (Boateng et al. 2009; Buyandelger, Miocic, Gunkel, et al. 2011). Further studies with the yeast two hybrid assay demonstrated a direct interaction of MLP with another Z-disk component Tcap (telethonin). Later, in a mouse model of MLP knock-out, a decline in Tcap signal was noticed, as observed by immunofluorescent staining. Similar results were obtained from human patients carrying a MLP mutation that caused the absence of communication with Tcap (Knöll et al., 2002). On the other hand, Tcap in the Z-disk binds to titin by very stable, unique palindromic bonds with the N-terminus of titin, forming an arrangement of one Tcap with two titin molecules (Zou et al. 2006). Titin, being a large elongated protein that is half-sarcomere long, interacts with multiple proteins exerting different effects and activating multiple signaling pathways. However, the primarily function of titin is related to its elastic properties. In the sarcomere titin serves as a length sensor (Granzier and Labeit 2002). To date, several mechanisms have been revealed to depict the role of this gigantic protein in cell
mechanics. Specifically, titin I-band received a lot of recognition in the study of mechano-sensation as it contributes to the cell passive tension (Linke et al. 1999). Muscle ankyrin repeat proteins (MARPs) bind to titin elastic I-band region and activate transcriptional regulation in the nuclei. This interaction with other proteins is augmented during mechanical stretch, as increased titin length makes it more accessible for protein binding (Miller et al. 2003). Another protein, four and a half LIM domain (FHL) is responsible for activating transcription and signaling pathways upon binding to titin I-band, suggesting a mechano-sensing role of this protein (Raskin et al. 2012). Several other molecules have been found in close proximity to titin and have been shown to translocate to the nucleus upon stretching the sarcomere (Dobner, Amadi, and Lee 2012). Genetic removal of I-band significantly decreases titin YM, increasing the sarcomere strain; at the same time it causes FHL upregulation and eventually leads to the development of a hypertrophic phenotype (Granzier et al. 2014).

The multimolecular complex of MLP, Tcap and titin is seen as a principal cardiac mechanical stretch sensor (Knöll et al. 2002). In the sarcomere, closely associated with titin are actin (thin) and myosin (thick) filaments. Contraction force is generated due to the energy-dependent conformational change in the actin and myosin which leads to dynamic attachment of actin to myosin and subsequent detachment in a contraction cycle (Chung et al. 2011; Geeves and Holmes 1999). Laterally generated force within the sarcomere is reinforced by multiple sarcomeres arrangement along the same axis in the myofiber and eventually, in the entire muscle. The mechanical equilibrium is additionally supported by a close link to the generic cytoskeleton that provides support and spatial organization.

1.5.8 Generic cytoskeleton

Cell cytoskeleton is a key player in maintaining cell structure and function; its interaction with different cellular components have been already mentioned in the previous paragraphs. Cytoskeleton functions include: cellular content organization via development of intracellular tension, cell connection with ECM via focal adhesion mechanical load sensing and transmission, regulating force generation, inciting cell motility and shape alterations, providing resistance to cell deformation and mechano-transduction. To carry out all of these roles the cytoskeleton is connected to numerous cross-linkers, stabilizers, motor-proteins and organelles (Fletcher and Mullins 2010). The spatial and temporal cytoskeletal network organization is maintained by constant polymerization and depolymerization of constituent
proteins, specifically actin and tubulin. The process of polymerization and depolymerization is coordinated by several mechanisms: polymerases accelerate the growth of filaments, depolymerizing factors remove certain molecules, nucleation factors initiate the growth of filaments, and capping proteins inhibit the polymerization of filaments (Saarikangas, Zhao, and Lappalainen 2010; Desai and Mitchison 1997). This shows that single filaments as well as the intact cytoskeletal network are dynamic nonlinear structures rather than a plain solid construction. Therefore, understanding the mechanisms of the filamentous protein network formation, maintenance and degradation is essential to understand the working of cell mechanics.

An elegant review from Parsons, Horwitz and Schwartz (2010) has shown that cell cytoskeleton responds to mechanical tension via ECM and focal adhesions, which causes network reorganization. On the other hand, cytoskeleton is also generating its own tension by the 3D network organization and attachment to the sarcolemma, (Vignaud, Blanchoin, and Thé 2012). Overall, it is fair to say that every mechano-transduction event involves the cytoskeleton (Burrige and Guilluy 2016; Parsons, Horwitz, and Schwartz 2010).

In the eukaryotic cells, cytoskeleton is formed by three biopolymer networks: actin filaments, intermediate filaments and microtubules. All three biopolymer molecules have different chemical and physical properties. Actin is a strongly conserved protein, present in large amounts in almost all eukaryotic cells and forming interactions with multitude of other proteins. It’s represented by three isoforms, α-actin, found in muscle cells and responsible for contraction; β- actin and γ-actin, present in muscle and non-muscle cells (Lee and Dominguez 2010). These isoforms can be found in two forms, globular (G-actin) and filamentous (F-actin). The latter one is characterized by the presence of barbed end with an attachment site for new monomers for filament elongation and pointed end where actin filament is shortened. Actin filament polymerization and depolymerization processes are highly regulated by a cohort of actin binding proteins (Winder and Ayscough 2005). Actin filaments exist in a form of a double-stranded helix, 7-9nm in diameter. They play a major role in cell migration, emphasizing their role in the movement initiation via filopodia or lamellipodia (Nemethova, Auinger, and Small 2008). In order to produce the movement or generate force actin forms a complex with myosin, where actin serves as a rail for myosin to slide on; this process is accompanied by ATP hydrolysis (Winder and Ayscough 2005). Apart from the role in the
dynamic regulation of cell structure, actin functions as a molecule guide; for example, it helps to deliver connexin 43 (Cx43) proteins to the cardiomyocyte membrane. Intermediate filaments (IF) are 10 nm in diameter fibers that are represented by 50 different genes, making this a very diverse group. Each filament undergoes a coordinated assembly process to finally form a polar protofilament, generally more stable than an actin filament or a microtubule (Kamm, Lammerding, and Mofrad 2010). In epithelial cells, intermediate filaments bind to desmosomes and hemidesmosomes at the plasma membrane, and that helps the cell to withstand mechanical stress (Garrod and Chidgey 2007). Nevertheless, in cardiomyocytes the presence of IF has been shown to be important in the myofibril maintenance by surrounding z-disc, sarcoplasmic reticulum and T-tubules (Tokuyasu et al. 1985). Principle component of the IF in cardiac myocytes, desmin, has been recently shown to connect the sarcomere and microtubules (MTs) (Robison et al. 2016). Microtubules are hollow tubules of 25nm outer diameter composed of α- and β- tubulin dimers. MTs are responsible for establishing cell shape in the interphase of cell division, whereas during mitosis MT serve as conduit for chromosome separation. Each MT is a highly dynamic short-lived filament in which a fast GTP-dependent turnover of tubulin monomers occurs within fast and slow growing ends (Cooper 2000). Recently, MTs have been suggested to be able to resist mechanical load. Using traction force microscopy it has been shown that MT balance contractile stress by carrying the compression and upon the MT disruption the stress is transferred to the underlying substrate (Stamenović et al. 2002). Multiple findings confirm the role of MT in balancing the cell tension in order to maintain cell shape (Wang et al. 2001; Wang, Butler, and Ingber 1993; Ingber et al. 2000). MT can be made more stable by making post-translational modification to the tubulin monomers; these modifications include detyrosination and acetylation. If the unmodified filament on average has a half-live of 10 minutes, a stable MT can last for hours (Portran et al. 2017; Burbank and Mitchison 2006; Wloga, Joachimiak, and Fabczak 2017). Stable MTs have been shown to be key regulators in cardiac myocyte mechanics (Robison et al. 2016; Kerr et al. 2015; Xu et al. 2017) and reported that detyrosinated MTs bind to sarcomeric desmin, which determines myocyte mechanical resistance (Robison et al. 2016). Moreover, improved imaging techniques have uncovered changes in MT geometry during contraction and resting state. Specifically, detyrosinated MT were shown to adopt sinusoidal shape (buckling effect)
during twitch and going back to linear shape in the resting state (Kerr et al. 2015; Robison et al. 2016).

However, the exact role of MT in cell mechano-sensitivity still remains controversial, as some groups suggest that MT cannot carry such a big load at a cellular level (Deguchi, Ohashi, and Sato 2006; Dogterom et al. 2005).

Strikingly, the above mentioned three filamentous polymers differ greatly in stiffness. Structural studies determined the persistence length (a value that represents the bending stiffness of a polymer) of the three filamentous networks. It was found that IF have persistence length on the nanometer range whereas actin filaments have persistence length of around 10 millimeters and MTs are even more stiff with persistence length of up to several millimeters (Lodish 2000). Furthermore, the cytoskeletal matrix components also differ in the viscoelastic properties, where microtubules exhibit the highest rate of deformation (Lin, Koenderink, MacKintosh, & Weitz, 2007).

### 1.6 Cardiomyocyte mechanical properties in disease

As discussed in chapters 1.1-1.2, proper cellular response to the mechanical cues from their microenvironment is necessary for the tissue development and maintenance. A large variety of mechanical stimuli may be connected to a wide range of forces depending on the tissue type. Therefore, each cell type needs to accommodate to the stress generated in its specific environment. In addition, air pressure and blood flow may contribute to the mechanical properties of extracellular environment to which the cell needs to adapt. Most physiological processes are dependent on the mechanical signals, for example translation and release of proteins, adhesion, migration and apoptosis (Yusko and Asbury 2014).

Firstly, local alterations in the mechanical properties of the microenvironment can induce pathological effect despite a properly functioning cellular structure (Jaalouk and Lammerding 2009). One of the first diseases to be shown to arise from a modified mechanical stimulus is atherosclerosis. Here, steady laminar blood flow is disturbed at the vessel bifurcation leading to an oscillatory shear stress that promotes an inflammatory reaction at the endothelial lining of the blood vessel eliciting a whole cascade of pathological events resulting in the atherosclerotic plaque formation (Chiu and Chien 2011). Another example of mechanical stress-related diseases is the loss of muscle and bone mass in the microgravity environment. Due to a significant reduction in the mechanical stimulus (gravitation) that acts on the
musculoskeletal system, its basic supportive and movement function is diminished. Bone has a unique structure formed by solid and flexible components. Stiff structure properties are provided by hydroxyapatite, a calcium-based inorganic component. However, in the absence of gravity force, bones are subjected to modified stresses that results in calcium hydroxyapatite breakdown and calcium migration from the bone into the bloodstream.

Secondly, diseases caused by disturbances in mechanical signal transmission due to random or inherited mutations are also very common in humans (Lang et al. 2017). A perfect example of such disease is deafness. Present in the ear, hair cells with stereocilia located at the upper surface are responsible for sensation and localization of the sound. However, severe deafness is demonstrated in patients with inherited mutations in the proteins involved in ear mechanotransduction. Usher syndrome type 1 is described as a multiple deficiency of balance, hearing and vision. A set of affected genes includes genes encoding adherence, motor and scaffolding proteins (Cdh23, Psdh15, Myo7a, Usch1c, Usch1g) (Morgan and Barr-Gillespie 2013).

Cell YM has been proven to play a key role in the cancer disease. A measure of cell optical deformability was used to distinguish normal from the cancerous cells in different types of tumours (Lekka et al. 2012), suggesting that this parameter may become a novel biological marker for cancer. In conclusion, cellular mechanics are receiving more and more recognition being a relevant factor in the pathogenesis of different diseases (Guck et al. 2005). Within the cardiovascular system, the possibility of altered mechanical sensing leading to cardiac pathophysiology is very high, mostly due to its very dynamic nature. Cardiac heart myopathies are linked to severe morbidity and mortality (Maron et al. 2006). One of the most prevalent heart diseases are dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM). It has been shown that 30% and 80% of the incidents, in DCM and HCM respectively, are due to the hereditary genetic alterations (Buyandelger, Miocic, Gunkel, et al. 2011). Current findings demonstrate that a variety of genes encoding sarcomeric and cytoskeletal proteins is affected by genetic mutations as a primary cause of familiar cardiac disease. So far, studies have shown that several proteins can be affected by mutations in the encoding genes and further leading to cardiac diseases. For example, proteins that are implicated in the force generation such as β-myosin heavy chain (β-MHC), troponin T, and troponin I (Kamisago et al. 2000; Ahmad et al. 2008; Niimura et al. 2002); molecules that are responsible for mechanosensing such as MLP, Tcap or titin (Buyandelger, Miocic, Gunkel, et al. 2011); and proteins that transmit signals to ECM like dystrophin (Lapidos, Kakkar, and McNally 2004). Ineffective
response to mechanical stimulus in mutated generic or sarcomeric cytoskeleton significantly impairs heart mechanics as well as contractility. In particular, proper cross talk between the mechanical input and cellular response is of great importance in heart remodeling and adaptation, as faulty structural proteins can block the remodeling. Enhanced mechanical load characterizes multiple heart diseases; it is shown to elicit detrimental morphological changes in the left ventricle (Gerdes 1992; Anversa, Ricci, and Olivetti 1986). For example, concentric hypertrophy of the heart, characterized by an increase in the ventricle wall thickness, is the consequence of pressure overload (Katz 2002). Moreover, overload can cause hypertrophy of the myocytes via the formation of new myofibrils that causes cross-section heart wall enlargement and reduction in the heart length to width ratio. (Grossman, Jones, and McLaurin 1975; Olivetti et al. 1988; Smith and Bishop 1985). On the other hand, eccentric hypertrophy develops mainly due to volume overload and it is indicated by addition of new sarcomeres to the already existing bundles of myofibrils in the cell, leading to increased heart length to width ratio and eventually thinner ventricle wall (Beltrami et al. 1995; Capasso, Fitzpatrick, and Anversa 1992). Further consequences include chamber dilation, changes in the mechanical wall input and adverse remodeling (Luk et al. 2009; Beltrami et al. 1995; Gerdes 1992). This demonstrates that the hypertrophic process can follow different patterns, each with a very precise myofibril structure formation during remodeling. Moreover, following pressure overload not only cardiac myocytes undergo changes, but also the ECM, which is mainly made up by fibroblasts. For example, the ECM component fibronectin was demonstrated to be expressed de novo in fibroblasts and an elevated integrin level was shown, suggesting transition into active secretory state (Ross and Borg 2001; Oliviero et al. 2000).

1.6.1 Left Ventricular Assist Device
Heart failure (HF) is a long and progressive condition in which the heart muscle is unable to effectively pump the blood needed for the whole body. According to a 25-year study, cardiovascular diseases cause 1 out of 3 deaths worldwide, therefore new treatments are desperately needed. Currently, implantable left ventricular assist devices (LVADs) are being used as a part of the therapy for HF as a bridge-to-transplant or a destination therapy. Several benefits of the LVAD transplantation has been shown that include reducing LV workload and increasing cardiac output. However, in some patients, adjustment to the post-implantation
stage has been widely recognized as difficult. This includes sleep difficulties, panic events, emotional distress or depression episodes. Despite psychological discomfort, emerging studies show that prolonged unloading of the heart can have detrimental effects instead of desired improvement (Sharp, Miller, and Al-Attar 2018).

Ibrahim et al., using a rat model showed that T-tubule network is severely disrupted in the advanced stage of HF and it can be partially restored after the removal of mechanical load. As a consequence of modifications in T-tubules that hold ion channels, distortion of calcium regulation event that induces contraction, known as Ca-induced Ca release (CICR) is noted. As suggested, 4-week mechanical unloading reduced calcium sparks and normalized time-to-peak calcium transient, which signifies a more normal phenotype (Ibrahim, Navaratnarajah, et al. 2012). However, it was found that the time of mechanical unloading matters notably, as removing mechanical load for 8 weeks has an adverse effect. Prolonged unloading was found to increase frequency and duration of calcium sparks, disrupt T-tubule network and eventually led to a cell atrophy phenotype (Ibrahim, Kukadia, et al. 2012). Another recent study has found that following mechanical unloading β2-adrenergic receptors (BAR) functionality was significantly affected, without affecting β1 adrenergic receptors. Also, L-type calcium channels activity was depressed (Wright et al. 2018).

1.7 Mechanical properties of cell-cell contacts
Structurally, tissues in the body need to withstand the applied deformation force; this is achieved by forming durable cell-cell adhesions that make the basis of the tissue integrity. Mechanical link between cells has been shown to be significant in a wide range of processes as early as at the very onset of embryo development; it includes all tissues and multiple cell types (Mammoto et al. 2010), wound migrating epithelial cells (Trepat et al. 2009), vessel endothelial cells integrity exposed to blood flow shear stress (Tzima et al. 2005) and epithelial cells in cancer metastasis (Bajpai et al. 2009). Therefore, we see that regulated control of the mechanical forces is of great importance for the integrity and cell-cell communication.

Heart muscle performance in normal conditions involves a coordinated work of all cell units in a dynamic fashion. A wide range of mechanical stimuli affect the heart during development, homeostatic as well as pathological stages and these stimuli need to be dealt with. During early stages of heart development, the appearance and regularity of myofibrils greatly relies on the cell-ECM adhesion (McCain et al. 2012). Later cell-cell adhesions take over cell-ECM
interactions and tight intercellular adhesions form at the cell edges, known as intercalated discs (IDs), as the tissue maturation favors the longitudinal, intercellular load transmission. The process of cooperative remodeling of the adhesion sites occurs during active contraction of the heart. This implies that the mechanical stimuli must be necessary for the assembly and disassembly of the cellular bonding (McCain et al. 2012). Studies carried out in rabbit hearts subjected to volume unload and overload have shown dynamic shifts that occur at the IDs and are related to the formation of sarcomeres (Lyon et al. 2015). This favors the notion that IDs are the mechano-transduction hot spots which, when formed, impact on muscle growth. But myocardium not only consists of electrically excitable myocytes that can generate action potential and produce mechanical force. Non-excitatory cells such as fibroblasts, epithelial and immune cells are equally important for the tissue development, integrity maintenance and restoration following damage (Camelliti, Borg, and Kohl 2005). This diverse group of cells notably outnumbers the excitable cardiomyocytes, but takes a smaller volume in the whole heart (Pinto et al. 2016). Additionally, changes in the mechanical properties of the surrounding microenvironment can alter the functional order of the cellular adhesion. Increased YM of the ECM makes focal adhesions more active as compared to the IDs as a compensatory pathway to maintain the tissue integrity (McCain et al. 2012). Thereby, it is important not to overlook the mechanical communication between non-myocytes and the ECM in the heart; it would be relevant to unravel the cellular cross-talk that takes place in normal and pathological states of the organ.

1.8 Junction between cardiomyocytes
Force generated within myocytes by the action of actin and myosin is transmitted laterally to the cell edge and further passed through intercalated discs (IDs) on to the next cardiomyocyte (Budnar and Yap 2013). Within the heart, cardiomyocyte end-to-end coupling via IDs is a highly regulated process. Proper coupling guarantees a rapid and undisturbative electrical signal propagation that leads to contraction and secures tissue integrity under constant and variable mechanical load generated during every heartbeat. This shows that IDs in their totality serve as a functional, mechanical and electrical continuum which is essential for the whole heart function (Vermij, Abriel, and van Veen 2017). The ID is formed by different adhesion protein complexes that physically join two cardiomyocytes and allow detection and processing of mechanical signals; where the most
important are adherens junctions and desmosomes. A third element, known as gap junctions, is responsible for unique electrical signal propagation between the two cells (Vermij, Abriel, and van Veen 2017; Li and Radice 2010).

Figure 4. Schematic demonstrating protein complexes at the cardiomyocyte intercalated disc. Three types of junctions are present at the cardiomyocyte edge, desmosomes, formed by desmoplakin, plakoglobin, plakophilin, desmoglein, desmocollin; gap junctions made up from Cx43 and adherens junctions composed of α-, β-, γ-catenin, p120, N-cadherin.

1.8.1 Adherens junctions
Mechanical coupling facilitated by adherens junctions (AJ; fascia adherens) is accomplished by a protein complex composed of cadherins and catenins. N-cadherin is the main cadherin isoform in cardiomyocytes. It is a transmembrane protein; one molecule of N-cadherin on one cell forms a homological connection with one molecule on the other cell. N-cadherin interacts with catenins via C-terminal region. There are three isoforms of catenins present in cardiac
myocytes: β- and γ-catenins (plakoglobin) that are connected to the cadherin cytoplasmic end and α-catenin that is a mediator between cadherin and actin cytoskeleton (Wilson et al. 2014). The role of cell-cell adhesion involving N-cadherin is very important in the development of the heart at the embryonic state (Radice 2013). The link from cadherin to catenin and to the actin filaments provides the possibility to transmit mechanical force from one cell to another, mediated by cell cytoskeleton. At an adheres junction, tension provided by actin filaments, is generated from both sides of the junction, so mechanical signaling can be transmitted in both directions. In addition, AJ can react to the force that pulls the junction outwards by increasing the amount of protein complexes assembled at the junction (Liu et al. 2010). These studies show that AJ can respond to mechanical load and are a part of the cardiac mechano-transduction mechanism, by promoting active cytoskeleton remodeling and mechanical signal transmission to the cytoplasm.

1.8.2 Desmosomes
Desmosomes represent second mechanical coupling complex at the intercalated discs of cardiac myocytes. This interface binding elements are the most powerful cell-cell coupling structures that can withstand the strongest force (Harmon and Green 2013). Desmosomes are present in different tissue types that are subjected to high mechanical stress, mostly epithelial, such as gastrointestinal mucosa lining the inside of gastrointestinal tracts, urinary tract, skin and cardiac myocytes (Groschwitz and Hogan 2009; Kowalczyk and Green 2013). Juxtaposed desmosomes are called macula adherens (MA). MA formation within the heart differs from the desmosomal junction between the epithelial cells (Patel and Green 2014). The overall MA structure is divided into two parts. Closely located desmocollin and desmoglein, known as desmosomal cadherins, are responsible for a close connection between two cells and form a dense structure called desmosomal plaque in the transcellular zone. The second part includes cytoplasmic molecules, such as plakoglobin (PG) and desmoplakin (DP) that connect the desmosomal plaque to IF, in particular desmin. The desmosomal junction is more robust compared to AJ in withstanding high force, as the complex is linked to more elastic IFs (Harmon and Green 2013).
1.8.3 Gap junctions
Gap junctions (GJ) also called nexus, are present in different tissues, allowing an easy flow of small molecules and electrical signals between cells. In the heart, they are important for the electrical impulse conduction. Six connexin proteins are necessary for the formation of one connexon. To make a functional pore, two connexons from the adjacent cells come in close proximity. It has been shown that GJ appear after the AJ formation, showing that mechanical stability comes before electrical connectivity when connection between two cardiomyocytes is formed (Rohr 2004; van Veen, van Rijen, and Opthof 2001; Segretain and Falk, 2014). Also, in a N-cadherin knock out mouse model, lack of GJ formation is noted, demonstrating a tight regulation between these two structures (Wei et al. 2005). Within the heart, GJ can be composed of three different connexin types, connexin-40 (Cx40), connexin-43 (Cx43) and connexion-45 (Cx45), where Cx43 is the most prevalent in ventricular myocytes. Studies carried out in different animal models prove that differences exist in connexin abundance and location between animal species. Also, a heterocellular GJ have been observed between cardiomyocytes and fibroblasts in the heart, although this is still a debatable subject (Camelliti, Borg, and Kohl 2005). Interestingly, Cx43 has been found to be able to connect to actin fibers and that promotes cell ‘spreading’ (Chen et al. 2015), suggesting a link between junctions and mechanical forces. Further studies demonstrated that apart from the function of ions and small molecules transportation, GJ can also play a role in cytoskeleton-dependent cell migration and polarization (Francis et al. 2011). During heart failure the first structures which disappear from the ID are GJs, eventually leading to decreased conduction velocity and ventricular arrhythmia (Li, Patel, and Radice 2006).

1.8.4 Gap junction formation
GJ are relatively dynamic structures. The main protein of GJ, Cx43, is constantly cycling between GJ and the cytoplasm. Single Cx43 molecules translocate from the endoplasmic reticulum to the Golgi apparatus with the help of chaperon protein such as GJA1-20k (Epifantseva and Shaw 2018). It has been found that half-life of a Cx43 molecule is 1-1.5 hours whereas time spent at the membrane was less than 2 hours (Smyth and Shaw 2012). At the GJ site six connexins oligomerize to form a hemichannel. Then it is packed into vesicles and loaded onto microtubules to be delivered to the ID. MT are indispensable molecular traffickers as they are constantly being polymerized and depolymerized and span across the whole length
of the cell. It has been shown that Cx43 transportation to the specific membrane sites is regulated by two microtubule-associated proteins, EB1 and p150GLUED. While at the plasma membrane these proteins were found to attach first to N-cadherin (Shaw 2014). This shows that AJ must be assembled prior to GJ at the ID. Further investigations on the Cx43 membrane trafficking have shown a direct role of filamentous actin in Cx43 delivery by non-sarcomeric actin immunoprecipitated with the GJ proteins. Also, both molecules were found by immunofluorescence to localize in the same areas in the nucleus as well as overlap in the ID area. However, traffic of the loaded proteins along actin filament does not follow the same pattern as MT-anchored traffic (Basheer et al. 2017). To follow up this alternative Cx43 cargo delivery and its interaction with microtubules more research is necessary to get more insight in to mechanism of F-actin-dependent GJ protein delivery.

1.8.5 ID as a joint complex
Apart from the three well-established protein complexes (AJ, GJ, desmosomes) sodium channels are also present at the intercalated discs (ID). Recent advances in studying cardiac coupling have challenged the current view on ID as a collection of separate junctional structures. It was recently found that proteins such as zonula occludens-1, ankyrin-G and synapse-associated protein play multiple roles in all adherens junctions, gap junctions, desmosomes and sodium channels at the cell-cell interface. Therefore, this suggests that ID functions as an integration unit and all the ID constituents rely on each other in order to maintain the electrical and mechanical communication between two cardiomyocytes (Agullo-Pascual, Cerrone, and Delmar 2014).

In the experiments with an engineered muscle tissue, stiffening of the surrounding ECM was shown to induce cell-cell coupling disassembly, which is compensated by the enhancement in the cell-substrate adhesion. In the myocardial infarction (MI), significant fibrosis and tissue stiffening is observed (Conrad et al. 1995). Additionally, higher integrin expression at the focal adhesions and cell-cell uncoupling is also a hallmark of cardiomyopathies (Dekker et al. 1998; Chen et al. 2016).

These experimental findings indicate that mechanical properties of the surrounding matrix (increased YM for example) can affect cell-cell coupling by triggering a compensatory mechanics via FA to maintain the mechanical load. This rises several questions. What is happening at the cardiomyocyte IDs during disease? Does the ID work as a one integral
structure? Can we invent the treatment techniques which will help to maintain the edge-to-edge coupling of myocytes during disease?

1.9 The role of fibroblasts in the heart
Fibroblasts are the most common cell type present in the connective tissue of all vertebrate organisms. This cell type of mesenchymal origin has been shown to be highly engaged in the heart formation at the embryonic stage. Proepicardial organ is a temporary structure that guides cells to different cardiac lineages. During transition of the proepicardium to the epicardium cells differentiate and therefore proepicardium is thought to be the main source of cardiac fibroblasts (Moore-Morris et al. 2016). Previously it has been thought that fibroblasts constitute the most abundant cell type in the whole heart, but recent studies uncovered that fibroblasts make up only 20% of the total heart cell number (Pinto et al. 2016). Fibroblasts are elongated, sparse cells with one nucleus in the middle. This cell type do not passively connect to the ECM, they are intimately involved in the ECM synthesis, maintenance and degradation by metalloproteinases (Spinale 2007). Apart from ECM formation, fibroblasts release a set of cytokines and growth factors that are involved in the immune response during heart injury (Kendall and Feghali-Bostwick 2014).

ECM contains multiple components: collagen, glycoproteins, proteoglycans, proteases, growth factors and cytokines (Frantz, Stewart, and Weaver 2010). It is acting as a scaffold and forms a milieu for cardiomyocytes and other cell populations in the heart. The formation of collagen matrix starts at the fetal developmental stage (Lockhart et al. 2011). During that time cardiomyocytes connect to ECM via integrins. Each muscle fiber becomes wrapped in a fine layer of endomysial connective tissue. To perform their functions, fibroblasts form a cellular network by connecting to each other and to the myocytes. By connecting directly to the ECM fibroblast can transfer mechanical force generated by the ECM to the cardiomyocytes.

1.10 Fibroblast alteration during disease
In a healthy heart, fibroblasts (FBs) remain in a steady state and the turnover of the ECM is minimal. Following an injury such as myocardial infarction (MI), hypoxic cardiac myocytes cease to contract and undergo cell apoptosis. The mechanical properties of the native ECM change, causing disintegration and temporary decrease in the tissue YM (Hegarova, Malek, and Kautzner 2016). Since adult cardiomyocytes are terminally differentiated and do not have
the ability to regenerate or proliferate, the loss of cells is replaced by a different cell type, usually fibroblasts, thus the regional myocardium mechanical properties change (Shinde and Frangogiannis 2014).

One of the hallmarks of myocardial remodeling is fibroblast phenotypic switching to myofibroblasts (MFBs), recognized by presence of α-smooth muscle actin (α-SMA). The fibroblast-to-myofibroblast transition is promoted by soluble growth factors such as transforming growth factor β1 (TGFβ1), platelet-derived growth factor, basic fibroblast growth factor, catecholamines, angiotensin II and mechanical cues (Kendall and Feghali-Bostwick 2014). Thirty days after MI, several cell types are present at the injury site such as endothelial cells and macrophages. Cytokine and growth factor secretion from these cells initiates MFB proliferation, mobility and enhanced collagen deposition and MMP production. In the MI, FB from the surrounding areas infiltrate the injury site and start to proliferate and deposit collagenous matrix, forming a scar tissue that changes the contractile and electrophysiological properties of the area (Czubryt 2012). At the same time the production of interleukin-1, interleukin-6, tumor necrosis factor by fibroblasts themselves stimulate even more MMP activation and incites the remodeling process (Liu, Sun, and Sader 2006). Additionally, newly formed actin stress fibers in myofibroblasts enable them to generate a stretching force that can be further transmitted to the ECM and modify the mechanical properties of the tissue leading to formation of a dense scar tissue with high YM (Li and Wang 2011).

Inflammatory and fibrotic reactions are indispensable to the cardiac repair but frequently the inflammation has a profound effect, more that the tissue needs. In this scenario fibroblasts are more vulnerable to different mechanical stresses and this can further result in enhanced stretch and elicit further newly deposited collagen cross-linking, which can contribute to the tissue YM becoming excessively elevated (Bayomy et al. 2012; B. Li and Wang 2011).
Figure 5. Schematic showing fibroblast to myofibroblast transition. Several factors activate this process such as a variety of cytokines and mechanical stress. Myofibroblasts are distinguished from other cells by a de novo formation of α-smooth muscle actin fibers.

1.11 Mechanical and structural make up of heterocellular cardiomyocyte-myofibroblast junctions

As described in the previous chapter, in MI, cardiac fibroblasts become myofibroblasts and due to excessive stimulation, scar tissue is formed. This fibrotic process forms a substantial part of the heart tissue that is deprived of cardiomyocytes and where contraction doesn’t occur. However, the area around the scar, known as infarct border zone (IBZ), is a region where collagen-producing myofibroblast come into contact with cardiac myocytes. In the IBZ contacts between fibroblasts and myocytes are frequent (Driesen et al. 2007). This contact formation is not found in a normal state of the heart, and such coupling has a potential to change the contractile behavior of cardiomyocytes.

MFB also express a range of channels at the membrane, this includes stretch-activated channels, chloride and potassium channels, recently found members of TRP family channels as well as sodium/calcium exchangers (Baum and Duffy 2011). If the contacts between the two cell types include an electrical coupling, it can change action potential propagation.

Mechanical cell-cell coupling via AJ has been seen between two myofibroblasts and between a myofibroblast and a myocyte (Thompson et al. 2011). These connections are mediated by different cadherins depending on the cell type. Changes in the cell phenotypes can effectively
alter the junctional protein level and distribution. Both, fibroblasts and cardiomyocytes express N-cadherin as the main component of AJ. On the other hand, myofibroblasts stimulated by TGF-β1 have been found to reduce the expression of N-cadherin, which is compensated by augmented OB-cadherin levels. This protein isoform displays the ability to transmit higher tension forces as compared to N-cadherin suggesting increased mechanical stress is laid down in the injured ECM and this is affecting tissue YM (Follonier et al. 2008). Controversially, GJ have been only noted by some researchers at the heterocellular interface (Camelliti, Borg, and Kohl 2005). This point will be covered in the next chapter as this allows to present the whole picture of CM-MFB electrical coupling.

1.11.1 Cardiomyocyte-myofibroblast functional interaction

Several studies show that fibrotic scar provides mechanical integrity to the injured heart tissue, however at the same time it is responsible for the induction of arrhythmia due to the disruption of action potential propagation wave (Czubryt 2012). Several research groups proposed a theory which describes direct cardiomyocyte-myofibroblast cell coupling via gap junctions with connexins as the major players. The direct CM-MFB contact via GJ has been proven by immunostaining and dye transfer assays in vitro (Dixon and Davies 2011; Zhang, Kanter, and Yamada 2009; Kakkar and Lee 2010). However, a definitive proof of a functional electrical coupling between cardiomyocytes and fibroblasts in vivo is still lacking. To date, the presence of heterocellular GJ has been shown only in an animal model (Camelliti, Borg, and Kohl 2005). Using a dye transfer assay functional heterotypic coupling have been illustrated in rabbit sinoatrial node. The reason for such a low successful rate in picking out the GJ at the heterocellular interface is potentially caused by a small size of the junction, which results in a very low signal when using fluorescence-based techniques. Thus, despite considerable in vitro data and one elegant in vivo study the presence of GJ at the heterotypic cell coupling is still questionable. In vitro experiments provided data that have been put in a mathematical model which shows that the coupling between cardiomyocytes and non-conductive myofibroblasts leads to arrhythmia. Recent data demonstrate that myofibroblasts indeed do express ionic currents that can contribute to the heart electrical activity. Using an optogenetic method myofibroblasts have been shown to contribute to the changes in action potential at the IBZ (Kostecki et al. 2019). Therefore, it is unlikely that ECM and MFB at the IBZ form a simple
barrier to action potential but rather distort the action potential propagation. Theoretically, myofibroblasts can control cardiomyocyte electrophysiology in two ways:

a) The remodeling of cardiomyocyte ion channels and gap junctions can be induced by specific paracrine factors released by MFBs following mechanical stimulation.

b) The remodeling of cardiomyocyte ion channels and gap junctions is induced by mechanical stretch exerted on cardiomyocyte by myofibroblasts via AJ which further activate mechanosensitive channels on CMs.

The scope of each of the above-mentioned signaling events is not fully clear and still needs to be investigated.

1.11.2 Cardiomyocyte-myofibroblast dynamism

At the IBZ, cardiomyocytes come into contact with infiltrating fibroblasts. Heterocellular coupling has negative consequences on the whole heart function as it leads to conduction slowing caused in part by CM membrane depolarization. However, not only changes in the electrophysiology has been observed following the CM-FB coupling.

In an in vitro model, Cx43 has been shown to promote dynamism in CM-CM, CM-MFB and MFB-MFB contacts (Schultz, Swiatlowska, Alvarez-Laviada et al., FASEB accepted). Using Scanning Ion Conductance Microscopy (SICM) in a repetitive scan mode, CM pair expressing high levels of Cx43 has been demonstrated to have a very low level of movement, CM-MFB with a lower level of Cx43 present higher dynamism, and MFB-MFB with the lower Cx43 level display a very high level of dynamism compared to the two previous study groups. Several Cx43 modifications were used to test the Cx43-dependent dynamism primary finding. For example, downregulation of Cx43 reduces cell-cell movement, whereas, uncoupling cell pairs using heptanol doesn’t have an effect. This suggests that Cx43 presence is needed for the movement and not the formation of functionality active GJ. Oxygen deficiency is known to alter Cx43 level. Therefore hypoxic conditions were employed in the next experiment demonstrating Cx43 internalization, which significantly decreased cell-cell movement (Wu et al. 2013). Also, a mimetic peptide that has been used previously in a cryoinjury model (Ongstad et al. 2013) was employed to enhance Cx43 expression at the cell-cell interface, and this resulted in an increased CM-MFB dynamism. This study uncovered a new role of Cx43.
contributing to the dynamism of intercellular contacts (Schultz, Swiatlowska, Alvarez-Laviada et al., FASEB in revision).

1.12 Cardiac hypertrophy
Myocardial infarction studies has shown that the profibrotic factors not only target MFBs. Resident CM acquire hypertrophy phenotype when they are exposed to abnormal concentration of growth factors such as β1 transforming growth factor -β1, fibroblast growth factor, insulin-like growth factorand humoral agents such as angiotensin II, endothelin-1 and nor-/ adrenaline (Gray et al. 1998; Schultz et al. 2002; Kinugawa et al. 1999). Additionally, CM hypertrophy can be initiated by mechanical stress. It has been shown that mechanical stretch is able to elicit a hypertrophy reaction in neonatal and adult cardiac myocytes (Blaauw et al. 2010; Rysä, Tokola, and Ruskoaho 2018), which results in the change of the phenotype and gene expression, without the need of the humoral input. Initially hypertrophy is necessary as a compensatory response, but prolonged hypertrophic signaling leads to heart failure (Lazzeroni, Rimoldi, and Camici 2016). Hypertrophy is characterized by the activation of a fetal genetic program that includes re-expression of fetal isoforms of proteins (beta-myosin heavy chain) and early gene sets (c-myc) (Cox and Marsh 2014). Two types of hypertrophy have been described:

a) concentric, manifests as increased LV wall thickness as new myofibrils are added on top due to the pressure overload.

b) eccentric, results in the new sarcomere insertion in the horizontal direction leading to the LV wall thinning, caused by the volume overload (Müller and Dhall 2013).

Notwithstanding which hypertrophy type is induced, both lead to the same effect; more muscle mass generates more powerful contractions. At the same time stronger contractions stimulate mechano-transduction process both within and between cells. Hypertrophy is not unique to MI, it has been observed in other heart diseases such as hypertension, ischemic heart disease and valve diseases. It is also worth noting that during pathological conditions different factors do not act individually, but very often simultaneously, sharing common pathways.
1.13 Angiotensin as a disease factor
Angiotensin II (Ang II) is an octapeptide, part of the Renin Angiotensin Aldosterone System (RAAS), and mainly responsible for vessel vasoconstriction and holding water-salt equilibrium. Ang II is produced in several steps. First angiotensinogen is produced in the liver and then it is converted to angiotensin I by renin, a kidney-produced enzyme. Next, angiotensin converting enzyme (ACE) is needed to obtain the final product. Ang II acts through two types of receptors, angiotensin type 1 receptor (AT1R) and type 2 receptor (AT2R). Generally, AT1R plays major role in many physiological processes, as compared to AT2R.

The major part of the RAAS is located in kidney, but small, local pools of RAAS components are found in different organs (Benigni, Cassis, and Remuzzi 2010). Ang II has been shown to have multiple functions.

It has been suggested that immediate effects of Ang II differ from its long-term effects. Ang II acts on cell cytoskeleton within minutes after being added to the cells (Wesselman and De Mey 2002). In doing so, Ang II may further regulate cell division, migration, force generation, intracellular organelles organisation and cell YM.

On the other hand, long term Ang II exposure has been found to exert opposite effects. For example, chronic Ang II exposure leads to cardiac hypertrophy (Schultz et al. 2002). Further consequences include congestive heart failure, increased chance of arrhythmia and/or contribute to myocardial ischemia (Gopinathannair et al. 2009). Interestingly, short term Ang II treatment has been demonstrated to have a beneficial role on cardiac myocytes, by slowing the cell contraction (Zhang et al. 2015). Another study of acute Ang II exposure reported a new role of the octapeptide, showing that it’s able to regulate of cell YM in glomerular cells and cardiomyocytes (Leite-Moreira et al. 2009; Lee, Patel, and Park 2011).

1.14 Aims of the study
As described in Chapter 1, cell mechanobiology is a new emerging area of research that deserves great attention. A number of studies investigated different cell mechanical parameters. New methods and tools introduced in recent years have permitted better understanding of cellular mechanics. In particular, cell YM examination becomes indispensable. To date, titin and collagen fibers have been shown to be the main contributors to cardiac tissue YM. However, other structures may be equally significant, in particular myocytes plasma membrane receptors and/or cytoskeleton.
Previous work in our lab for the first time used Scanning Ion Conductance Microscopy to map cell YM in control and failing heart muscle cell. This research led to suggesting submembrane mitochondria play an important role in altered cell mechanics (Miragoli et al. 2016). Therefore, the general hypothesis of my work is:

**Mechanical properties of a single cardiomyocyte adapt to the working conditions through the coordinated work of different intracellular elements and this balance is severely disrupted in disease.**

In this work I will mainly focus on measuring cell YM using high-resolution Scanning Ion Conductance Microscopy. Major advantage of this technique is in the non-contact mode of measurement, which is a great benefit in studying living cell mechanics. In the present study I aim to:

- Investigate cellular elements that contribute to the cell YM in healthy cardiomyocytes;
- Study changes in cell YM in different disease models and explore what cell structural elements are responsible for observed alterations;
- Examine mechanical properties at the cell junctions with further analysis of protein levels and distribution;
- Investigate a novel role of Cx43 in cell-cell dynamics.
Chapter 2

General Materials and Methods
2.1 Animal cardiomyocyte models

Animal maintenance and utilization was carried out accordingly to the criteria stated in Scientific Procedures Act 1986 (ASPA, 1986), ASPA 1986 Amendments Regulations 2012, which also includes the EU directive 2010/63/EU. Different rat and mouse models were used for this project in order to investigate different aspects affecting cell mechanics.

2.1.1 Rat models

2.1.2 Neonatal rats
Animals were purchased from Charles River Laboratories. 1-3 day old Sprague-Dawley rat pups were used for the experiments.

2.1.3 Adult rat control and heart failure model
Male wild type Sprague Dawley rats (250-300g) were obtained from Charles River Laboratories (UK). Animals were kept at a 12-hour light-dark cycle, 25°C and fed with standard food at self-regulated times. Rats usually housed 4- 5 per cage.

A model of chronic Myocardial Infarction (MI) was generated by Dr. Catherine Mansfield (Lyon et al. 2009). MI was elicited by proximal coronary ligation in adult male Sprague-Dawley rats. Pressure-volume tests were carried out 16-weeks after the operation using 2-F Millar microconductance catheter (SPR838; Millar Instruments). To perform these studies in vivo animals were anesthetised with 5% isoflurane. Recorded data were analyses using PVAN 3.6 software (Millar Instruments). Blood samples for calibrating the equipment and B-type natriuretic peptide test were taken, afterwards animals were prepared for cell isolation.

2.1.4 Adult rat Partial Mechanical Unloaded Heart Model (PMUH)
Male adult Lewis rats (150-250g) were purchased from Charles River Laboratories (UK). Animals were kept at a 12-hour light-dark cycle, 25°C and fed with standard food at self-regulated times. Before the operation animals were sedated with 5% isoflurane and kept 1,5 % throughout the surgery via nosecone. Syngenic, adult rats were randomly assigned to be a donor or a recipient. Organs were transplanted in the abdominal region where ascending

Figure 6. Myocardial Infarction rat model generated by a 16-week coronary artery proximal ligation.
aorta from the incoming organs was sutured to the recipient abdominal main artery. Blood flow from the coronary arteries goes to LV via coronary sinus by pulmonary circulation. This leads to partial heart unloading as the ejected fraction by LV is only the coronary return. (Ibrahim, Navaratnarajah, et al. 2013). In this work, two types of PMUH models were studied

a) PMUH in a control rat model generated by Dr Peter T. Wright
1- healthy heart (control)
2- load-deprived heart (unloaded)

![Partial Mechanical Unloaded Model](image)

Figure 7. Partial Mechanical Unloaded rat model. A healthy heart from a syngenic Lewis rat has been transplanted to the abdomen of a second rat. Ascending donor animal aorta has been anastomosed to the recipient abdominal donor aorta. Circulating blood enters the ascending aorta and is delivered to the right atria through a network of small capillaries. Along with the heart, pair of lungs is transplanted. However, they do not oxygenate the blood. Following an 8-week period of unloading both hearts were harvested.

b) PMUH in a MI rat model generated by Sean O. Bello
1- failing heart (MI)
2- load-deprived MI heart (unloaded MI)

Model of a failing heart was anastomosed to a healthy recipient abdominal aorta. Blood circulation as described in the previous model. Following an 8-week period of unloading, load deprived heart was harvested. Unloaded MI model mimics the role of left ventricle assist device (LVAD) in human patients were damaged left ventricle does not have enough force to pump the blood due to a pathological event.
Chapter 2 Materials and Methods

2.1.5 Mouse models

2.1.6 Mouse C57BL/6 Wild Type

Male adult wild type mice C57BL/6 were purchased from Charles River Laboratories (UK). Animals were kept at a 12-hour light-dark cycle, 25°C and fed with standard food at self-regulated times. Three-month-old mice at an average weight of 24-28g were used for cardiomyocyte isolation.

2.1.7 Caveolin-3 (Cav3) Knockout (KO) Model

Animals were kindly provided by Ravi C. Balijepalli (University of Wisconsin, USA). Animals were kept at a 12-hour light-dark cycle, 25°C and fed with standard food at self-regulated times. Three-month-old mice at an average weight of 24-28g were used for cardiomyocyte isolation. Crossing floxed mice with and α-MHC-Mer-Cre-Mer mouse allowed to generate a cardiac specific KO model. Tamoxifen-inducible (3 consecutive days) Cre recombinase cuts out Exon 2 for an efficient Cav3 removal. One mg intraperitoneal tamoxifen injections were administered for 3 consecutive days. Animals were sacrificed 21 days after tamoxifen injection (Wright et al. 2018). C57BL/6 mice were used as a wild type control.

Figure 8. Left ventricular assist device (LVAD) implanted to a heart as temporary (Bridge to Transplant) or a permanent (Destination Therapy) solution. Here, connecting left ventricle and aorta.
2.1.8 MDX mouse as a Duschenne Muscular Dystrophy Model

Animals were kindly provided by David Kass from John Hopkins University, Baltimore. Mice were purchase from Jackson Laboratories, USA. C57BL/10ScSn mice with an mdx allele were crossed with Utrntm1ked utrophin KO. Utrntm1ked mutation was obtained by exon 7 disruption using PGK-Neo cassette Cre-loxP system. Obtained construct was then electroporated into F1-Kitl+ R1 embryonic stem cells. Cells with incorporated construct were injected into C57BL/6 blastocysts. DBA/2J mice were used to inbreed the strain and further obtain a homozygous colony. Animals housing conditions were the same as for the wild type. Average weight of adult mice ranged between 24 and 26g. Cell isolation protocol used as in control C57BL/6 wild type mice.

2.2 Human cardiomyocyte models

All human samples were obtained following patient consent. During the hospital-lab transportation the samples were preserved in cardioplegia buffer including Na⁺ 110 mM, Mg²⁺ 16 mM, Cl⁻ 160 mM, K⁺ 16mM, Ca²⁺ 1.2 mM, which is a common solution used during open-heart surgeries in order to protect the myocardium. Samples were placed in plastic containers and kept on ice throughout all the time.
2.2.1 Human donor
Control samples were obtained from hearts rejected from a transplant surgery due to human patient unsuitability with the approval of NHS BT with REC approval reference: 16/LO/1568.

2.2.2 Ischemic/dilated cardiomyopathy tissue sample
Heart samples from the dilated (DCM) and ischemic cardiomyopathy (ICM) heart transplant patients were obtained. Patient details were anonymized and kept secure accordingly to the human tissue act (Human Tissue Act 2004, UK). Samples were used under the approval of Brompton Harefield & NHLI Ethics Committee Biobank REC 09/H0504/104+5.

2.3 Cell isolation and culture
2.3.1 Isolation of neonatal rat cardiomyocytes and fibroblasts
Newborns were sedated using 5% isoflurane and cervical dislocation was performed with decapitation as a secondary Schedule 1 confirmation. Neonatal rat ventricle cardiomyocytes (NRVM) and fibroblasts from Sprague-Dawley neonatal rats were isolated according to Neonatal Heart Dissociation kit (Mitenyl Biotec). Briefly, hearts were harvested from the newborn rat pups and transferred to PBS. After the atria removal tissue was chopped into small pieces and incubated for 45 minutes with an enzyme mix included in the dissociation kit. After each 15min, samples were vortexed for 40sec. Next, samples were spun down RT, 5min, 10000 x g; enzymatic mix was substituted with fresh culture media. One day old cardiomyocytes and fibroblasts were taken at this step for junctional Young’s modulus experiments. The rest of the cell suspension was transferred to two T-75 tissue culture flasks (CytoOne), and incubated in 37°C, 1% CO₂ for fibroblasts to attach. 1 hour later supernatant with unattached ventricle cardiomyocytes was taken out and cells were plated on laminin-coated dishes for specific experiments. Flasks containing only fibroblasts were topped up with media and left for 7 days in culture to induce fibroblast-myofibroblast transition.

For the cell isolation media supplemented with 10% calf serum (NCS, Biosera), 1% Vitamin B12 (Sigma), 1% L-glutamine (Sigma) and 1% antibiotics (Sigma) in MI99 media (MI99, Sigma) was used. Culture conditions: 37°C, 1% CO₂ incubator.

2.3.2 Isolation of adult rat cardiomyocytes and fibroblasts from control, myocardial infarction and partial mechanical unloading model
Adult Sprague-Dawley/Lewis rats were anaesthetized (5% isoflurane) and cervical dislocation was performed as Schedule 1 killing method. Hearts were harvested and placed in a cold, oxygenated Krebs including 0.94mM MgSO4, 4.7mM KCl, 119mM NaCl, 25mM NaHCO3, 11.5mM glucose, 1.2mM KH2PO4 and 1mM CaCl₂, pH=7.4. Langendorff method was used for cell isolation. Primary wash-out solution contained higher calcium concentration in order to establish a rhythmic heartbeat. After removing the remaining blood (60-90sec) the heart was perfused 5 minutes with a low calcium solution including chelating agent, nitrilotriacetic acid (NTA) to stop the excitation-contraction coupling due to the lack of calcium ions. Hearts were perfused sequentially with Krebs+Ca³⁺ solution, low calcium solution (12-15μM CaCl₂, 120mM NaCl, 5.4mM KCl, 5mM MgSO4, 5mM pyruvate, 20mM glucose, 20mM taurine, 10mM HEPES, 5mM NTA) and collagenase (1 mg/ml), hyaluronidase (0.6 mg/ml) diluted in enzyme buffer (12-15μM CaCl₂, 120mM NaCl, 5.4mM KCl, 5mM MgSO4, 5mM pyruvate,
20mM glucose, 20mM taurine, 10mM HEPES, 150μM Ca\(^{2+}\) ) for 5 minutes, 5 minutes and 10 minutes respectively. Afterwards left ventricle (LV) was separated from the whole tissue and portioned to smaller pieces. Tissue pieces were shaking in 35°C 100% O\(_2\) for 5 minutes in the same solution, filtered through a linen mesh and incubated 8-10min. Lastly, received cell suspension was centrifuged RT, 2min, 600rpm; supernatant was removed and cells resuspended in a fresh low calcium solution.

Collected supernatant during the isolation was further used for adult fibroblast isolation. Collected sample was centrifuged at RT, 5 minutes, 10 000 x g. Next, supernatant was removed and the remaining pellet was washed three times with phosphate-buffered saline solution (PBS, Thermo Fisher), last wash was followed by a centrifugation step as mentioned before. Received pellet was resuspended in fresh DMEM (Gibco) culture media and plated for cell culture. Next day fibroblasts were supplemented with fresh media.

Isolated cardiomyocytes were plated on a laminin-coated 25-mm plastic dishes (CytoOne, Starlab) or 13-mm glass coverslips (Thermo Scientific, VWR International) and used for further experiments. For adult cardiomyocyte culture, 37°C and 5% CO\(_2\) incubation, a M199 media enriched with creatine 5mM (Sigma), taurine 5mM (Sigma), carnitine 5mM (Sigma), 1g bovine serum albumin (Sigma), ascorbate 100mM (Sigma) and penicillin/streptomycin 100mM were used.

### 2.3.3 Isolation of ventricular cardiomyocytes from adult control, Cav3 knock out and mdx mice

Cell isolations were carried out by Peter O’Gara and Navneet Bhogal. Animals were sacrificed by cervical dislocation under 5% isoflurane, anaesthetic agent. Hearts were harvested, placed in ice-cold Krebs solution (composition mentioned before) and attached to the Langendorff perfusion system through cannulated aorta. The heart was firstly washed with Krebs buffer until the wash out buffer was clear and then switched to low enzyme solution (composition mentioned before), 5 min, to stop the heartbeat. Afterwards, the heart was perfused 1 minute with proteinase XXIV (diluted in enzyme solution, 0.36mg/ml; Sigma) and 3 minutes with collagenase XXIV (diluted in enzyme solution, 1mg/ml; Sigma). Both of these enzymes cause extracellular matrix (ECM) disruption. A significant increase in flow rate indicates a successful sample digestion. Further isolation was continued with the ventricles only. Tissue was chopped into small pieces and left for a 5-10 minutes gentle agitation step. Additional step of pipetting up and down was added to further breakdown the leftover tissue. Lastly, digested sample was filtered and suspended in a 1% BSA enzyme solution.

### 2.3.4 Isolation of human donor and ischemic/dilated cardiomyopathy cardiomyocytes and fibroblasts

Prior the isolation Krebs-Ringer (KR) solution (5mM MgSO4, 5.4mM KCl, 120mM NaCl, 20mM Glucose, 10mM HEPES, 5mM NTA, 20mM taurine, 5mM pyruvate, 6.7mM nitrilotriacetic acid, pH 7) was prepared and kept in the fridge for up to two weeks.

Cell isolations were performed by Dr. Jose Sanchez Alonso-Mardones using previously published protocol (Sanchez-Alonso et al. 2016). LV tissue samples of control, ICM and DCM hearts were transferred from a cold cardioplegia to an ice-cold KR solution. Using a small razor blade fat and connective tissue was removed and muscle tissue was cut into cubic fragments of 1mm\(^3\). Fragments were then washed in 10ml of oxygenated Ca\(^{2+}\)-free KR solution at 35°C.
three times for 3 minutes, 9 minutes in total. Next, fragments were incubated in a protease XXIV solution (0.36mg/ml, Sigma; diluted in KR w/o nitrilotriacetic acid and supplemented with 200nM CaCl$_2$) with shaking for 25 minutes in 35°C. Next, the sample was filtered through a piece of gauze and the tissue leftovers were transferred to a collagenase XIV solution (1mg/ml, Sigma; diluted in KR w/o nitrilotriacetic acid and supplemented with 200nM CaCl$_2$) for three gentle shaking steps at 35°C for the duration of 15 minutes, 10 minutes and 10 minutes. After each shaking step falcon tubes were spun down for 2 minutes, at 600rpm, RT and supernatants were removed and collected for fibroblast isolation, whereas pellets were topped up with a fresh solution. The final pellet containing myocytes was used for plating. Supernatant collected throughout the isolation was used for fibroblast isolation, as follows. Combined supernatant from previous steps was centrifuged for 5 minutes, at 1000 rpm, RT, supernatant removed and the pellet washed three times with PBS; each wash was followed by a centrifugation step as mentioned before. The pellet was resuspended in fresh culture medium and plated for cell culture. Next day fibroblasts were supplemented with fresh media.

Isolated cardiomyocytes were plated on a laminin-coated 25-mm plastic dishes (CytoOne, VWR) or 13-mm glass coverslips (Thermo Scientific, VWR International) and used for further experiments. For adult cardiomyocyte culture, 37°C and 5% CO$_2$ incubation, a M199 media enriched with creatine 5mM (Sigma), taurine 5mM (Sigma), carnitine 5mM (Sigma), 1g bovine serum albumin (Sigma), ascorbate 100mM (Sigma) and penicillin/streptomycin 100mM was used.

<table>
<thead>
<tr>
<th>Transplant date</th>
<th>Pathology</th>
<th>Gender</th>
<th>Age</th>
</tr>
</thead>
<tbody>
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<td>16.03.2016</td>
<td>ICM</td>
<td>Male</td>
<td>65</td>
</tr>
<tr>
<td>23.11.2016</td>
<td>ICM</td>
<td>Male</td>
<td>67</td>
</tr>
<tr>
<td>2.05.2017</td>
<td>DCM</td>
<td>Male</td>
<td>47</td>
</tr>
<tr>
<td>23.01.2017</td>
<td>DCM</td>
<td>Male</td>
<td>49</td>
</tr>
<tr>
<td>28.01.2018</td>
<td>DCM</td>
<td>Female</td>
<td>47</td>
</tr>
<tr>
<td>5.12.2017</td>
<td>donor</td>
<td>Male</td>
<td>39</td>
</tr>
<tr>
<td>13.06.2017</td>
<td>donor</td>
<td>Male</td>
<td>48</td>
</tr>
</tbody>
</table>

*Table 1. Details of human samples from donor and failing hearts (ischemic and dilated cardiomyopathy).*
2.4 Chemical compounds

<table>
<thead>
<tr>
<th>Chemical compound</th>
<th>Concentration</th>
<th>Supplier</th>
<th>Catalogue #</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenylephrine</td>
<td>10μM</td>
<td>Sigma</td>
<td>61-76-7</td>
</tr>
<tr>
<td>blebbistatin</td>
<td>10μM</td>
<td>Tocris</td>
<td>1760</td>
</tr>
<tr>
<td>cytochalasin D</td>
<td>5,10 μM</td>
<td>Sigma</td>
<td>C8273-1MG</td>
</tr>
<tr>
<td>viblastine</td>
<td>1,2 μM</td>
<td>Sigma</td>
<td>1256/10</td>
</tr>
<tr>
<td>mβ-cyclodextrin</td>
<td>1mM</td>
<td>Sigma</td>
<td>332615-5G</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>20 μM</td>
<td>Tocris</td>
<td>1158/5</td>
</tr>
<tr>
<td>Losartan</td>
<td>100 μM</td>
<td>Abcam</td>
<td>Ab120997</td>
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<tr>
<td>SB-431542</td>
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<td>Tocris</td>
<td>1614</td>
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<tr>
<td>Y-27632</td>
<td>5 μM</td>
<td>Sigma</td>
<td>Y0503</td>
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<td>Tubastatin A</td>
<td>2 μM</td>
<td>Tocris</td>
<td>6270/10</td>
</tr>
<tr>
<td>Taxol</td>
<td>10 μM</td>
<td>Sigma</td>
<td>Y0006</td>
</tr>
<tr>
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<td>Tocris</td>
<td>0610</td>
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</tr>
<tr>
<td>Na/H exchanger inhibitor</td>
<td>1 μM</td>
<td>Sigma</td>
<td>A5585-25MG</td>
</tr>
</tbody>
</table>

Table 2. List of pharmacological compounds used in the performed studies.

2.5 Modified Scanning Ion Conductance Microscopy

Scanning Ion Conductance Microscopy (SICM) is a microscopy technique that allows to obtain the topography map of analyzed sample in a non-contact mode (Korchev et al. 1997). Also, pipette hopping mode movement allows to scan even most complex 3D structures at a high-resolution up to 20nm (Novak et al. 2009). The SICM is able to generate an image of a nonconductive sample (e.g. cellular membrane) as the working principle is based on the ion flow between electrolyte-filled sharp, borosilicate nanopipette and electrolyte-filled bath. As the nanopipette get closer to the sample the current decreases due to the reduced space for the ion flow. The nanopipette approaches the sample until it reaches a previously defined current value. The current value relies on the probe (nanopipette inner metal element) resistance (Rp), which is a combination of a nanopipette resistance and access resistance (R Ac) (Fig.10). The latter variable is a function of the sample-probe distance and the geometrical, electrical and chemical properties of the analyzed sample.

To secure a fixed sample-probe distance, that is equal to the inner pipette radius, feedback system moves the pipette up proportionate to the drop in current through the pipette when the pipette approaches the sample. By raster scanning the sample in X and Y dimensions a topographical image of the surface of the sample is obtained.
To allow imaging of samples that have complex topography, new hopping mode was introduced recently, in which at each imaging point the pipette starts approaching to the sample from the same vertical position well above the sample. The nanopipette is attached to a three x-y-z computer-controlled piezoelectric stage. Maximum x-y distance is 100 µm allowing to obtain an imaging area as big as 100 x 100 µm. However, for the purpose of this study 10 x 10 µm size images were acquired. Stage movement was controlled by a high-voltage amplifier (Piezosystem). The voltage value of 90-190mV was applied to measure the current. Ag/AgCl nanopipettes electrodes used in the circuit were connected to a headstage of Multiclamp 700B (Molecular Devices). The main scanning part was assembled to Nikon Inverted Microscope (Nikon Corporation).

A detailed method of measuring cell Young’s modulus is described in Chapter 3.
P-2000 Brown-Flaming laser puller (Sutter Instrument Company, San Rafael, CA) was used to make the pipettes from the borosilicate glass microcapillaries (inner diameter: 0.58mm; outer diameter 1.0mm) Intracel, Herts, UK). Pipette (20-30 MΩ resistance) with a bigger opening as compared to standard SICM pipettes was used in order for the mechanical stimuli application. Scanning solution including 144 mM NaCl, 10mM HEPES, 1mM MgCl₂ 5Mm KCl, 7.4 pH was used as an internal and external electrolyte-source solution.
2.6 Cell-cell dynamism analysis
Control and treated CM-CM, CM-MFB cell cultures were prepared for the cell-cell contact dynamism analysis. Using SICM, 60 µm x 60 µm images were generated. Next, a 15 µm x 15 µm cell-cell interface area was chosen and repeatedly scanned for 40 minutes in a ‘loop scan mode’. Generated images were analyzed in CellTrack software (Sakan, Ferhatosmanoglu & Coskun, 2008), where randomly allocated points around the cell-cell contact were placed. Based on the time needed to acquire image and the image pixel size, an average of the cell-cell movement (µm/min) was possible to calculate. Experiments were performed in RT. Cells were immersed in scanning solution (144 mM NaCl, 10 mM HEPES, 1 mM MgCl₂, 5 mM KCl, 7.4 pH).

2.7 RNA isolation and Real Time PCR
1) Sample preparation
Fresh or frozen cells (1 x 10⁷/sample) were used for the isolation. RNA isolation was performed according to PeqGOLD Total RNA Kit. Firstly, cells were spun down (1500 x g, 3 min) and supernatant was removed. 400 µl/sample of Lysis Buffer was added. To remove the DNA the lysate was transferred into a DNA Removing Column placed in a 2 ml eppendorf tube and spun down (12000 x g, 1 min). The centrifuged lysate from the 2 ml tube was mixed with an equal volume of 70% ethanol and vortexed. The mixture was added to a new PerfectBind RNA (PBR) Column placed in a 2 ml eppendorf tube and spun down (10 000 x g, 1 min). The flow through is disposed as the RNA is bound to the column and will be washed in the next steps. 500 µl/sample of RNA Wash Buffer was added to the PBR column and spun down (10 000 x g, 15 sec). An optional DNAse digestion step was performed.

\[
\begin{align*}
DNase I Digestion Buffer & \quad 73.5 \mu l \\
DNase I & \quad 1.5 \mu l \\
Total volume per sample & \quad 75 \mu l
\end{align*}
\]

Prepared mix was added to the PBR column and left for 15 minutes at RT. Next, 400 µl/sample Wash Buffer I was added to the column, incubated 5 minutes at RT and spun down (10 000 x g, 15 sec). 600 µl/sample of RNA Wash Buffer II was added to the PBR column and spun down (10 000 x g, 15 sec), repeated twice. Flow through was discarded and columns were spun down (10 000 x g, 2-3 min) in order to dry. 25 µl/sample of RNAse free dH₂O was added and spun down (5 000 x g, 1 minute) to elute the RNA. Samples concentration were measured using NanoDrop 8000 8-sample Spectrophotometer (ThermoFisher) and the company provided software ND 8000 1.0.0.

2) Real Time PCR (RT PCR)
One step RT PCR using SYBR Green I Qiagen kit was used, where reverse transcription and the PCR reaction are taking place in one tube. Components and used volumes/sample:

\[
\begin{align*}
2x QuantiTect SYBR Green & \quad 12.5 \mu l \\
RT-PCR Master Mix & \quad 0.25 \mu l \\
Forward primer & \quad 0.25 \mu l \\
Reverse primer & \quad 0.25 \mu l \\
QuantiTect RT Mix & \quad 0.25 \mu l \\
RNAse free water & \quad 2 \mu l
\end{align*}
\]
Chapter 2 Materials and Methods

RNA template 9.75 µl
Total reaction volume 25 µl

General reaction mix was dispensed to 96-well PCR plate (STAR LAB) and the RNA templates were added.

Rpl4 was used as a house keeping gene (Qiagen). Primer were either designed using Primer3Plus software or found in literature (Table 3) and synthetic oligonucleotides were obtained (Sigma). PCR reaction was performed in Eppendorf Realplex2 Mastercycler.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Isoform</th>
<th>Species</th>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Source</th>
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<td>rat</td>
<td>F</td>
<td>GACACTTACAGTTGGAGATTGC</td>
<td>(Makarenko et al. 2004)</td>
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<td></td>
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<td>R</td>
<td>TGTTAAATTGACTCAGGGGCTTC</td>
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<td>F</td>
<td>CTCT GGACA GCA CCACA GTG</td>
<td>(Ecarnot-Laubriet et al. 2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R</td>
<td>GTA AGCC CT CCAAACCC AAT</td>
<td></td>
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<tr>
<td>Tcap</td>
<td>rat</td>
<td></td>
<td>F</td>
<td>GTCAAGTGCTGAGGAGAGCGAG</td>
<td>designed</td>
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<tr>
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<td></td>
<td></td>
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<td>GCCAAAGAAACTCCAGGTACAC</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>R</td>
<td>TCACAGAACACCTCTGATTCC</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. List of used primer sequences for respective genes.

2.8 Protein isolation and Western blotting

Cells were washed with cold PBS and kept on ice throughout the whole experiment to avoid protein degradation. Cell lysis was performed using RIPA buffer (Sigma) and protein inhibitor (Sigma). Next, cells were scraped, sonicated for 1 min, 0°C and left for a 15 minute gentle agitation step. Afterwards, lysates were centrifuged for 15 min, 4°C, 12 000 x g. Protein concentration was assessed according to Pierce BCA Protein Assay Kit protocol (Thermo Fisher) and Beckman Coulter Detection Platform (Beckman Coulter) was used to read absorbance values.

Protein lysates were mixed with water, sample buffer (BIO-RAD), boiled for 5min, 95°C and resolved on sodium dodecyl sulfate (SDS) polyacrylamide gels. Usually 12% resolving gel (H2O, 30% acrylamide mix, 1.5M Tris pH 8.8, 10% SDS, 10% ammonium persulfate, TEMED) and 5% stacking gel (H2O, 30% acrylamide mix, 1M Tris pH 6.8, 10% SDS, 10% ammonium persulfate, TEMED) were prepared. Protein marker (PageRuller Plus Prestained Protein Ladder, ThermoFisher) was loaded on each gel to discriminate the band molecular weight. Depending on the analyzed protein, a quick 10 minute Trans-Blot Turbo System (BIO-RAD) or an overnight transfer system was used. Membranes were blocked in 5% skim milk (Sigma) or 5% bovine calf serum (BCA), both diluted in TBST and incubated with primary antibodies (Table 4) diluted in milk overnight at 4°C. Prior adding secondary antibodies, membranes were rinsed 3 times with tris-buffered saline + Tween 20 (TBST) buffer, 10 minutes each. After 1hour of HRP-linked secondary antibody incubation, membranes were again washed 3 times with TBST. ECL Western blotting Detection Reagent (GE Healthcare) or (BIO-RAD) kit was used to visualize the bands. Membranes were imaged on the Syngene G:Box.

Depending on the experiment, analyzed samples were normalized to GAPDH, α-actinin or β-actin. Image J software was used to quantify protein level.

TBST buffer: 1M Tris pH 8, sodium chloride, Tween 20%, H2O.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Dilution</th>
<th>Species</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-GAPDH</td>
<td>1:1000</td>
<td>rabbit</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>anti-β actin</td>
<td>1:5000</td>
<td>mouse</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-Cx43</td>
<td>1:1000</td>
<td>rabbit</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-α actinin</td>
<td>1:1000</td>
<td>mouse</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-β tubulin</td>
<td>1:1000</td>
<td>mouse</td>
<td>Biolegend</td>
</tr>
<tr>
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<td>1:1000</td>
<td>mouse</td>
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</tr>
<tr>
<td>anti-acetylated tubulin</td>
<td>1:1000</td>
<td>mouse</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>anti-detyrosinated tubulin</td>
<td>1:600</td>
<td>rabbit</td>
<td>Merck Milipore</td>
</tr>
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</table>

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>HRP-linked anti-mouse</td>
</tr>
<tr>
<td>HRP-linked anti-rabbit</td>
</tr>
</tbody>
</table>

Table 4. Primary and secondary general antibody list used in the Western blot.

2.9 Cell and cell-cell structural analysis

2.9.1 TetramethylRhodamine, Methyl Ester (TMRM) live staining

Cells were plated on 35-mm laminin-coated glass petri dishes (MatTek) and left for 40 minutes to attach. Cells were incubated with 100 nM TMRM diluted in the culture media for 10 min, 37°C and afterwards washed gently 3 times with physiological solution. Live cell imaging was performed at the Facility for Imaging by Light Microscopy (FILM) at Imperial College London. Zeiss Laser Scanning Microscope 780 Confocal inverted (Carl Zeiss, Germany) Plan Apochromat 63x/1.4 oil immersion at room temperature. Z-stack images were acquired at a distance 1µm. Further analysis was performed using ImageJ and a mitochondria morphology plugin (Dagda et al., 2009). Three top z-stack slices were used for the analysis in each cell. Selected cell area for analysis is further binarized and single or clustered mitochondria are calculated.

2.9.2 Immunocytochemistry

Cells were plated on laminin-coated 13-mm glass coverslips. Next, the preparations were washed with PBS, fixed for 10 minutes with 4% PFA (Sigma), 5 minutes 100% ice cold methanol or both, washed 3 times with PBS and incubated 15 minutes with 0.5% Triton-X to permeabilize cell membranes. In order to block unspecific binding coverslips were kept for 1 hour in blocking buffer, 10% horse serum (Gibco)/ 10% calf serum (Thermo Fisher) / 1% BSA (Sigma). Next, samples were incubated with primary antibodies (Table 5) for 2 hours and secondary antibodies (Table 5) for 1 hour with a triple PBS wash in between. DAPI or Hoechst 33342 was used to stain the nuclei and incubated together with secondary antibodies. Stained samples were mounted with Prolong Gold (Thermo Fisher) or Mowiol (Sigma) mounting media. Cell imaging was performed in the facility specified before, using Zeiss Laser Scanning...
Microscope 780 Confocal inverted (Carl Zeiss, Germany) Plan Apochromat 63x/1.4 oil immersion at room temperature. When necessary z-stack images were acquired at a distance 1µm. Data was collected using provided software ZEN 2011 (Carl Zeiss, Germany) and analyzed using ImageJ (Schindelin et al.2011) with additional plugins or a novel cell-cell adhesion analysis software, Junction Mapper (Brezovjakova et al., eLife in revision)

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Species</th>
<th>Supplier</th>
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<td>actin-phalloidin</td>
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<td>Sigma</td>
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<td>Hoechst 33342</td>
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<td>x</td>
<td>Thermo Fisher</td>
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<td>DAPI</td>
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<td></td>
<td>Thermo Fisher</td>
</tr>
<tr>
<td>anti- Cx43</td>
<td>1:1000</td>
<td>rabbit</td>
<td>Sigma</td>
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<tr>
<td>anti- Cx43</td>
<td>1:1000</td>
<td>mouse</td>
<td>Merck Milipore</td>
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<tr>
<td>anti-β tubulin</td>
<td>1:1000</td>
<td>mouse</td>
<td>Biolegend</td>
</tr>
<tr>
<td>anti-α tubulin</td>
<td>1:1000</td>
<td>mouse</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>anti- acetylated tubulin</td>
<td>1:1000</td>
<td>mouse</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>anti- detyrosinated tubulin</td>
<td>1:600</td>
<td>rabbit</td>
<td>Merck Milipore</td>
</tr>
<tr>
<td>anti-vimentin</td>
<td>1:3000</td>
<td>chicken</td>
<td>Thermo Fisher</td>
</tr>
<tr>
<td>anti-α SMA</td>
<td>1:1000</td>
<td>mouse</td>
<td>DAKO</td>
</tr>
<tr>
<td>anti-β catenin</td>
<td>1:50</td>
<td>rabbit</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>anti-T cadherin</td>
<td>1:100</td>
<td>mouse</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>anti- desmoplakin</td>
<td>1:10</td>
<td>mouse</td>
<td>Gifted from D.Garrod, University of Manchester</td>
</tr>
<tr>
<td>anti- desmin</td>
<td>1:100</td>
<td>mouse</td>
<td>Invitrogen</td>
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<table>
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<th>Secondary antibody</th>
<th>Dilution</th>
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<tr>
<td>Alexa Fluor 488</td>
<td>1:1000</td>
<td>donkey anti-mouse</td>
<td>Thermo Fisher</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>1:1000</td>
<td>goat anti-rabbit</td>
<td>Thermo Fisher</td>
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<tr>
<td>Alexa Fluor 546</td>
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<td>Thermo Fisher</td>
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<td>Alexa Fluor 546</td>
<td>1:1000</td>
<td>donkey anti-rabbit</td>
<td>Thermo Fisher</td>
</tr>
<tr>
<td>Alexa Fluor 647</td>
<td>1:1000</td>
<td>donkey anti-chicken</td>
<td>Thermo Fisher</td>
</tr>
</tbody>
</table>

Table 5. Primary and secondary general antibody list used in the immunocytochemistry

2.9.3 Transmission Electron Microscopy
Adult rat cardiomyocytes were fixed with 2.5% glutaraldehyde (Sigma) in 0.2M cacodylate buffer (0.2M cacodylic acid, 0.2M NaOH, pH 7.2) for at least one day in RT. Next, cells were spun down 500 rpm for 15 minutes, embedded in 2% agarose (Sigma) post-fixed with 2.5% glutaraldehyde (Sigma) in 0.2M cacodylate buffer at RT for at least one day and processed for embedding. First, agarose blocks were stained with 4% osmium tetroxide for 1 hour at RT (Sigma). Then a consecutive solutions of increasing methanol concentration were used, followed by two incubations with pure methanol, then two incubations with propylene oxide. Last, the sample was incubated in increasing concentrations of Araldite (Sigma) in propylene oxide, followed by overnight incubation in pure Araldite. Blocks of Araldite were cured at 60°C for two days.
Prepared blocks were cut in 0.1µm sections using Leica Ultracut microtome (Leica, UK) and stained with 5% uranyl acetate (Sigma) for 7 minutes and 5% led citrate (Sigma) for 5 minutes. Images were acquired using Transmission Electron Microscopy (Hitachi H7000 (Hitachi High-Technologies, Japan).

2.9.4 Cell measurement software

Cellular contacts are of great importance in tissue integrity. However, in the pathological state they undergo several structural changes. To precisely analyze the cell-cell contact composition in different control and disease models, new Junction Mapper software had been used (Brezjovakova et al., eLife in revision). This semi-automated analysis tool allows to precisely characterize non-linear cellular junctions using different parameters. To date, junction quantification was performed using two approaches, based on intensity levels, but doesn’t take into account the morphometric structure of the cell contacts. Second method requires time-consuming manual junction indication by the user. To overcome these hurdles, recently developed semi-automated software that allows to quantify a non-linear and frequently fragmented cell interface was applied in this work. Following immunofluorescent sample staining of the junctional marker and the protein of interest located at the cell adhesions, generated images were analyzed in Junction Mapper.

Cell co-cultures were fixed after specific pharmacological manipulation. Acquired immunofluorescent images were uploaded. Membrane protein staining was used as a reference to generate a skeletal map. As CM-CM and CM-MFB not always form straight junctions, those were manually adjusted where necessary. Individual cell was chosen for further quantification. Next step includes selecting the dilation number, which defines how big area at the membrane will be recognized by the software during the quantification. Cell corners were defined to indicate single junctions. Afterwards, threshold for the membrane protein and Cx43 were selected and kept constant through out all the analysis. Lastly, generated raw values were used to calculate specific morphometric characteristics.
Chapter 2 Materials and Methods

Figure 13. Schematic demonstrating step-by-step protein quantification using Junctional Mapper software.

To obtain a robust cell interface analysis, based on intensity, area and length, a variety of parameters are distinguished. Here, proteins at the CM-CM and CM-MFB interfaces were calculated using three parameters selected in the software. Detailed parameters description is present in chapter 6.

2.10 Parachute assay

In order to assess the functionality of formed gap junctions between cells, parachute assay was performed. Neonatal fibroblasts (control, 4PB/Latrunculin B treated, virally transfected) were plated on 25mm plastic dishes. Cells were incubated with 5µM Cell Tracker Orange-AM/Far Red Cell Tracker-AM (Thermo Fisher) and 1µM Calcein-AM (Thermo Fisher) for 30 minutes in 37°C, trypsinized and parachuted onto neonatal cardiomyocytes in 1:2 ratio to form heterocellular contacts. Fibroblasts were treated for 48hrs with 2µm/ml 4-phenylbutyric acid (4-PB). For the Cx43 knock down, fibroblasts were transfected with a lentiviral vector expressing shRNAs to silence Cx43 and eGFP to detect the transfected cells, prior the dye staining. Cells were co-cultured for 24 hours and next imaged using Zeiss Laser Scanning Microscope 780 Confocal inverted (Carl Zeiss, Germany) Plan Apochromat 63x/1.4 oil immersion at room temperature.

Figure 14. Schematic demonstrating parachute assay. Green-yellow fibroblasts (donors) are labelled with two dyes, Cell Tracker as a non-permeable dye and Calcein-AM as a permeable dye. Cardiomyocyte Calcein-AM-acceptor cells are depicted in green.

2.11 Viral Transduction

Prior transduction adult rat cardiomyocytes were washed twice with supplemented M199 media. Next, cells were counted and plated ~15.000 cells per 25-mm laminin-coated dishes. m-Cherry Profilin-1, m-Cherry Cofilin-1, m-Cherry Control adenoviral constructs were used. Virus MOI =200. Cells were kept in culture for 48hrs.

2.12 Statistics

All the graph and statistical analysis were performed using GraphPad Prism 5 (GraphPad Software Inc., California). Firstly, data distribution was assessed using Kolmogorov-Smirnov test. If the experiment included only two group, following the normal distribution Student t-
test was performed. If the Gaussian distribution was not observed, Mann-Whitney test was used. One-way ANOVA (parametric or non-parametric) was used if more than two groups were included in the experiment. For the parametric score, ANOVA 1-way analysis of variance and Tukey post-hoc test were used. For the non-parametric score, Kruskal-Wallis test and Dunn’s multiple comparison post-hoc test were used. Number of analyzed samples is included in the figure legend underneath each figure, in the format: number of cells/cell pairs analyzed per isolation (n) / total number of isolations (N), number of all analyzed junctions / total number of isolations (N) or total number of isolations (N). All data is presented as standard error of the mean (SEM). Significance was approved at the p-values 0.05 (*), 0.01 (**), and 0.001(**).
Chapter 3

SICM as a novel tool to measure cell nanomechanics
3.1 Introduction
Chapter 3 focuses solely on the adaptation of commonly used SICM to study cellular mechanics and its further application. In recent years, cellular mechanobiology has attracted a lot of interest. Now, it is widely accepted that the biomechanical properties play an important role in cell biology, particularly cells from the heart muscle are fully capable of sensing and generating mechanical forces (Sequeira et al. 2014). Several features contribute to the cellular response to mechanical stimuli, among them cell stiffness measured as cell Young’s Modulus (YM) has become one of the prevailing parameters studied in recent years.

As it has been shown that in different conditions, healthy and diseased states, this can change remarkably constituting an ongoing adverse modification (Borbély et al. 2005; Dague et al. 2014; Chen et al. 2018; Zile et al. 2015). YM is defined as the amount of force per unit area (referred to as stress) required to induce 100 % extension (referred to as strain) is known as Young’s modulus [YM; Pa=N/m²]. YM is effectively a measure of the resistance of the material to deformation. Alternatively, mechanical properties could be also described simply by ratio of force per unit of extension (N/m), referred to as stiffness, which is a term familiar to non-specialists, and therefore much more often used instead of YM. In fact, one can recalculate YM into stiffness and back if dimensions of the extended/compressed piece of material are known. To measure this feature at the cellular level, several different techniques emerged in the recent years. To choose one, we need to take into consideration three different parameters in order to perform an appropriate experimental study; durability of the sample, length of the experiment and the size of the sample. Most commonly used techniques for the study of cell mechanical behavior are magnetic and optical tweezers, cantilever sensing, optical stretchers, micropipette aspiration and atomic force microscopy (described in chapter 1).

One of the most widespread techniques for investigation of YM transverse alterations in cells is Atomic Force Microscopy, member of scanning probe microscopy (SPM) family. A remarkable advantage of this microscopy tool is the ability of imaging living cells at a nanoscale resolution (Li et al. 2017). However, a prominent limitation, which is physical interaction with the sample, can affect the measurement and possibly distort the sample. Scanning Ion Conductance Microscopy is another representative of SPM family, where a 50 nm lateral and 10 nm vertical spatial resolution can be reached (Korchev et al. 2000) The new hopping scan mode allows to obtain three-dimensional topography images of highly convoluted biological
samples with a high resolution up to 20 nm (Novak et al. 2009), allowing to visualize a number of noteworthy details. The working principle is based on the ion flow between the electrode, placed inside an electrolyte-filled glass nanopipette, and an electrode situated in the bath. The flow of ions through the nanopipette tip is reduced when the nanopipette tip approaches sample surface. Nanopipette position above the sample is controlled by the feedback system, while hopping from one imaging point in the x-y plane to another so that the nanopipette tip stops at a particular distance from the sample (typically tip radius away from surface), thus securing a non-contact scanning mode. Few advantages should be pointed out using this technique:

- it can image both, live and fixed cells or tissues, without the need of a special sample preparation
- there is also no concern regarding sample damage or contamination due to the non-contact working mode

All these merits make SICM an exquisite tool to study live cells with a variety of different applications. SICM has been widely used in combination with many other techniques, portraying as a very powerful tool. Smart patch-clamp technique is a combination of SICM and patch-clamp that allows to monitor the activity of ion channels localized in the different cell membrane microdomains which has been successfully applied to the study of L-type calcium channels (Gorelik et al. 2002). To study localization and function of β-adrenergic receptors, which play a regulatory role in the failing heart, SICM was combined with Förster resonance energy transfer (FRET) (Nikolaev et al. 2010). Recently, a new angular approach SICM has been developed, which allows to scan cell areas such as adult cardiomyocyte intercalated discs that had been inaccessible so far (Shevchuk et al. 2016). One of the first applications of SICM in the mechanosensitive studies on adult rat cardiac myocytes were performed by Miragoli et al., (2016). Loaded with Fluo-4-AM, calcium sensitive dye, cells were scanned to obtain the topography map and localize myocyte-specific structures. A pressure pulse was applied to chosen areas on the sarcolemma. Membrane indentation in the grove location resulted in a confined calcium activation, whereas hydrojet-dependent movement of the membrane at the crest area effected in whole cell calcium spread. On the other hand, cells isolated from a myocardial infarction rat model due to their significant structural and functional changes
generate aberrant calcium response irrespective of the hydrojet application. Together these data indicate localized mechanical properties that are lost in the pathologic phenotype.

### 3.2 SICM adaptation to measure cell Young’s modulus.

#### 3.2.1 Setup modification

On the contrary to the system used in Miragoli et al., where the hydrojet was applied only to selected points on the cell surface once the image of cell topography has been obtained, recent modification of SICM allows to map Young’s modulus of a whole area simultaneously with topography imaging (Clarke et al. 2016; Rheinlaender and Schäffer 2013; Miragoli et al. 2016).

Tubing inserted to the top part of the nanopipette holder delivers aerostatic pressure that propels the inner pipette solution forming a hydrojet mechanical stimuli. Pressure value is applied manually by a syringe throughout the whole scan at a constant 15kPa value, monitored by a pressure meter (PM1000, World Precision Instruments) (Fig. 15 A,B).
Figure 15. Scanning Ion Conductance Microscopy schematic. A) SICM imaging setup that includes Ag/AgCl electrode, feedback system, ground electrode, pressure port delivering aerostatic pressure to the nanopipette, hydrojet as a mechanical stimuli, sample and objective. B) Pressure application system including a syringe, pressure meter and a tubing system delivering aerostatic pressure. Positive pressure value is displayed on the meter screen. C) Close up of the non-contact SICM working principle in measuring cell YM. D) Exemplar topography and Young’s Modulus Maps.

Standard SICM pipettes used for imaging are around 150 nm diameter wide (~100 MΩ resistance) in order to generate a high-resolution image. However, to effectively apply a hydrojet of pressure, wider pipettes (250-350 nm) are needed, where simultaneously cell YM map and surface images can be obtained (Fig.15 C,D). Displacement of the membrane due to the pressure of pipette solution is registered by the nanopipette approaching the membrane at each imaging point.

3.2.2 Micro- and nanoscale structures

In this work, the main objective was to measure YM of adult cardiac myocytes in different models using SICM. Heart muscle cells present a high degree of variability in the cell size, an
average 110 µm in length and 40 µm in width is observed (Fig.16). As reported by Gorelik et al. (2006) distance between two neighboring crests is 2 µm. Whereas, T-tubule opening is equal 200 - 400 nm and gets more and more narrow as it gets deeper into the cell. Using high spatial resolution SICM a detailed topography map of cardiac myocytes can be generated. Each measurement is taken in the cell center in a 10 µm x 10 µm area. Within that area three crests and three z-groove regions are analyzed.

Figure 16. Scalability. Adult cardiac myocytes present a high rate of variability in terms cell size. 10 µm x 10 µm scan size (red box) is carried out in the cell centre.

### 3.2.3. Working principle based on method by Rheinlaender & Schaffer

In order to obtain a topography image, the pipette travels vertically to the sample and stops at a previously predefined reduced current amount (SP₁ = 0.7 %). Upon reaching the reduction in current, z-axis position is considered as cell height and demonstrated as topography map. (Fig.17 A). To measure the YM described in Rheinlaender and Schaffer (Rheinlaender and Schäffer 2013) positive pressure \( p_0 \) is delivered to the nanopipette and applied during imaging. After reaching the SP₁ nanopipette travels further down until the current comes to the second
reduction point, 1% current (to setpoint $SP_1 = 1\%$), simultaneously recording the change in distance $d_1$ (Fig. 17 B) and finally stops at the last setpoint $SP_2 = 2\%$ (2% from the $SP_T$, Fig. 17 C) recording the distance from $SP_T$ as $d_2$. This operation is replicated for every imaging point from a selected area. The current-distance curve between setpoint 1 and 2 in the study from Rheinlaender and Schaffer can be estimated as a straight line, where the slope is the transverse YM of the analyzed sample. Overall, the steeper the current-distance curve, the stiffer the sample.

The equation below was used to calculate the YM in Rheinlaender and Schaffer

$$E = \frac{p_0 A}{s_R \left( \frac{s_R}{s} - 1 \right)}$$

(Eq.1)

where $s_R$ is the current-distance curve slope between $SP_1$ and $SP_2$ measured at infinitely stiff surface (culture dish YM is stiff enough) as reference; $s$ is the current-distance curve slope calculated while approaching the sample surface surface; $p_0$ is the constant, positive pressure applied to the nanopipette; $A$ is a constant representing the geometry of the nanopipette.

---

**Figure 17.** Images generated during a sample scan according to Rheinlaender and Schaffer method. From the left, cell topography and two displacement views. Top image panel shows fibroblast, bottom image panel depicts cardiomyocyte.
Methodology used in this work modified the Rheinlaender and Schaffer method by calculating the slope from the equation

\[ s = \frac{1}{d_2 - d_1} \left[ \% \text{ nm}^{-1} \right] \]  

(Eq.2)

where \(d_2\) and \(d_1\) are displacements at SP₂ and SP₁ (Fig. 1). Equation 1 is then presented as

\[ E = \frac{p_0 A}{d_2 - d_1} - \frac{1}{d_{2R} - d_{1R}} \]  

(Eq.3)

where \(d_{2R}\) and \(d_{1R}\) are displacements calculated on a stiff surface (reference) for example PVC plastic or glass of the culture dish. The \(d_{2R}\) and \(d_{1R}\) values can be measured at the dish area free from cells from the same or a different dish as long as the same nanopipette is used.

### 3.2.4 Practical guide to calculate the YM in the SICMImageViewer software

In the SICMImageViewer double-clicking a topography image will open any associated displacement images recorded at higher setpoints (Fig.18). These associated files are identified by extension “…SP1t.img” and “…SP2t.img”. After opening the images, go to menu Analyze → Stiffness → Rheinlaender and Schaffer Method.
3.2.5 Correction for the effect of topography slope on Young’s modulus cell measurement

Cells forming different tissues differ from each other not only functionally but also phenotypically. The disparity is mainly due to the fulfilled function. Red blood cells are known for their characteristic biconcave shape. Roundly shaped white blood cells are able to squeeze in between tightly coupled epithelial cells during an inflammation event. Adult ventricular cardiomyocytes are present as rectangular shaped cells. Both, the internal and external cardiac myocyte structure is complex. Unlike some cells, cardiomyocyte outer membrane organization is represented by different structures. Z-grooves are grooves in the cell sarcolemma that are formed by regularly located transverse tubules (TT). TT form a network of membrane invaginations that open to the extracellular milieu via TT openings. Each TT is separated from the next one by a bulge-like structure called crest. Based on the electron
microscopy images of adult cardiomyocytes we can observe that such area is occupied by subsarcolemmal mitochondria. These membrane-embedded structures shape an uneven cell surface that potentially can affect SICM YM measurements.

It has been reported that the sample slope affects the way the ionic current drops when nanopipette approaches the sample (Thatenhorst et al. 2014). Thatenhorst et al., have shown that the effect on the current-distance dependence can be corrected simply by measuring the tip sample distance in the direction perpendicular to the inclined surface instead of along the nanopipette. Since the Young’s modulus measurement established by Rheinlaender & Schäffer (2013) relies on the current-distance relationship, the effect of slope needs to be taken into account. Otherwise measurement of irregular surface with structures of different heights may result in underestimating the YM of the sample (lower YM). To minimize the measurement inaccuracy a local slope correction parameter was introduced to the software (Fig.19).

\[ d_n = d \times \cos \alpha \]

Where \( d \) is the surface-nanopipette distance measured along z-axis; \( d_n \) is the surface-nanopipette distance measured along direction perpendicular to surface; and \( \alpha \) is the angle of the slope.

Following each scan, slope correction parameter is applied. Based on the sample topography mapping, sloppy areas are selected and further multiplied by \( \cos \alpha \). Young’s Modulus is calculated as mentioned before.

*Figure 19. Slope correction schematic for the flat and inclined sample surfaces.*
3.5 Discussion

Mechanical properties have been recognized as a major regulator of cell structure and function, thus studying these parameters became more common in recent years, as more techniques became available. Optical tweezers are probably the first tools that have been developed to measure cell mechanics and are still used on a daily basis. The working principle is based on trapping the sample in a laser beam and manipulating it using small forces as with traditional tweezers (Ashkin 1970). A major advantage is the absence of mechanical handling of the specimen during the measurement. However, the use of high intensity light effects in local heating that can modify the enzymatic reactions in the cell as well as modify the viscosity. Also, lack of selectivity is common and any particle close to the laser will be caught by the optical system, which means that not just one specimen can be analyzed at a time that in turn will affect the result (Neuman and Nagy 2008). Another known technique is micropipette aspiration that is considered as a straightforward technique to measure tension, Young’s modulus and viscoelasticity. Employed force acts in opposite way to that present in AFM. The working principle is based on tracking the movement of the samples surface while it’s aspirated inside a long glass tube that causes the specimen to extend. However, the throughput of this technique is very low as only one cell can be analyzed one at a time that can take up to 10 minutes. These two experimental techniques allow to examine mechanical properties of a whole cell and very often used for erythrocytes and neutrophils (Barns et al. 2017; Lee, Patel, and Park 2011). In order to examine more precise mechanical parameters within the cells, AFM is the most common technique currently used that allows to locally map cell YM with the topography. Sample can be analyzed at a nanometer scale by using a piezo-controlled cantilever with a tip at the end. Despite wide range of cantilevers with different degrees of softness, this technique is still not suited to very steep samples, which can cause image artefacts. Previous reports outlined slow speed as a limitation of this technique, but since then new modifications were incorporated to resolve this problem (Schitter and Rost 2008). Nevertheless, the major concern is the working mode that comes into direct contact with the sample to determine the surface contours, which can affect the sample. Big interest in cell biomechanics is the main driver of the new study techniques development. Another emerging tool is the high resolution SICM that can precisely discern cell YM. It has been adapted to investigate cell mechanics by adding a pressure port to deliver aerostatic pressure.
which subsequently utilizes a hydrojet as a mechanical stimulus. The first use of SICM to measure cell mechanics was performed by Sanchez et al. using two modes. Firstly, stimulating the cell by a direct pipette contact that deforms the membrane due to a stronger impulse. Second, using a stream of solution propelled from the pressurized pipetted at a chosen time (Sánchez et al. 2007). Recently, Miragoli et al., used SICM to firstly resolve the cell topography and then investigate local YM by a hydrojet application in a selected point. However, to get more insight into the cellular mechanoproperties we introduced a new pressure application mode. In this course, topographical and Young’s modulus map can be obtained simultaneously. This technique advancement allows also to study soft samples with a nanoscale resolution and virtually no physical contact in vertical and lateral directions. Moreover, the steep sample limitation was overcome by implementing a slope correction while scanning. Nevertheless, in order to use SICM to study cell mechanics, the sample doesn’t need special preparation, but it has to be an adherent specimen, which limits the cell type that can be analyzed. Additionally, similar to AFM, the imaging size is restricted to 100 µm. Since it has been shown that cell mechanics differ between cancer and healthy cells, measuring cell YM have been proposed as a diagnostic indicator of diseased state. Study from Seifert et al (2015) compares AFM and SICM in the microvilli of epithelial cells. However, there is no published work comparing AFM and SICM in cardiac myocytes, as this is the first study to show the study of YM in these cells.

In our experiments, SICM measurements of YM in healthy and failing adult rat cardiac myocytes give values comparable with YM measurements by AFM (Lanzicher et al. 2015; Chen et al. 2018; Yoshikawa et al. 2013). Interestingly, the AFM technique gives more variability in YM values; that is perhaps due to the differences in cantilever tip shape, applied mathematical model, and experimental design. Along with this studies a new technique using an optical system has been introduced to measure these properties (Eldridge et al. 2017). Quantitative phase imaging system does not rely on cell deformability, but measures the disorder strength, parameter indicating the level of cell heterogeneity, that correlates with cell stiffness. Similarly to SICM, adherent cells do not require extra labelling protocol. Final measurements are presented as Young’s modulus profiles of each cell.

The growing body of evidence shows how dynamic are cell biomechanics. Along with the range of available tools adapted to measure the same property, variability in obtained results appear when comparing between different techniques. The reason for the observed disparity is not
only due to the difference in used tools but it’s also highly dependent on not homogenous cell membrane, different cell stage and the experimental conditions. Deciphering cellular mechanical properties carries a big potential for getting more insight into different diseases that have been shown to be mechano-dependent (Borbély et al. 2005; Hayashi and Iwata 2015). Therefore, presented work in this thesis is focused on investigating cellular mechanoproperties using SICM.
Chapter 4

Investigating cell mechanics in a single-cell cardiomyocyte model
4.1 Introduction
Chapter 4 will investigate the role of mechanical stimuli on cellular Young’s modulus (YM) at the single cell level in a control model. Cardiac myocytes isolated from left and right ventricle will be compared and further experiments will be focused on left ventricular cells as the building blocks derived from the main force generating heart unit.

Myocardium experiences considerable mechanical stress throughout its lifespan (Jacot, Martin, and Hunt 2010). As a result, cardiac tissue is very adaptive to the mechanical load. However, myocardial tissue functionality is region specific, as individual chambers perform different tasks. Left ventricle is known to be the main force generator in the heart by pumping the blood at a high pressure, as a consequence it’s composed of a bigger muscle mass as compared to right ventricle. Ventricle walls are muscular structures folded in a spiral course (Wei-Ning Lee et al. 2012). By dissecting individual heart muscle layers we don’t observe diametrically running fibers, on the contrary, muscle strands are oriented in different directions. Therefore, transverse and longitudinal directionality of YM should be examined. Longitudinal YM has received a lot of recognition mostly due to a range of available techniques, such as optical tweezers or carbon fibers (Dai and Sheetz 1995; Nishimura et al. 2006; Neuman and Nagy 2008). On the other hand, investigating cell YM in the transverse direction has been only possible to study by Atomic Force Microscopy (Sen, Subramanian, and Discher 2005; Crick and Yin 2007; Kuznetsova et al. 2007). Here, a new high resolution, contactless scanning technique has been used to explore cardiomyocyte mechanics. It has been shown that tissue YM is markedly increased in pathological conditions (Sumita Yoshikawa et al. 2013; Borbély et al. 2005; Zile et al. 2015). A number of studies also demonstrated higher YM in single cells. This topic will be investigated in more details in the next chapter. However, to study the diseased models, a basic knowledge of the cell mechanical properties from a control model is needed.

Cardiac myocytes are notably distinct cells that comprise several potential mechanosensitive elements due to the kinetic nature of the myocardium. This cell type is able to sense different magnitudes of mechanical load and efficiently respond to it in the process called mechanotransduction. Environmental mechanical cues are converted to biochemical signaling pathway which eventually exerts structural and functional alterations that serves as an adaptive feature to the applied load (Lyon et al. 2015). Next step is to dissect from the entire population of mechanosensitive elements, those proteins that additionally change cellular
YM. Sarcomere (cardiac-specific cytoskeleton), as the basic cardiomyocyte unit is considered as a focal point of mechanotransduction (Russell et al. 2010). Other cellular elements involved in the mechanosensation at the cellular level include intercalated discs, cardiomyocyte-cardiomyocyte connections, cell membrane and cardiac myocyte generic cytoskeleton that is composed of actin filaments, intermediate filaments and microtubules (Beedle et al. 2015; Skwarek-Maruszewska et al. 2009; Vermij, Abriel, and van Veen 2017; Nishimura et al. 2006; Robison et al. 2016). Cell membrane is a collection of different sarcolemma-embedded elements that include ion exchangers, channels, and receptors. Specifically, in cardiac myocytes the sarcolemma is characterized by two distinctive structures, T-tubules as transverse, cell-deep invaginations, and crest domains. Both of these structures contain previously mentioned cell-specific elements. Within the crest domain, flask-shaped modules known as caveolae are found. A role in different signaling pathways has been described in the literature (Deurs et al. 2003). However, a mechanosensitive feature has been also proposed (Nassoy and Lamaze 2012; Echarri and Del Pozo 2015).

All eukaryotic cells contain a set of proteins forming cellular scaffold that play several major roles such as intracellular cargo transportation, shape maintenance at rest and movement, protein organization within the cell and withstanding the deformation force (Alberts et al. 2002). Steady actin filaments elongation is suited to the produced and received force. Cardiac myocytes as fast contracting cells contain two pools of actin protein, force-producing sarcomeric form that is in close proximity to myosin in the sarcomere, and non-sarcomeric which connects different protein complexes within the cell (Sequeira et al. 2014). Second, the most prominent cardiomyocyte cytoskeletal contributor is the microtubular network. Despite controlling the cellular machinery in a similar manner as actin, conflicting data has been published regarding the role of microtubular network in cell mechanics (Brangwynne et al. 2006; Nishimura et al. 2006; Zile et al. 1998; Granzier and Irving 1995; Robison et al. 2016).

This chapter will test the hypothesis:

Cardiomyocyte YM correlates with cell size, and the location of the cell within the heart; this is determined by intracellular structure of individual cells.

Aims:

- To compare YM of VMs from the right and the left ventricles.
- To find out the correlation between the cell size and the YM value.
• To dissect the role of specific cellular components such as actin, microtubules and cholesterol in the maintenance of the YM, using selective inhibitors.

4.2 Materials and Methods

4.2.1 Cardiomyocyte isolation
To study cardiomyocyte YM, cells were isolated from different animal models. Adult rat cardiomyocyte isolation from left and right ventricles were performed by Mr. Peter O’Gara (Imperial College London). Control and transgenic Cav3 knock out adult mouse cardiac myocytes were isolated by Navneet Bhogal (Imperial College London).

4.2.2 Membrane mechanics studies

4.2.2.1 Scanning Ion Conductance Microscopy-based Young’s modulus examination
In order to analyze cell membrane YM, SICM technique was used to obtain sample topography. Simultaneously, a constant, 15kPa, pressure was applied during the scan to examine membrane compliance. The magnitude of air-pressure was controlled by a pressure monitor. Pressure was delivered to the pipette holder inlet through a tubing system and in the form of a hydrojet mechanical stimuli pushes cell membrane in a contactless mode. Collected information were converted to three digital projections, topography image and two current-reduced images (SP1 and SP2) that were further used to analyze the YM properties of the cell. Nanopipettes had resistance between 20 and 35 MΩ. Topography images of a 10 x 10 µM scan area were typically acquired and analyzed in a custom-made software, SICM Image Viewer (Y. Chen, Sukhorukov, and Novak 2018). During the analysis two cardiac-specific sarcolemma domains were distinguished, Z-groove and crest, as described (Gorelik et al. 2006).

4.2.2.2 Cell size-Young’s modulus correlation determination
It is well known that variability between single cardiomyocytes is very common, even between cells isolated from the same animal. We sought to determine how YM relates to the cell size. Freshly isolated adult rat cardiomyocytes were plated on a laminin-coated 25-mm dishes and left for 40 minutes to attach. Next, randomly chosen single cells were imaged using VLC media player (VideoLAN Organization, France) and a camera that is integrated in the SICM system. Following acquiring the image, each cell was scanned to determine the cell-specific YM values.
Obtained cell size images were analyzed using ImageJ (Schindelin et al. 2012). Each cell area was selected individually, and the ‘Analyze Measure’ tool was used to measure cell area. Scale was set to 1 pixel = 10µm. Next, YM value for every cell area was calculated in SICM Image Viewer, and plotted against the area size.

4.2.2.3 Cell culture-induced changes in Young’s modulus
It’s widely known that isolated cardiomyocytes undergo structural changes in culture by losing their integrity. One of the major changes in vitro is loss of T-tubules. Therefore, we next aimed to check if culture conditions affecting cell structure also project on mechanical properties. Left ventricle cells were plated on 25-mm laminin-coated dishes and left for 40 minutes to attach. Unattached cells were removed and the sample dishes were topped up with M199 supplemented culture media. Cells were kept in the incubator (37°C and 5% CO₂) for 24 and 48 hours. After the respective time of incubation cells were scanned using SICM and images analyzed using SICM Image Viewer.

4.2.2.4 Pharmacological modification of intracellular components to examine the contribution to cell Young’s modulus
To study the role of cardiomyocyte intracellular components in the determination of YM, protein-specific inhibitors were used. In particular, two major scaffolding proteins were studied, actin and tubulin. Freshly isolated left ventricle adult cardiomyocytes were plated on 25-mm laminin-coated dishes and left for 40 minutes to attach. Non-adherent cells were removed from the dish and fresh supplemented M199 culture media was added. To study the role of actin fibers in cell mechanics 5 and 10 µM cytochalasin D was added up to the culture media. Control and treated dishes were kept for 2 hours in 37°C and 5% CO₂. Cytochalasin D (Sigma) is a cell-permeable component that inhibits actin polymerization. It binds to the (+) barbed end of filamentous actin (F-actin) and prevents from binding new globular actins (G-actins) (Fig.20).
Chapter 4 Investigating cell mechanics in a single-cell cardiomyocyte model

Figure 20. Actin filament (F-actin) assembly process. Actin-binding proteins, cofilin and profilin, play a crucial role in the filament regulation. Cytochalasin D a pharmacological compound inhibits adding globular acts (G-actin) to the (+) barbed end.

To look at the role of microtubules in adult cardiomyocyte YM, culture media was supplemented with vinblastine 1 and 2 μM (Sigma). Control and treated dishes were kept for 1 hour in 37°C and 5% CO₂. Vinblastine is a commonly anti-cancer drug used in chemotherapy that slows down cell growth by preventing the assembly of tubulin dimers by binding to free tubulin (Fig. 21).

Figure 21. Cartoon showing the microtubule formation. These hollow tubular structures 25nm in diameter are formed by new a- and B-tubulin dimers assembly. Shifting from growing (A) to shrinking (B) mode is called ‘catastrophe’. Pharmacological compounds, Vinblastine (C) and Taxol (D) affect MT stability. The former binds outside and blocks protein polymerization. The latter, binds inside the tubule and stabilizes MT.

To verify whether actin and microtubules follow a common pathway, which influences YM, 5 μM cytochalasin D and 1 μM vinblastine have been added to the culture media and incubated in 37°C and 5% CO₂ for 2 hours to observe if a cumulative effect of the two drugs exists.
4.2.2.5 Visualization of tubulin structure

Microtubules were visualized using immunocytochemistry. Cells were plated on 13-mm coverslips and fixed in ice-cold methanol for 5 minutes. 10% horse serum (Gibco) diluted in PBS was used as a blocking buffer for 1 hour. Next, samples were incubated 2 hours in RT with anti-mouse B-tubulin antibody in a 1:1000 concentration (Biolegend). Following the primary antibody incubation, samples were washed 3 times in PBS and then incubated with secondary antibody Alexa Fluor 488 (Thermo Fisher) donkey anti-mouse 1:1000 and Hoechst 33342 (Thermo Fisher) 1:1000 for 1 hour. All antibodies were diluted in the blocking buffer. After the last incubation, cells were washed 3 times with PBS and 3 times with water. Samples were mounted using ProLong Gold (Thermo Fisher) mounting media. Z-stacks or single images were acquired using 63x oil lense inverted ZEISS 780 Laser Scanning Confocal Microscope at Imperial College FILM Facility. Acquired images were quantifies using ImageJ.

4.2.2.6 Adenoviral transduction

A more in-depth study on the role of actin was performed using an overexpression system of actin binding proteins, coflin and profilin. 150.00 adult rat LV cells were plated on 25-mm laminin-coated dishes. After 40 minute of incubation, unattached cells were washed off with the supplemented M199 culture media. Cells were individually transduced with adenoviruses containing m-Cherry Profilin-1, m-Cherry Cofilin-1, and m-Cherry Control adenoviral constructs at 200 MOI per cell (Kooij et al. 2016). Cells were cultured in in 37°C and 5% CO₂ for 48 hours.

4.3 Results

4.3.1. Cardiomyocytes from the left ventricle are slightly stiffer than from the right

Environmental conditions exerted evolutionary changes of the heart and chambers shape to meet specific vertebrates demands. Within the conically-shaped mammalian heart, depending on the location, myocardial chambers experience different load magnitudes that are regulated by the performed function. Right ventricle (RV) and left ventricle (LV) are both responsible for ejecting the blood from the heart to the succeeding arteries. Yet, differences are observed between the ventricles in the functionality and morphology; furthermore, blood exerts different pressure on the ventricle walls. At first, we aimed to determine whether there
is a difference in cell YM between RV and LV cells. The results show that the RV cells have a YM about ~20% lower than LV cells but this difference is not statistically significant (RV Z-groove 1.73±0.07 kPa, crest 1.71±0.087 kPa vs control Z-groove 1.99±0.25 kPa, crest 2.29±0.26 kPa, p>0.05) (Fig. 22). Cells in the LV also present local differences on the cell surface with a trend towards stiffer crest versus Z-groove, a trend that is not present in the cells from RV.

![Figure 22. Scanning Ion Conductance Microscopy YM measurement in control right and left ventricle adult rat cardiac myocytes. A) 10µm x 10µm topography [µm] and Young’s modulus maps [kPa] B) YM in Z-groove and crest domains in panel A. Data represented as mean ± SEM. Number of measured samples: 6-8 cells / 3-4 isolations. Data were analysed using non-parametric Mann-Whitney student t-test, * p<0.05, ** p<0.01, *** p<0.001.]

**4.3.2 Cell size does not correlate with the YM**

High level of variability is present between cardiac myocytes within single cell isolation. Cells do not display perfectly rectangular shapes and contain two nuclei. We sought to determine whether cell size correlates with YM in the freshly isolated adult rat left ventricular cells. Following cell preparation, images of randomly chosen cells were taken and then each cell was scanned to obtain YM value. As shown in the representative pictures, cells of different size were analyzed. However, no distinct correlation was found between cell size and YM, coefficient of determination ($R^2$) is equal to 0.0018 (Fig. 23). This result suggests there is no link between cell size and cell YM.
4.3.3 Changes in cardiac myocytes Young’s modulus after cell culture
Cardiac myocytes are known to undergo changes in in vitro conditions that lead to cell dedifferentiation. To see whether YM property changes soon after putting CM in culture or is it a rather constant trait and requires a longer period of time to undergo change, we measured YM at different times in culture. Adult rat LV cardiac myocytes were kept in culture conditions, supplemented M199 media in 37°C and 5% CO₂ for 24 and 48 hours. Obtained results show that along with the previously reported data on cell structural changes, cell YM is also altered (Poulet et al., in preparation). After 24 hours YM is increased (24 hours Z-groove 2.35±0.17 kPa crest 3.16±0.3 kPa vs control Z-groove 1.99±0.25 kPa crest 2.29±0.26 kPa, p>0.05), moreover, after 48 hours cells become even stiffer (48 hours z-groove 3.04±0.21 kPa crest 3.7±0.21 vs 24 hours Z-groove 2.35±0.17 kPa crest 3.16 kPa, p>0.05) (Fig.24). However, no change between Z-groove and crest is observed.
Figure 24. Scanning Ion Conductance Microscopy YM measurement in control and cultured adult LV rat cardiac myocytes. Freshly isolated cells were plated on laminin-coated dishes, left for 40 minutes to attach and scanned immediately. A) 10µm x 10µm topography [µm] and Young’s modulus maps [kPa] B) YM values in Z-groove and crest domains in panel A. Data represented as a mean ± SEM. Number of measured samples: 6-8 cells / 3-4 isolations. Data were analysed using non-parametric ANOVA test, * p<0.05, ** p<0.01, *** p<0.001.

4.3.4 Cardiomyocyte generic cytoskeleton as a cell mechanics-balancing network

Cardiac myocytes are the building blocks of the heart, the most dynamic organ in the whole body. Therefore, load-dependent machinery needs to adjust to a highly changeable environment. Here, two major generic cytoskeletal components of cardiomyocytes have been studied.

4.3.4.1 Actin filaments contribute to cardiomyocyte Youngs modulus

Adult rat LV cardiomyocytes were incubated with cytochalasin D, which inhibits actin filament polymerization. This drug is widely used in different actin-focused studies. Two concentrations, 5µM and 10µM were tested. Control and treated samples were incubated for 2 hours in supplemented M199 culture media in 37°C and 5% CO₂. SICM analysis show that both treatment conditions affect cell YM, significantly softening the cells with 5 µM cytochalasin D already decreasing cell YM by 33 % (5µM CytD Z-groove 1.7±0.16 kPa, crest 2.01±0.16 kPa vs 10µM Z-groove 0.94±0.17 kPa, crest 1.25±0.23 kPa vs control Z-groove 2.6±0.27 kPa, crest 3 ±0.78 kPa, p<0.05) (Fig.25). No difference between Z-groove YM has been observed.
Figure 25. Scanning Ion Conductance Microscopy YM measurement in control and cytochalasin D-treated adult rat cardiac myocytes. Control cells were kept in culture for 2 hours, respective to the pharmacological treatment. Cells were treated with 5 and 10 µM for 2 hours. A) 10µm x 10µm topography [µm] and Young’s Modulus maps [kPa]. B) Graph representing values in Z-groove and crest domains in panel A. Data represented as mean ± SEM. Number of measured samples: 6-8 cells / 3-4 isolations. Data were analysed using non-parametric ANOVA test, * p<0.05, ** p<0.01, *** p<0.001.

To obtain a bigger picture on the relationship between actin cytoskeleton and cell YM, this filament network has been remodeled. Actin assembly is controlled by two indispensable proteins, profilin that adds up new globular actin at the barbed (+) end of a filament, whereas cofilin, disassembles actin filaments from the pointed (-) end (Figure 20). Adult rat LV cells were transduced with adenoviral constructs: m-Cherry Profilin-1, m-Cherry Cofilin-1 and m-Cherry Control. Following transduction, cells were kept in culture for 48 hours and then YM was measured. According to the obtained results regulation of actin dynamics by profilin overexpression significantly increased cell YM (Profilin Z-groove 4.37±0.4 kPa, crest 4.87±0.43 kPa vs transduced control Z-groove 2.25±0.18 kPa, crest 2.11 ±0.77 kPa, p< 0.001) (Fig.26). Unexpectedly, actin disassembly by cofilin overexpression did not change cell YM (Cofilin Z-groove 2.34±0.36 kPa, crest 2.4±0.33 kPa vs 2.25, p>0.05). In addition, no difference was found between Z-groove and crest.
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Figure 26. Scanning Ion Conductance Microscopy YM measurement in 48 hour control and transduced adult rat LV cardiomyocytes. Control cells were transduced with m-Cherry empty virus and kept in culture 48 hours, respective to the viral over expression. Cells were incubated with the adenovirus for 28 hours. A) 10μm x 10μm topography [μm] and Young’s modulus maps [kPa]. B) Graph representing YM values in Z-groove and crest domains in panel A. Data represented as mean ± SEM. Number of measured samples: 6-8 cells / 3-4 isolations. Data were analysed using parametric ANOVA test, * p<0.05, ** p<0.01, *** p<0.001.

4.3.4.2 Microtubular network regulates myocyte Young’s modulus

Next, adult rat LV cells were tested on the role of microtubules in governing cell response to mechanical stimuli. Vinblastine, a specific microtubule polymerization inhibitor was used. Cells were incubated with the compound at two concentrations, 1 μM and 2 μM for 1 hour in supplemented M199 culture media in 37°C and 5% CO₂. Microtubule disrupting agent shows a gradual decrease of cell YM (1μM Z-groove 2.16±0.27 kPa crest 2.28±0.27 vs 1 hour control Z-groove 2.77±0.4 kPa, crest 2.72±0.33 kPa, p>0.05) reaching a marked reduction at the concentration of 2 μM (Z-groove 1.26±0.3 kPa ±0.11 kPa, crest 1.19±0.11 kPa vs 1 hour control Z-groove 2.77±0.4 kPa, crest 2.72±0.33 kPa, p<0.001) (Fig.27 A,B). No differences between Z-groove and crest was indicated.
Figure 27. Scanning Ion Conductance Microscopy YM measurement in control and vinblastine-treated adult rat LV cardiac myocytes. Control cells were kept in culture for 1 hour, respective to the pharmacological treatment. Cells were treated with 1 and 2 µm for 1 hour. A) 10µm x 10µm topography [µm] and Young’s modulus maps [kPa]. B) Graph representing values in Z-groove and crest domains in panel A. C) Representative confocal micrographs, control, 1uM and 2uM vinblastine stained with anti-B tubulin antibody (green) and Hoechst 33342 to illustrate nuclei (blue). Scale bar 10uM. Data represented as as mean ± SEM. Number of measured samples: 6-8 cells / 3-4 isolations. Data were analysed using non-parametric ANOVA test, * p<0.05, ** p<0.01, *** p<0.001.

To further investigate microtubule dynamics, we assessed the polymerized β-tubulin network based on immunocytochemistry. Quantification results indeed demonstrate that after 24 and 48 hours the tubulin protein level is enhanced (Fig.28). This result also matches the increased cell YM in an in vitro conditions (see chapter 4.3.3).
4.3.5 Caveolae-dependent cell Young’s modulus
Cardiac myocytes apart from two distinct sarcolemma domains, Z-groove and crest, contain cholesterol-rich microdomains known as caveolae (Fig.28 A). These surface Ω-shaped pits contain a number of receptors and channels such as L-type calcium channels, Na⁺ channels and Na⁺/Ca²⁺ exchangers (Balijepalli and Kamp 2008). Specifically, in cardiomyocytes these elements are hold together by a scaffolding protein caveolin 3 (Cav3) (Wright et al. 2014). Being a reservoir for different protein complexes caveolae play a multifunctional role in cardiac cells. Interestingly, previous research links caveolae to mechanosensitive signaling pathways (Nassoy and Lamaze 2012; Echarri and Del Pozo 2015). In order to explore a potential role of caveolae in cardiomyocyte mechanics, adult rat LV cells were treated with 1mM methyl-β cyclodextrin (MβCD) to remove membrane cholesterol and cholesterol-rich
caveolae (Fig. 29 A, B). Cells were exposed to the pharmacological treatment for 30 minutes in supplemented M199 culture media in 37°C and 5% CO₂. Significant increase in cell YM has been observed following the treatment (MβCD Z-groove 3.59±0.21kPa, crest 4.62±0.27kPa vs control Z-groove 1.84±0.15, crest 2.13±0.17 kPa, p<0.001) (Fig. 29 C, D).

**Figure 29.** Cell YM following cholesterol depletion. Scanning Ion Conductance Microscopy measurement in control and cyclodextrin-treated adult rat LV cardiac myocytes. Cells were treated with 1mM for 30 minutes. A) Representative electron microscopy images of control versus cholesterol-depleted cells. B) Representative immunofluorescent images of control and cyclodextrin-treated cells stained for actin. Scale bar 10 µm. C) 10µm x 10µm topography [µm] and Young’s modulus maps [kPa] D) Graph representing values in Z-groove and crest domains in panel A. Data represented as mean ± SEM. Number of measured samples: 6-8 cells / 3-4 isolations. Data were analysed using non-parametric Student t-test, * p<0.05, ** p<0.01, *** p<0.001
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MβCD depletes membrane cholesterol including disruption of caveolae. In addition, to verify whether caveolae contribute to cell mechanics, a cardiac-specific Cav3 knock out mice were used. Adult cardiomyocytes were isolated from the hearts of these mice and YM was measured. Based on the obtained results genetic removal of caveolae only significantly increases cell YM in the Z-grooves, but not at the crest (Cav KO Z-groove 2.59 ± 0.16kPa, crest 3.05± 0.17kPa vs control Z-groove 3.48± 0.26kPa, crest 3.7 ± 0.29kPa, p<0.05)(Fig. 30).

**Figure 30.** Scanning Ion Conductance Microscopy YM measurement in control and Cav3 KO mouse. Both cell types were plated on laminin-coated dishes, left for 40 minutes to attach and scanned immediately. **A)** Representative immunofluorescent images of control and cyclodextrin-treated cells stained for actin. Scale bar 10µm. **B)** 10µm x 10µm Young’s modulus maps [kPa]. **C)** Graph representing Young’s modulus values in Z-groove and crest domains in panel A. Data represented as as mean ± SEM. Number of measured samples: 6-7 cells / 3 isolations. Data were analysed using non-parametric Student t-test, * p<0.05, ** p<0.01, *** p<0.001.
4.4 Discussion
In this chapter I show that if polymerization of either actin or microtubules was inhibited, cell YM decreases. Whereas, cholesterol depletion demonstrates an opposite effect. Removal of Cav3 leads to a drop in YM, but only in z-groove.

4.4.1 Analysis of YM dependence on cardiomyocyte size and localization within heart tissue.
Adult ventricle rod-shaped cardiac myocytes present a high degree of size variability within the same population. Correlation studies on the size and YM have shown no association between these two parameters. Moreover, available literature to date has not reported the comparison of YM between adult cardiac myocytes from different ventricles. Therefore, here I tested the differences in the mechanical properties of the region-specific cardiomyocytes. I showed that cells from the RV display lower YM as compared to the LV (Fig. 22). However, despite a ~20% lower values, the observed changes are not significant possibly due to the variability between ventricular myocytes. However, specialized complexity in the geometry of the heart has been developed to efficiently deliver blood to the body. Functional heterogeneity among all four chambers is a well-known phenomenon (Molina, Heijman, and Dobrev 2016). Interestingly, recent work from our group shows differences in cardiomyocyte contractility following the β2-adrenoceptor stimulation between ventricular myocytes the apex and the base of the heart (Wright et al. 2018). Further work on other mechanical properties is needed to obtain a wider view on the mechanical properties of region-specific cardiac myocytes. Next, pharmacological treatment was employed to show specific contribution of each cellular element to assess the impact exerted on cell YM. As a matter of fact, all analyzed cellular components in this work that were tested by pharmacological manipulation demonstrated alterations in cell YM.

4.4.2 The effect of cell culture on cardiomyocyte YM
In this section I aimed to evaluate if culture time changes cardiac myocytes YM in an in vitro model. Initial investigations have shown that YM is already affected after 1 hour of cell culture and continues to increase after 48 hours. This could be explained by the modulation that occurs in cells isolated from their physiological environment. Data from our lab have shown a significant drop in the T-tubule density during culture(Gorelik et al. 2013). Performed
experiment shows that not only YM is a dynamic parameter but also demonstrates that each chronic pharmacological treatment will require its respective control of the same culturing time.

4.4.3 Effect of actin cytoskeleton on cardiomyocyte YM

The findings here show that disrupting actin fibrillar network by cytochalasin D, which binds to the barbed end of actin filaments, reduces cell YM by 35%. This suggests that actin is an important contributor to cardiomyocyte mechanical properties (Fig. 25). It has been shown in different cell types such as fibroblasts, carcinoma cell lines, and neutrophils that actin contributes to cell mechanics using actin-targeting drugs (Ting-Beall, Lee and Hochmuth 1995; Xuan et al., 2018, Ujihara et al.,2012). Actin is present in 6 different isoforms, but not all are found in the heart muscle cells. Cardiomyocyte ultrastructure is formed by two types of cytoskeleton. A generic network is formed by γ- and β-actin as structural component, which is present at cell-cell contacts, z-discs and costamers (Balasubramanian et al. 2010). Whereas, a cardiac-specific cytoskeleton is formed by α-actin as a part of the sarcomeric contractile unit. Therefore, by using actin targeting drug, assembly of all actin isoforms is inhibited. This is also confirmed by results as both, Z-groove and crest YM went down, maintaining the same domain YM relation as in control cells. Actin cytoarchitecture is controlled by a set of specific actin-binding proteins (Lee and Dominguez 2010). In order to build on previous findings, profilin and cofilin have been virally overexpressed to further investigate actin dynamic influence on cell mechanical properties. In our experiments, profilin upregulation, that affects G-actin binding to filaments, significantly increases cardiomyocyte YM. On the other hand, overexpressing cofilin, which disassembles actin filaments, did not change cell YM (Fig. 26). Kardos et al., (2009) showed that profilin and cofilin determine the speed of adding new G-actins. Cofilin-actin interaction results in a more packed ATP binding site that is less accessible. An opposite outcome is observed at the profilin-actin binding region, where a more open and accessible ATP pocket has been reported. These results show that a specific amount of actin is needed to maintain the mechanical properties of myocytes.

4.4.4 Microtubules impact on cardiac myocyte YM

Similarly to actin, microtubules also take part in the cytoskeletal organization. Colchicine is a pharmacological compound commonly used to target microtubular network, but in addition
to its main effect on microtubules, a potential effect on calcium current density and intracellular transient have been suggested (Kerfant, Vassort, and Gómez 2001). Also, dimers of colchicine are shown to activate adenyl cyclase as a potential G protein analog (Gómez, Kerfant, and Vassort 2000). Therefore, to investigate the role of MT network and the effect of the removal of the cytoskeletal component, I used the microtubule-specific drug vinblastine, an alkaloid that also inhibits tubulin polymerization (Stanton et al. 2011), resulting in a softening of the cell. The complex structure of microtubules spans the entire cell cytoplasm and it is thought to provide cellular highways to deliver proteins to their destinations or acting as permanent pit stops for molecule allocation (Watanabe, Noritake, and Kaibuchi 2005; Basheer et al. 2017). However, microtubular contribution to cell mechanical properties have not been fully explored. One of the first experiments on the role of microtubular network in cardiac mechanical surrounding was shown by George Cooper. His seminal work in this field has greatly contributed to our understanding on the role of this complex network in myocardium stiffness and visco-elastic properties (Tsutsui, Ishihara, and Cooper 1993; Harris et al. 2002; Zile et al. 1999). In an end stage of the failing heart a densification of microtubules is observed that affects cardiomyocyte contractility. Using colchicine this effect is ceased as shown in animal models. However, throughout all these years a list of studies has been published showing contradictory results. Therefore, current view on the role of microtubules in cell mechanics is still debatable. Nishimura et al., (2006) investigated two aspects determining cardiomyocyte mechanics, cellular and shear stress mechanics in a control, colchicine-treated and taxol-treated myocytes (increase of microtubules). According to the obtained results, only the longitudinal direction of the shear stress deformation has shown difference in YM. Interestingly, no change in transverse YM has been observed following the shear stress application. Similar results were obtained by Lammerding presenting MT anisotropy (Lammerding et al. 2003). Increased tubulin protein level in different cardiac diseases gained a lot of interest among different research groups, ultimately trying to find a link between enhanced MT level and mechanical stress relation. A more detailed discussion focusing specifically on the role of MT network in the pathological context will be covered in chapter 5. However, findings in this chapter show a significant impact of MT on the transverse YM in control heart muscle cells. Also, in the cell culture an increase in cell YM as well as microtubule staining is observed (Fig. 27), further suggesting the role of microtubules in cardiac mechanics.
4.4.5. The effect of lipid bilayer on cardiomyocyte YM

Equally to actin and microtubules, cell lipid membrane plays a major role in cell mechanics as it acts as a cell protecting barrier from the external environment.

To target lipid rafts on the sarcolemma we used methyl-β-cyclodextrin (MβCD) which is the most commonly used cholesterol sequestering compound (Yancey et al. 1995). Adult rat ventricular myocytes subjected to a MβCD treatment for 30 minutes exhibit higher YM (Fig. 29).

Studies of cell mechanical properties (YM) after cholesterol depletion were performed in early 2000s in aortic epithelial cells and fibroblasts (Hissa et al. 2013; Byfield et al. 2004), showing surprising results. Cholesterol removal was predicted to decouple sarcolemma from the underlying cytoskeleton and soften the cells, however these studies indicated increased YM. Cell lipid bilayer containing several complexes embedded within the membrane allowing to pass through differently charged atoms, proteins and molecules is tightly connected to cell cytoskeleton (Cooper 2000). For the first time Kwik et al., (2003) suggested that the increased YM results were due to the submembrane F-actin cytoskeletal network strengthening following cholesterol depletion, rather than cholesterol depletion itself. Further work showed that disrupting actin filaments impeded increased membrane YM after the removal of cholesterol (Byfield et al., 2004; Sun et al., 2007). Moreover, as suggested by recent studies this stiffening effect is possibly due to actin-binding proteins located in the membrane domains. Following cholesterol depletion these proteins relocate in the sarcolemma, forming new connections with the membrane and the cytoskeleton (de Oliveira Andrade 2016). A very recent study from Zhang et al., (2019) has also shown that cholesterol depletion results in YM increase in chinese hamster ovary cells, confirming the previously published results in other cell types (Zhang, 20019).

4.4.6 The role of caveolae in cardiomyocyte YM

The main goal in this paragraph was to investigate the plasticity of the membrane to accommodate mechanical cues, governed by caveolae microdomains. In this work, a cardiac-specific Cav3 knock out mouse has been used to investigate the effect on cell YM. Obtained results show increased YM in the Z-groove domain but not in the crest in the Cav3 cardiac
deficient mouse (Fig. 30). Caveolae are membrane-embedded invaginations that comprise a number of different protein complexes important for the cardiomyocyte function. Their flask shape is tightly regulated by the membrane tension as they flatten during stretch and come back to native shape in a steady state (Sinha et al. 2011). Therefore, it has been proposed that these microdomains may also orchestrate cardiac mechanosensation (Echarri and Del Pozo 2015; Nassoy and Lamaze 2012). A common technique to image and quantify the flask-shaped structures is electron microscopy as shown in Fig.28 A. (Burton 2017, Ariotti 2014, Wright 2018). In the heart muscle cells these microdomains maintain their Q-shape due to the expression of caveolin-3, cardiac specific caveolae-associated protein. Two other caveolins -1 and -2 are widely present in different cell types such as smooth muscle cells, epithelial cells, adipocytes and fibroblasts, but their presence in adult cardiac myocytes is still a matter of debate. (Balijepalli and Kamp 2008). The loss of Cav-2 has been rescued by the re-expression of Cav-1 in epithelial cells (Davalos et al., 2010). However, such mechanism cannot be proposed in adult cardiac myocytes due to the contradictory results on the presence of other caveolins. Therefore, the non-significant change in the Cav3 KO crest domain is possibly due to the low number of cells.

Future work will need to focus on whether caveolae removal elicits similar changes in the actin cytoskeleton and actin binding proteins as seen in the cholesterol-treated cells. Role of caveolae in cardiomyocyte mechanics may be determined by their close proximity to the dystrophin-glycoprotein complex (DGC) that binds to mechanoresponsive actin cytoskeleton on one end and ECM on the other end. Some studies associate DGC with Cav3, as in a Cav3 KO mouse model DGC immunofluorescent staining is modified (Galbiati et al. 2001). On the other hand, in the animals with an absence of one of the DGC components, Cav3 staining is altered as well (Côté, Moukhles, and Carbonetto 2002). A functional connection has been shown in patients suffering from limb girdle muscular dystrophy that possess a dominant Cav3 mutation and this leads to a significant decline in α-dystroglycan (Herrmann et al. 2000). Based on the obtained data in this chapter I can suggest that caveolae play a role in cell biomechanics, which is consistent with the previous reports. Still future work is needed to investigate this potential mechanosensor further.
Chapter 5

Alterations of cell mechanics in disease
5.1 Introduction
As a continuation of Chapter 5, this section will equally concentrate on mechanical properties at a level of single cells. However, the findings from the previous chapter will be now explored in the context of different cardiac pathologies, such as Duchenne Muscular Dystrophy, Myocardial Infarction and load-deficient Myocardial Infarction in animal models as well as healthy and pathological human samples.

Current statistics show that cardiovascular diseases (CVD) is a class of diseases constituting the number one cause of morbidity and mortality worldwide (World Health Organization 2017). One of the reasons for these alarmingly numbers is the complexity of the origin and progression of cardiovascular diseases, frequently also linked to other organs (Buddeke et al. 2017; Kendir et al. 2018). Apart from standard approaches based on physiology and genetics, mechanical aspects also should be taken into consideration as the regulators of organ shape and maintenance. In fact, more and more cardiac diseases appear to have a link to disrupted biomechanics (Ohayon et al. 2008; Cheng et al. 1993; Takahashi et al. 2013). Therefore, cardiac mechanobiology is arising as a major player in the whole cardiovascular system. More detailed studies have demonstrated that friction force exerted by the blood stream is sensed by endothelial cells lining the vessels (Chatterjee 2018). Also, the onset of atherosclerosis has been linked to the changes from laminar to the oscillatory blood flow (Nigro, Abe, and Berk 2011). Cumulative evidence on the heart pathology show altered organ mechanics. Extensive studies show that prolonged changes in the mechanical load can switch from adaptive to maladaptive mechanosensitive responses (Lyon et al. 2015). Mechanical stress has been reported to activate different signaling pathways in vivo that act on the cell function (Yasukawa et al. 2001; Knöll et al. 2002). Therefore, a hypothesis of altered mechanical stimulation in the pathological conditions can be extrapolated.

Duchenne Muscular Dystrophy (DMD) is a genetic disease that due to an incomplete dystroglycan complex assembly (lack of dystrophin) losses mechanical connection between cell cytoskeleton and ECM. Most of the attention is focused on disease progression in skeletal muscles, but adverse changes have been also observed in the heart (D’Amario et al. 2017). Ex vivo model of dystrophin-deficient mice (mdx) show lower YM of the whole organ (Barnabei and Metzger 2012). However, studies on the single cell level demonstrate contradictory
results effecting in reduced or increased longitudinal stiffness in mice models (Yasuda et al. 2005; Barnabei and Metzger 2012). Still, little attention is directed to YM in the transverse direction at the cellular level. Several questions come up: is YM modified in the transverse direction? Is it different in all cardiac-specific domains or only in selected ones? It is known that heart failure with preserved ejection fraction (HFpEF) constitute for half of the heart failure incidents (Gladden, Linke, and Redfield 2014). These patients demonstrate increased organ stiffness. Several studies have been conducted to decipher the culprit/s of this notable change. Modification in the structure and formation of the ECM collagen had been observed and suggested to alter tissue mechanics as an external cardiomyocyte environment effector (Zile et al. 2015; Kasner et al. 2011). Other cardiac mechanobiology studies have stressed out titin modifications as a major player (LeWinter and Meyer 2013). Titin is present in two isoform N2B and N2BA and known by far as the biggest protein in the organism, stretching from the Z-disc to the M-line, therefore serving as a multiprotein communicator. Constituting an attractive mechanistic target due to its length and location. The role in cell mechanics of this gigantic protein has been demonstrated experimentally by modifying one of the titin regions, the I-band that has shown to be responsible for the whole protein passive stiffness (Anderson and Granzier 2012). Now it is well documented that titin contributes to cell stiffness by its specific isoform ratio and phosphorylation level. At the adult stage N2B, the stiffer form, is highly expressed as compared to N2BA, the softer form, in a human and rat model. On the other hand, titin is also targeted by three kinases: PKA and PKG that lead to reduced cell stiffness and PKC exerting an opposite effect (LeWinter and Granzier 2010). It has been reported that cardiomyocytes isolated from dilated heart patient myocardium display higher stiffness that is accompanied by a higher level of titin stiffer form (van Heerebeek et al. 2006). Other work on mechanical performance of myocardium strips from HFpEF show no change in the titin isoform ratio, but a hyperphosphorylation of PKC at the PEVK titin region, which increases passive stiffness (Zile et al. 2015). On the other hand, Borbely et al, presented that cardiomyocytes from patients with HFpEF despite showing a higher level of more compliant N2BA titin isoform than N2B, display higher stiffness which was explained by a more important role of titin phosphorylation changes (Borbély et al. 2009). Collectively, the interplay between titin modifications present a complex regulations of titin passive stiffness and all potential contributors should be closely examined in each study. Recent work form Zile et al. shown for the first time in a human patient study group that tissue stripes stiffness
change is due to the titin phosphorylation and increased fibrillar collagen, corroborated by finding the same results in animal models (Zile et al. 2012; Zile et al. 2015). By far these two elements are widely recognized as the modulators of stiffening during heart failure. However, cardiac pathology can also arise from mechanical stress alteration exerted on single cardiomyocytes that result in cell damage (Chien 1999). Myocyte Z-disc apart from a pivotal role in the proper contractile machinery assembly and cell organization has also been proposed as one of the heart muscle cells mechanotransductive pathways. Knoll et al. has introduced a mechanical stretch sensor machinery composed of three proteins titin, Tcap (telethonin) and MLP (CRP3) located at the sarcomere edges (Knöll et al. 2002). Therefore, closer examination of these elements in different diseased models with altered YM would give us more insight into cardiac mechanics.

As seen in previous chapter, a spread structure of microtubules within the cell impacts cell mechanics. Cooper’s group was the first to posed a hypothesis that microtubular network contributes to cardiomyocytes mechanics and impedes contraction in hypertrophy model (Tsutsui et al. 1994; Tagawa et al. 1997). Since then, several studies have been conducted on the sarcomere motion which led to a discrepancy in the field on whether microtubules are essential for cell mechanoproperties or not. Eventually, a dogma that microtubules do not contribute to cardiac biomechanics was established (as discussed in the Chapter 4). Although, in the failing heart a higher level of microtubules have been documented (Miragoli et al. 2016; Cooper 2006; Chen et al. 2018). Recent advances in experimental techniques have demonstrated a nodal function of microtubule post-translation modifications in cell properties. To gain more, new mechanistic understanding of these MT modifications, closer investigation in different disease models is needed as well as potential mutual relation.

Another emerging game-changer in cardiac mechanics is a widely studied hormone, angiotensin II. This octapeptide is much known for its role in vasoconstriction and salt-water homeostasis, but also exerts a wide range of indirect beneficial and negative effects (Taubman 2003). Ang II is a part of RAAS system located in kidneys but small local systems have also been found in different organs including the heart. To date, several studies proved that upregulated Ang II concentration is the initiator of different cardiac pathologies (Hernández et al. 2014; Sadoshima and Izumo 1993). Previously documented findings from Leite-Moreira, identified reduced cardiac YM at a cell level and in an ex vivo experiment following a 15-minute Ang II
stimulation (Leite-Moreira et al. 2009). Though, presented signaling pathway that potentially leads to reduced YM is still not clear.

In short, accumulation of several adverse functional, structural and mechanical changes leads to advanced stage of heart failure that poses a huge risk to patient health. The final treatment for heart failure is heart transplantation. Due to an insufficient donor number, patients may receive a mechanical circulatory assist device as a bridge to transplant or alternatively as a destination therapy when the stage of disease is examined as not advanced. By far, LVADs (left ventricular assist devices) have been considered as a recovery to the injured heart by removing the excessive load. In order to study the role of LVAD in the failing heart a partial mechanical unloaded rat model was generated that reflects the condition that takes place in human patients. Recent more in-depth studies have revealed that prolonged mechanical unloading results in heart atrophy and changes in calcium handling (Wright, et al. 2018; Ibrahim, et al. 2012; Ibrahim, et al. 2013). However, the alterations in cellular cardiomyocyte mechanics in this model are still unknown.

This chapter will test the hypotheses:

- Mechanical properties are significantly altered in the loaded as well as unloaded failing rat heart and Duchenne Muscular Dystrophy mouse model.
- Angiotensin II regulates mechanics of cardiomyocytes

Aims:

- To measure YM in the MI rat, partial mechanical unloaded rat model and Duchenne Muscle Dystrophy mouse model.
- To examine if the moderated load affects mitochondria, generic and cardiac-specific cytoskeleton using molecular biology techniques such as staining, immunostaining, PCR, Western Blot
- To investigate the effect of Angiotensin II on cardiomyocyte YM; using specific inhibitors evaluate the role of specific signaling pathways.
5.3 Materials and Methods

5.3.1 Cardiomyocyte isolation

In order to examine cell YM cells were isolated from various animal models. We compared control rats versus 16-weeks post Myocardial Infarction (MI) rats, as well as with Partial Mechanical Unloaded Heart Model, which was implemented in control and MI hearts (Lyon et al. 2009; Ibrahim, Navaratnarajah, et al. 2013). Transgenic dystrophin knockout (MDX) adult mouse cardiac myocytes were also used (Jackson Laboratory, USA). Details on the model generation and cell isolations are described in chapter 2.1.

<table>
<thead>
<tr>
<th></th>
<th>Animal</th>
<th>Body weight [g]</th>
<th>Heart weight [g]</th>
<th>Lungs weight [g]</th>
<th>Heart/Lungs weight ratio</th>
</tr>
</thead>
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<td>398</td>
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</tr>
<tr>
<td></td>
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<td></td>
</tr>
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<td></td>
<td>528</td>
<td>1.63</td>
<td>1.59</td>
<td>1.02</td>
</tr>
</tbody>
</table>

*Table 6. Body, heart and lungs weight from control and 16-weeks post Myocardial Infarction rats.*

Cardiomyocytes from human left ventricular heart tissue donor and end-stage ischemic and dilated cardiomyopathy were isolated using protocol described in Chapter 2.1.

5.2.2 Effect of dystrophin knock out on cardiac myocyte Young’s modulus

Adult mouse ventricular cells from an MDX model were plated on laminin-coated 25-mm dishes and scanned as well as YM-analyzed as described in Chapter 4.2.2.1. Culture media was removed and substituted with the scanning solution (144mM NaCl, 10mM HEPES, 1mM MgCl$_2$, 5Mm KCl, 7,4 pH).
5.2.3 Impact of the 2-hour and 24-hour Angiotensin II treatment on biomechanics in healthy cardiac myocytes

5.2.3.1 Young’s modulus determination

Adult rat left ventricular cardiomyocytes were plated on laminin-coated 25-mm dishes. Cells were left 40 minutes for attachment. Next, dead cells were washed away and samples incubated for 2 hours in 37°C and 5% CO₂ with supplemented M199 culture media and Ang II. Respective 2-hour control was prepared. Different Ang II concentrations were used to determine a dose response curve. Subsequently, different pharmacological manipulations were performed according to the schematic protocols below (Fig.31).

- **Losartan** is a specific Ang II type 1 (AT1) receptor inhibitor that blocks Ang II binding to the receptor.
- **Y-27632** is a selective ROCK (Rho kinase) inhibitor, acting on the kinase catalytic site.
- **Taxol** (paclitaxel) is a plant alkaloid that stabilizes microtubules by preventing from depolymerization due to the high-affinity binding to β-subunit (schematic in chapter 4.2.2.3)
- **SB-431542** as a selective TGFβ1 inhibitor that competitively binds to ATP kinase site.
- **Vinblastine** is a microtubule blocker as it binds to tubulin (schematic in chapter 4.2.2.3).

![Diagram showing pharmacological application protocols](image)

*Figure 31. Pharmacological application protocols used in the Angiotensin II-dependent Young’s modulus study experiments.*

Lastly, to determine potential temporal difference, a 24 hour of 20 µm Angiotensin II treatment was also performed. Following the treatment time samples were scanned and YM-analyzed as described in Chapter 4.2.2.1.
5.2.3.2 Microtubule protein quantification

After the adequate pharmacological treatment, culture media was removed, cells were washed with cold PBS and incubated ~ 1 minute with the lysis buffer and afterwards scraped of the culture dish. Samples were further sonicated 1 minute, 0°C and left for a 15 minutes gentle agitation on ice. Lastly, samples were centrifuged 15 minutes 4°C and supernatant was collected. Sample protein concentration was quantified using BCA assay. Samples were resolved using SDS polyacrylamide gel electrophoresis (SDS PAGE). 5% stacking and 12% resolving gels were prepared. Protein transfer was performed using a 10-minute Trans-Blot Turbo System for the detyrosinated and acetylated tubulin proteins with membranes blocked in a 5% skim milk. Whereas, overnight transfer was conducted for α- and β-tubulins, which membranes were incubated with 5% BCA as a blocking buffer. All primary antibodies were kept ON in 4°C. Incubated membranes were washed 3 times with TBST and transferred to secondary antibodies for 1 hour in RT. Membranes were washed 3 times with TBST and visualized. Protein bands were quantified using image J. Briefly, all bands present on a specific membrane were selected, plotted onto a histogram and peak area quantified. All the samples were normalized to respective reference gene.

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>Species</th>
<th>Supplier</th>
</tr>
</thead>
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<tr>
<td>anti-α tubulin</td>
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<td>mouse</td>
<td>Santa Bio Cruz</td>
</tr>
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<td>anti-β tubulin</td>
<td>1:1000</td>
<td>mouse</td>
<td>Biolegend</td>
</tr>
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<td>anti-detyrosinated tubulin</td>
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<td>rabbit</td>
<td>Merck Milipore</td>
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<tr>
<td>anti-acetylated tubulin</td>
<td>1:1000</td>
<td>mouse</td>
<td>Santa Bio Cruz</td>
</tr>
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<table>
<thead>
<tr>
<th>Name</th>
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<th>Species</th>
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<td>horse</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>HRP-linked anti-rabbit</td>
<td>1:1000</td>
<td>goat</td>
<td>Cell Signalling</td>
</tr>
</tbody>
</table>

Table 7. Primary and secondary Western blot antibody list used in chapter 5.2.3.2.

5.2.3.3 Examination of microtubular network organization

Beta-, detyrosinated and acetylated tubulin cell organization was examined using immunofluorescent staining in the same set of samples as Western blot analysis.

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Species</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-β tubulin</td>
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<td>mouse</td>
<td>Biolegend</td>
</tr>
<tr>
<td>anti-acetylated tubulin</td>
<td>1:1000</td>
<td>mouse</td>
<td>Santa Cruz Biotechnology</td>
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<tr>
<td>anti-detyrosinated tubulin</td>
<td>1:600</td>
<td>rabbit</td>
<td>Merck Milipore</td>
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<table>
<thead>
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<th>Secondary antibody</th>
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<td>Alexa Fluor 488</td>
<td>1:1000</td>
<td>goat anti-rabbit</td>
<td>Thermo Fisher</td>
</tr>
</tbody>
</table>

Table 8. Primary and secondary immunocytochemistry antibody list.
Cells were fixed in 4% PFA for 10 minutes, 5 minutes ice cold methanol or both. Next, samples were washed 3 times with PBS and incubated with 0.5% Triton-X. After 15 minutes cells were washed once with PBS and incubated for 1 hour in a blocking buffer (10% horse serum or 1% BSA). Next, primary antibodies were added for 2 hours, washed once with PBS and incubated with secondary antibodies for 1 hour. After the staining procedure samples were mounted using Prolong Gold. Preparations were imaged using Zeiss Laser Scanning Microscope 780 inverted Confocal. Acquired images were analyzed using Image J. Each stained sample was quantified by the intensity threshold and standardized to the respective cell size. Microtubule submembrane network was quantified based on the z-stack first top image (Fig. 3). Whereas, cell center measurements were based on 3 middle stacks. As shown in the cartoon below.

Figure 32. Immunofluorescence quantification schematic.

5.2.4 Impact of the 2-hour Angiotensin II treatment on biomechanics in cardiac myocytes from a MI model

5.2.4.1 Young’s modulus measurements
Adult rat left ventricular cardiomyocytes from an MI model were plated on laminin-coated 25-mm dishes. Cells were left 40 minutes to attach. Next, dead cells were washed away and samples incubated for 2 hours in 37°C and 5% CO2 with supplemented M199 culture media and 20 µm Ang II. Next, cell YM was measured using SICM as described in Chapter 4.2.2.1.

5.2.4.2 Examination of microtubular network organization in MI treated cells
β-, detyrosinated and acetylated tubulin cell organization was examined using immunofluorescent staining according to the protocol described in Chapter 5.2.3.3. As submembrane and central quantification has shown similar results in previous Chapter, further quantification in Image J is carried out only from the cell center.

5.2.5 Role of the mechanical load on cell mechanics in healthy and failing cardiomyocytes

5.2.5.1 Young’s modulus measurement
Adult rat left ventricular cardiomyocytes from a i) mechanically unloaded control, ii) MI and iii) unloaded MI models were isolated and plated on laminin-coated 25-mm dishes. Cells were left 40 minutes to attach. Next, cell YM was measured using SICM as described in Chapter 4.2.2.1.
5.2.5.2 Mechanical-stretch complex genes expression analysis
Cardiac myocytes respond to mechanical stretch. A complex of three proteins, titin-MLP-Tcap located at the Z-disc has been shown to be a sensory machinery responding to mechanical stimuli. Gene expression of these three proteins had been analyzed. RNA isolation and Real Time Polymerize Chain Reaction (RT-PCR) has been performed according to detailed protocol in Chapter 2.7. PCR products were detected using the SYBR Green I dye. Primer sequences used in the following experiment are stated in the table below.

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<th>Gene</th>
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<th>Species</th>
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<th>Source</th>
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<td></td>
<td>R</td>
<td>TCCTGGTTACCCACACCCCTC</td>
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</tbody>
</table>

Table 9. Primer sequences used.

5.2.5.3 Mechanosensitive proteins quantification
Western blot technique has been used to determine the level of MLP, Tcap, desmin, detyrosinated and acetylated tubulin according to the protocol in the Chapter 5.2.3.2. All five protein gels had been transferred using 10 minute Trans-Blot Turbo System and blocked in a 5 % skim milk.

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>Species</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1:1000</td>
<td>mouse</td>
<td>Santa Bio Cruz</td>
</tr>
<tr>
<td>anti-MLP</td>
<td>1:500</td>
<td>rabbit</td>
<td>gift from</td>
</tr>
<tr>
<td>anti-desmin</td>
<td>1:1000</td>
<td>goat</td>
<td>gift from</td>
</tr>
<tr>
<td>anti-detyrosinated tubulin</td>
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<td>rabbit</td>
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<tr>
<td>anti-acetylated tubulin</td>
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<td>mouse</td>
<td>Santa Bio Cruz</td>
</tr>
<tr>
<td>HRP-linked anti- mouse</td>
<td>1:1000</td>
<td>horse</td>
<td>Cell Signalling</td>
</tr>
<tr>
<td>HRP-linked anti-goat</td>
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<tr>
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<td>1:1000</td>
<td>goat</td>
<td>Cell Signalling</td>
</tr>
</tbody>
</table>

Table 10. Primary and secondary Western blot antibody list used in chapter 5.2.5.3
5.2.5.4 Mitochondria quantification

Working cardiac myocytes need to generate a great deal of energy to sustain the excitation-contraction process in the whole heart. Mitochondria are known to be the energy factories delivering ATP for that purpose. However, removing mechanical stimuli from the heart can potentially alter the energy-producing organelles as the call for enormous amounts of ATP is no longer needed. To visualize the mitochondria tetramethylrhodamine methyl ester (TMRM) a permeable dye that accumulates in active mitochondria was used. Mitochondria were analyzed in 4 groups: control, unloaded, MI and unloaded MI cardiomyocytes. Cells were plated on glass MatTek dishes and left 40 minutes to attach. Afterwards they were stained with 100nM TMRM for 10 minutes. Live cell imaging was performed using Zeiss Laser Scanning Microscope 780 Confocal inverted (Carl Zeiss, Germany). Z-stack images were acquired with a 1 µm distance. Image analysis were performed using Image J mitochondria morphology plugin (Dagda et al. 2009) (Fig.33). Two middle stacks were used for mitochondria (single and clusters) number and area analysis. The area of interest was selected on each image and it was binarized and quantified. Organelles number was normalized to cell size in each measurement.

![Image](image_url)

*Figure 33. Schematic depicting steps of mitochondria analysis. A) Uploading original image; B) Binarizing image; C) Software mitochondria selection and quantification*

5.2.5.5 Microtubular network visualization

Microtubules in control, MI and MI + vinblastine-treated samples were visualized using immunofluorescent staining according to the protocol described in Chapter 2.9.2.

5.2.5.6 Pharmacological treatment of MI cells

To investigate the contribution of actin-myosin cross bridges, cell were treated with 10 μM blebbistatin as an inhibitor of myosin heads in an actin-detached state for 20 minutes at RT. Next cells were scanned and YM analyzed. Pharmacological compound had been also added to the bath during the scanning experiment.
Cells isolated from a MI model were also treated with cytochalasin D (described in details in Chapter 4.2.2.3) in order to determine the contribution of actin cytoskeleton to cell mechanics in the failing heart. Cells were incubated with 5 μM cytochalasin D for 2 hour incubated at 37°C with 5% CO₂.

To closely investigate the role of microtubules and its post-translational modifications. MI cells were exposed to different drugs

- 2 μM vinblastine (described in Chapter for 4.2.2.4) for 1 hour at 37°C with 5% CO₂
- 10 μM Parthenolide (detyrosinated MT blocker; PTL) for 2 hours at 37°C with 5% CO₂;
- 2 μM Tubastatin A (acetylated MT agonist; Tub A), HDAC6 deacetylase inhibitor for 3 hours at 37°C with 5% CO₂

5.2.6 Investigating Young’s modulus in cells from human samples

Adult human left ventricular cardiomyocytes from donor, ischemic and dilated cardiomyopathy were isolated and plated on laminin-coated 25-mm dishes. Cells were left for 1 hour to attach. Next, samples were scanned and YM-analyzed as described in Chapter 4.2.2.1.

5.3 Results

5.3.1 Dystrophin removal decreases cell Young’s modulus

Duchenne Muscular Dystrophy (DMD) is a genetic defect represented as loss of dystrophin protein leading to muscle weakening and ultimate loss. Isolated myotubes from skeletal muscles in an DMD animal model demonstrate decreased membrane cytoskeletal YM by the resistance to indentation (Pasternak, Wong, and Elson 1995). Moreover, alteration in the myocardium in the form of myopathies and ventricular dilation also had been reported in DMD. Using a non-contact scanning microscopy technique this study sought to identify whether a difference in cell YM is present and whether cardiomyocyte membrane domains, Z-groove and crest exhibit YM disparity between each other. YM analysis revealed decreased values in the MDX mouse model (MDX Z-groove 1.44±0.11 kPa crest 1.7±0.13kPa vs control Z-groove 2.59±0.16 kPa crest 3.05±0.19kPa, p<0.001) (Fig.34). Stiffer crest versus Z-groove is noted in the healthy mouse cardiomyocytes (p<0.05). However, dystrophin removal diminishes this difference.
5.3.2 Investigating cardiomyocyte mechanics in a MI model

5.3.2.1 Failing cardiomyocytes exhibit increased Young’s modulus

Following a pathological insult in the heart, cardiomyocyte undergo several structural and functional changes. A common observation in the cardiac pathology is the disruption of the calcium handling and loss of the membrane T-tubules (TTs) structures (Yano, Ikeda, and Matsuzaki 2005; Lyon et al. 2009). This structural remodeling will affect all the proteins associated with the TTs, including L-type calcium channels, one of the main players of the excitation-contraction coupling in cardiomyocytes. On the other hand, not only cardiomyocyte electrophysiological features are modified, changes in the mechanical properties have also been noticed in the cardiac pathologies at the cellular levels (Miragoli et al. 2016; Dague et al. 2014). Firstly, YM measurement have been performed to compare the healthy and diseased left ventricular cardiac myocytes from a 16-week MI Sprague-Dawley rat model that was performed by a LAD ligation. Obtained results show a clear increase in YM in the MI model (MI Z-groove 3.5±0.2kPa crest 4.13±0.75kPa vs control Z-groove 1.99±0.25 kPa crest 2.29±0.26 kPa, p<0.001) (Fig.35). Also, a significant difference in observed between Z-groove and crest in the failing model (p<0.05).

Figure 34. Scanning Ion Conductance Young’s modulus measurement in dystrophin-deficient mouse model. A) 10µm x 10µm Young’s Modulus maps[kPa] and surface maps [µm] B) Graph representing YM values in Z-groove and crest domains. Data represented as mean ± SEM. Number of measured samples: 7 cells / 4-5 isolations. Data were analysed using non-parametric Mann-Whitney student t-test, *** p<0.001.
Figure 3.1. Scanning Ion Conductance Microscopy YM measurement in control and Myocardial Infarction rat model. A) 10 µm x 10 µm Young’s Modulus YM maps [kPa] and surface maps [µm]. B) Graph representing YM values in Z-groove and crest domains. Data represented as mean ± SEM. Number of measured samples: 6-8 cells / 3-4 isolations. Data were analysed using non-parametric Mann-Whitney student t-test, * p<0.05, ** p<0.01, *** p<0.001.

5.3.2.2 Heart failure affects mitochondria number and size

During the heart failure cardiomyocyte undergo changes in the shape and size that cause modification in the shape of the whole chamber and wall stress. Along with these morphological changes several structural alterations take place within single cardiomyocyte. Miragoli et al. has shown that in the 16 week post MI model mitochondria lose their regular organization (Miragoli et al. 2016). Based on the TMRM staining, we show that in the same diseased model mitochondria number significantly decreases, but the area of each element increased. Based on the electron microscopy images, these results could be explained by the fact that in the MI model mitochondria clump underneath the membrane forming clusters (Fig.3.6).
Figure 36. Mitochondria analysis in control and 16 weeks post Myocardial Infarction rat model. A) Left, representative electron microscopy images. Scale bar 500 nm. Right, TMRM mitochondria-specific staining. Scale bar 10 μm. B) Mitochondria quantification graphs. Data represented as mean ± SEM. Number of measured samples: 5-7 cells / 3 isolations. Data were analysed using non-parametric Mann-Whitney student t-test, * p<0.05, *** p<0.001.

5.3.2.3 In the failing heart mechanical stretch sensor machinery mRNA expression is reduced but not all the protein levels

To assess the changes in the titin-Tcap-MLP as a mechanical stretch complex, genes expression levels were assessed using RT-PCR technique. Results showed a significant reduction in the Tcap mRNA level following the pathological insult (Fig.37 A). Whereas, MLP demonstrates a trend towards decrease (Fig.37 B). Titin protein is expressed by two isoforms, N2B and N2BA. Current results demonstrate a significant drop in the N2B isoform and an apparent increase of the N2BA isoform, however not significant. This is also reflected in the graph showing N2B/N2BA isoform ratio (Fig.37 C). As a follow-up, new titin accompanying gene RBM20 was also checked. As expected, a trend towards decrease in the MI cells is observed (Fig.37 D).
Figure 37. Gene expression analysis using RT-PCR in A) Tcap, B) MLP, C) titin and D) RBM20. Data represented as mean ± SEM. Cells were isolated from 3 animals. Statistical test non-parametric Mann-Whitney student t-test, * p<0.05, ** p<0.01, *** p<0.001.

Further, the protein levels of Tcap and MLP were measured by Western blot. Protein levels do not necessarily match the PCR results due to post translational modifications that are likely to happen. Obtained results show a significant drop in the Tcap protein, however no apparent difference in the MLP levels (Fig.38). Due to high molecular weight of titin (3kDa) the protein detection is very challenging. Additionally, available protocols are only optimized for the tissue and not for the cell samples used in this study. Despite much effort has been taken to assess titin protein level, the experiment was unsuccessful and titin protein levels are not reported in this thesis.
5.3.2.4 Defective actin-myosin detachment in the MI model

Actin-myosin cross-bridging constitutes the basis of muscle contraction as the main force generators inside the cell (Huxley 1969). To explore the possibility of the modified interaction between these two proteins 10 μM of blebbistatin, a specific cross-bridging inhibitor, was used to investigate their interplay in the diseased model. Results show that the inhibition of the actin-myosin interaction in the MI model significantly reduces cell YM, showing that only partial actin detachment from the myosin heads is happening (MI+blebbistatin Z-groove 2.22±0.19kPa crest 2.25±0.16 vs MI Z-groove 3.5±0.21 kPa crest 4.13±0.75 kPa, p<0.001).

Interestingly, treating healthy cells with blebbistatin notably increases cell YM (blebbistatin Z-groove 3.68±0.27kPa, crest 3.63±0.23 kPa vs control Z-groove 1.99±0.25 kPa, crest 2.29±0.26 kPa, p<0.001) (Fig.39).
5.3.2.5 Cytoskeletal components contribute to cell Young’s modulus in the MI model

Experimental finding from the Chapter 4 demonstrate that two major generic cytoskeleton components, actin and microtubules, contribute to cell YM at a baseline level. Here, the possibility of YM regulation was explored using the same specific pharmacological treatments, cytochalasin D and vinblastine that inhibit actin and microtubule polymerization, respectively. Results confirm that both structural elements significantly reduce cell YM and remove the difference between Z-groove and crest domains observed in MI cells (cytochalasin D Z-groove 2.44±0.2 kPa, crest 2.66±0.21 kPa, p<0.001 vs vinblastine Z-groove 1.26±0.1 kPa, crest 1.39±0.1 kPa, p<0.001 vs MI Z-groove 3.5±0.21 kPa, crest 4.13±0.25 kPa) (Fig.40).
5.3.2.6 Microtubule PTMs contribute to cell mechanics in control and failing model

Due to the contradictory results on the role of MT network in the cell mechanics (Nishimura et al. 2006; Harris et al. 2002; Robison et al. 2016; Cooper 2009), next step in this subsection was to investigate the role of microtubule-related changes in cardiomyocyte mechanics. Recently a lot of attention was drawn to post-translational modifications (PTMs) of MTs that contribute to cell biomechanics. Using WB technique, the level of detyrosinated (deMT) and acetylated (acMT) tubulin were determined first. As shown in Figure 41, detyrosination of MT is significantly higher in MI cells. Also, MT acetylation shows a trend towards increase. Along with the MT analysis, desmin protein levels were also measured as it was reported as a detyrosinated MT – sarcomere anchor protein (Robison et al. 2016). As expected, desmin levels have also been increased in the diseased model.
Figure 41. Beta-tubulin, acetylated (acMT), detyrosinated MT (deMT) levels in control, 16 weeks post Myocardial infarction treated and non-treated samples. A) Representative images of WB. B) Quantification graphs of panel A. Data represented as mean ± SEM. Cells were isolated from 4-8 animals. Statistical test non-parametric Mann-Whitney student t-test. ** p<0.01.

Following the results on the protein level, I sought to determine the role of each MT pool in the YM increase seen in failing myocytes. Control and MI cells were treated with Tubastatin A (Tub A), that blocks the deacetylation process, resulting in more acMT. Another set of control and MI cells was treated with PTL that specifically blocks the detyrosination reaction, giving less deMT. Following pharmacological interventions in control cells, that both treated groups become softer (Tub A Z-groove 2±0.13 kPa, crest 1.87 ± 0.11 kPa, p<0.01 and PTL Z-groove 1.95± 0.13 kPa crest 2.27±1.18 kPa, p<0.05 vs control Z-groove 2.6±0.19 kPa, crest 3±0.3 kPa). Similar softening effect is also observed in MI-treated cells (Tub A Z-groove 2.19±0.19 kPa, crest 2.45±0.26 kPa, p<0.001 and PTL Z-groove 1.99±0.15 kPa, crest 2.13±0.14 kPa, p<0.001 vs MI Z-groove 3.5±0.2 kPa, crest 4.13±0.75 kPa) (Fig.42).
Figure 4.2. Scanning Ion Conductance YM measurement in control, control-treated, MI and MI-treated adult cardiomyocytes. Cells were treated with 2 µM Tubastatin A for 3 hours and 10 µM Parthenolide for 2 hours. Control cells were kept for 2 hours in culture, respective to pharmacologically treated cells. Tubastatin A promotes MT acetylation. PTL reduces MT detyrosination. A) 10µm x 10µm Young’s Modulus maps [kPa] and surface maps [µm]. B) Graph representing YM values in Z-groove and crest domains. Data represented as mean ± SEM. Number of measured samples: 7 cells / 4 isolations. Data were analysed using non-parametric Mann-Whitney student t-test, * p<0.05, **p<0.01, *** p<0.001.

Findings by Asthana et al. (2013) showed that there is a relationship between deMT and acMT. However, similar experiment from Quinones et al., (2011) did not find the same relation. Here I aimed to verify if such cross-talk is present in adult rat cardiomyocytes from a 16 weeks post MI model. MI cells were treated with Tub A and PTL. Next, immunofluorescent staining was performed to analyze MT PTMs content (Fig. 43). As expected, Tub A treatment markedly increased acMT level, but, interestingly, caused deMT to drop. PTL treatment resulted in a deMT decrease, but it did not affect the acMT pool.
Figure 43. Acetylated and detyrosinated MT analysis in the MI and MI-treated cells. Cells were treated with 2 µM Tubastatin A for 3 hours and 10 µM Parthenolide for 2 hours. A) Representative immunofluorescent micrographs, acMT (yellow) and deMT (white). B) Quantification graph in MI and Tub A treated MI cells. C) Quantification graph in MI and PTL treated MI cells. Scale bar 10 µm. Data represented as mean ± SEM. Number of measured samples: 5-6 cells / 3 isolations. Data was analysed using non-parametric Mann-Whitney student t-test, **p<0.01, *** p<0.001.
5.3.3 Investigating cardiomyocyte mechanics in load deficient healthy and failing hearts

5.3.3.1 Removing mechanical load reduces cell Young’s modulus in control and MI model

Balanced mechanical load in the heart is of great importance for its proper function. Cardiac pathologies such as hypertrophy is characterized by an increased load (Ruwhof and van der Laarse 2000). Whereas, patients with an implanted LVADs are subjected to unloading of the heart. The latter case is known to bring improvement but closer investigation of this treatment in an appropriate animal model show progressive adverse effect after a longer period of implantation (Wright et al. 2018; Ibrahim, Navaratnarajah, et al. 2012). There is a lack of studies focused on the role of LVADs in cardiac mechanics. To investigate how load-deficiency will affect adult cardiomyocyte mechanical properties, partial mechanical unloading of healthy and failing hearts was examined. Lewis syngenic rats were used to generate this study model (as described in chapter 2.1.4). Experimental findings show that by removing mechanical load from control (unloaded Z-groove 0.93±0.1kPa, crest 2.15±0.11kPa vs control Z-groove 1.6±0.1, crest 2.15±0.11kPa) and MI (unloaded MI Z-groove 1.7 ±0.11kPa, crest 2.3±0.13 kPa vs MI Z-groove 3.13±0.56 kPa, crest 3.86± 0.52 kPa) cells, YM values decrease significantly (Fig.44).
Figure 4. Scanning Ion Conductance YM measurement in control, 16 weeks post Myocardial Infarction and unloaded rat models. A) 10µm x 10µm Young’s modulus [kPa] and surface maps [µm] B) Graph representing YM values in z-groove and crest domains. Data presented as mean ± SEM. Number of measured samples: 7-9 cells / 2-4 isolations. Data were analysed using non-parametric Mann-Whitney student t-test, * p<0.05, *** p<0.001.

5.3.3.2 Removing mechanical load reduces mitochondria number and normalizes the area

As the unloaded cardiomyocytes do not beat, the need for energy-harboring compound, ATP is also lower. This can potentially indicate that mitochondria undergo certain changes. To explore this possibility cardiac myocytes were stained with mitochondria-specific dye TMRM, imaged and next analyzed as in subsection 5.3.2.2. In terms of mitochondria area, the two samples: control and unloaded control cells, presented no difference (Fig.45). However, as already shown for the failing hearts in Sprague Dawley rats in the chapter 5.3.2.2, an increase of the mitochondria area is also observed in the Lewis strain. Interestingly, following the unloading of the failing heart the area of the mitochondria goes back to control values. However, in terms of mitochondria number that were normalized to cell size, unloading a heart is always followed by a decrease in mitochondria numbers.
Figure 45. Mitochondria analysis in control, 16 weeks post Myocardial Infarction and unloaded rat models. A) Representative electron microscopy images (left) and TMRM mitochondria-specific staining (right). Scale bar 500nm, on the left and 10 µm on the right. To accommodate the cell images, unloaded MI TMRM image shows a smaller scale than other images. B) Mitochondria quantification graphs. Data represented as mean ± SEM. Number of measured samples: 5-7 cells / 3 isolations. Data were analysed using non-parametric Mann-Whitney student t-test, * p<0.05, *** p<0.001.
5.3.3.3 Load deficiency differently regulates mRNA expression and protein level of mechanical stretch sensor elements

As mentioned before mechanical stretch sensor complex is composed of 3 mechanosensitive proteins, Tcap, MLP and titin. As the experimental findings from this study has already shown that YM is reduced in the unloaded model, it was interesting to check if and how the mechanosensitive protein level changes in the unloaded cell samples. To investigate that mRNA and protein levels were measured. No significant change is seen at the mRNA and protein level in the Tcap and MLP samples compared to healthy cells. However, in the failing cells both, MLP and Tcap levels are decreased at the mRNA level. Tcap protein measurements demonstrate similar decreased level in the MI model, but no change in the MLP protein. Removing mechanical load doesn’t change mRNA or protein levels of Tcap and MLP as compared to failing cells (Fig.46 A,B, F-H).

Titin mRNA level also doesn’t show any differences when comparing the unloaded control to the control samples. As already shown in chapter 5.3.2.3, titin in the MI model presents disrupted isoform expression levels with trend towards higher N2BA and lower N2B amount. However, following unloading the titin isoform distribution start to resemble the pattern seen in healthy cells. When looking at the RBM20 mRNA expression there is no change between control and unloaded control models. MI cells present a trend towards lower level of RBM20 mRNA, but after unloading RBM20 level increased to a level similar to healthy samples (Fig.46 C,D).
Figure 46. Gene and protein analysis in control, Myocardial Infarction and unloaded models. A) Tcap, B) MLP, C) titin isoforms (N2B and N2BA) and D) RBM20 mRNA expression levels. E) Western blot protein bands and quantification graphs of F) Tcap and G) MLP. Data presented as mean ± SEM. Number of measured samples 2-3 isolations. Data were analysed using non-parametric Mann-Whitney student t-test, ** p<0.01.
5.3.3.4 Microtubular network is significantly modified following the load removal

Recent experimental findings show how MT network contribute to cardiomyocyte mechanics. Additionally, intermediate filament, desmin has also been shown to support the mechanical role of MT (Robison et al. 2016). Therefore, I tested if this is also the case in the MI and the unloaded MI models. In control cells, the level of both PTMs doesn't change after unloading, in contrast, in MI cells, a decrease was found in deMT levels and an apparent reduction of acMT levels following unloading. On the other hand, unloading the hearts does not change the desmin protein levels significantly in either control or MI cells (Fig.47).

![Image of Western blot analysis]

**Figure 47.** Desmin, detyrosinated and acetylated tubulin protein level analysis in control, 16 weeks post Myocardial Infarction and unloaded rat models. A) Western blot protein bands. Quantification graphs of B) acetylated MT, C) detyrosinated MT and D) desmin. Data represented as mean ± SEM. Cells were isolated from 4-8 animals. Data were analysed using non-parametric Mann-Whitney student t-test, *p<0.05, **p<0.01.

5.3.4 Angiotensin II role in cardiomyocyte mechanics

5.3.4.1 Short-term angiotensin II reduces cell Young’s modulus

Angiotensin II is a hormone peptide that exerts different direct and indirect effects, including a role in cell mechanoproperties (Leite-Moreira et al. 2009; Lee et al. 2010). Here, short-term Ang II treatment (2-hour incubation) has been tested on cardiomyocyte mechanics and a signaling pathway was further demonstrated. In order to determine the Ang II concentration
that provokes a change in adult rat cardiomyocyte mechanics, a dose response curve has been carried out (Fig. 4.8). Strong change in YM is noticed at the concentration of 20uM (control Z-groove 2.6±0.19 kPa; crest 3±0.29 kPa vs. Ang II Z-groove 2±0.12 kPa; crest 2.1±0.15 kPa, p<0.001) that was used for further experiments.

![Ang II response curve](image)

*Figure 4.8. Scanning Ion Conductance measurement in Angiotensin II-treated samples. Ang II dose response curve representing YM values in Z-groove and crest domains. Cells were incubated with Ang II for 2 hours. Data presented as mean ± SEM. Number of measured samples: 7 cells / 3-4 isolations. Data were analysed using non-parametric Mann-Whitney student t-test, *** p<0.001.*

Ang II mostly acts through Angiotensin Type 1 receptor (AT1R), to verify whether the softening effect is exerted through this pathway a specific AT1R inhibitor, Losartan, has been used (Allen, Zhuo, and Mendelsohn 2000; Wassmann and Nickenig 2004). Firstly, I checked that the inhibitor itself does not change YM as compared to untreated control cells (Losartan Z-groove 2.58±0.17 kPa, crest 2.86±0.19 kPa) (Fig. 4.9). When Ang II treatment cells were pre-incubated with Losartan, the softening effect of Ang II was abolished, confirming the importance of AT1R in the Ang II effect pathway (Ang II Z-groove 2±0.12 kPa, crest 2.1±0.15 kPa vs. Ang II + Losartan Z-groove 3.02±0.7 kPa, crest 3.22±0.19 kPa, p<0.05). Previous work form Leite-Moreira suggests that Na⁺/H⁺ exchanger is a major player in the Ang II-dependent cell mechanics (Leite-Moreira et al. 2009). Therefore, the Na⁺/H⁺ exchanger blocker 5-[(N-methyl-N-isobutyl)-amiloride (MIA) was used to verify its role. MIA treatment did not show difference as compared to control by itself (MIA Z-groove 2.75±0.17 kPa, crest 3.13±0.25 kPa vs control Z-groove 2.6±0.19 kPa, crest 3±0.29 kPa), neither adding MIA to already Ang II treated cells (MIA + Ang II Z-groove 1.82±0.11 kPa, crest 1.91±0.12 kPa vs Ang II Z-groove 2±0.12 kPa, crest 2.1±0.15 kPa).
Chapter 5 Alterations of cell mechanics in disease

5.3.4.2 TGFβ1 and Rho kinase as elements of the Angiotensin II signaling pathway

To determine a possible signaling cascade, an extensive literature review has been done. Current findings show a cross talk between Ang II and TGFβ1 in the myocardium in different stages (Kupfahl et al. 2000; Wenzel et al. 2001; Sadoshima and Izumo 1993). To test whether this interaction is involved in the cardiac biomechanics, cells were treated with SB-431542, an explicit TGFβ1 receptor blocker. Following the treatment, cell YM was brought back to control values showing another player in the Ang II-dependent YM reduction (Ang II Z-groove 2±0.12 kPa, crest 2.2±0.15 kPa vs Ang II + SB-431542 Z-groove 2.6±0.11 kPa, crest 3±0.15 kPa, p<0.001). According to Wesselman and de Mey, Ang II can have an indirect effect on key cell cytoskeletal elements through the multifactorial Rho kinase (ROCK) known to be involved in

Figure 49. Scanning Ion Conductance Microscopy YM measurement in control, Angiotensin II-, MIA- and Losartan-treated cardiomyocytes. Cells were incubated with 20 μM Angiotensin II for 2 hours, 100 μM Losartan for 30 minutes and 1 μM MIA for 2,5 hours. Control cells were kept for 2 hours in culture, respective to pharmacologically treated cells. A) 10µm x 10µm Young’s Modulus [kPa] and surface maps [µm]. B) Graph representing YM values from Z-groove and crest domains. Data presented as mean ± SEM. Number of measured samples: 8-9 cells / 3-4 isolations. Data were analysed using non-parametric ANOVA Kruskal-Wallis test, * p<0.05, ** p<0.01, *** p<0.001.
cell shape and movement (Wesselman and De Mey 2002). To explore the possibility of the role of ROCK in these cells response, adult cardiomyocytes were treated with Y-27632, a specific ROCK inhibitor (Fig. 50). Interestingly, blocking the kinase and treatment with Ang II increases cells YM even more than in control values (Ang II + Y-27632 Z-groove 4.1±0.32 kPa, crest 4.2±0.29 kPa vs. Ang II Z-groove 2.0±0.12 kPa, crest 2.2±0.15 kPa, p<0.001).

Figure 50. Scanning Ion Conductance Microscopy YM measurement in control, Angiotensin II-, SB-431542-, Y-27632-treated cardiomyocytes. Cells were incubated with 20 µM Angiotensin II for 2 hours, 10 µM SB-431542 for 2 hours and 5 µM Y-27632 for 1 hour. Control cells were kept for 2 hours in culture, respective to pharmacologically treated cells. A) 10µm x 10µm Young’s Modulus maps [kPa] and surface maps [µm] B) Graph representing YM values from Z-groove and crest domains Data presented as mean ± SEM. Number of measured samples: 8 cells / 3 isolations. Data were analysed using non-parametric ANOVA Kruskal-Wallis test, ** p<0.01, *** p<0.001.

5.3.4.3 Angiotensin II acts via microtubules on cells Young’s modulus
It has been reported that ROCK inhibition can induce MT expression (Takesono et al. 2010; Amano et al. 2003). This suggests that ROCK generally known for the actin regulation, can also have an impact on MT. Cells were treated with Taxol, a compound that acts as a MT stabilizer. Obtained results show that the softening effect induced by Ang II is lost (Ang II+taxol Z-groove 4.3±0.21 kPa; crest 4.54±0.24 kPa vs Ang II Z-groove 2±0.12 kPa, crest 2.1±015 kPa, P<0.01)
Lastly, to test if the decreased YM is due to reduced level of MT, cells were treated simultaneously with vinblastine, which blocks MT polymerization, and Ang II. Double pharmacological treatment presents no increase or decrease in YM, as compared to Ang II treated samples, confirming that Ang II induced changes in cell mechanics are MT-dependent (Ang II + vinblastine Z-groove 1.91±0.14 kPa, crest 2.08±0.12 kPa vs Ang II)(Fig.51).

Figure 51. Scanning Ion Conductance Microscopy measurement in control, Angiotensin II-, Taxol, vinblastine-treated cardiomyocytes. Cells were incubated with 20 μM Angiotensin II for 2 hours, 10 μM Taxol for 3 hours and 2 μM vinblastine for 1 hour. Control cells were kept for 2 hours in culture, respective to pharmacologically treated cells. A) 10μm x 10μm Young’s Modulus [kPa] and surface maps [µm]. B) Graph representing YM values from Z-groove and crest domains. Data presented as mean ± SEM. Number of measured samples: 8 cells / 3 isolations. Data were analysed using non-parametric ANOVA Kruskal-Wallis test, ** p<0.01, *** p<0.001.

5.3.4.4 Microtubule post-translational modifications regulate cardiomyocyte mechanics in Ang II treated cells

Previous paragraph confirmed that MT are the main regulators of cell YM following Ang II treatment. Additionally, me (chapter 5.3.3.3) and others (Robison et al. 2016; C. Y. Chen et al. 2018) have shown the significance of MT PTMs in cardiac mechanobiology. Therefore, in this section I aimed to quantify the level of α-, β-tubulin, acMT and deMT using western blot and
immunofluorescence in normal myocytes following Ang II treatment, alone or in combination with taxol or SB-431542 (Fig.5.2).

Western blot was used to measure the level of α-, β-tubulin, acMT and deMT in four different conditions. No changes are present in the α- and β-tubulin. On the other hand, acetylated and detyrosinated pools depict a tendency towards reduced PTM’s levels following Ang II treatment. This effect is lost when cells were additionally treated with Taxol or SB-431542.

Figure 52. Alfa-, beta-, detyrosinated and acetylated tubulin protein level analysis in control and treated cardiomyocytes. A) Western blot protein bands B) Quantification graphs. Data represented as mean ± SEM. Cells were isolated from 4 animals. Data were analysed using parametric ANOVA test.

Immunofluorescence was used as a secondary method to visualize and quantify the polymerized tubulin proteins. Samples were stained for β-tubulin, acMT and deMT in the same
set of samples and Z-stacks of images of each cell were acquired using confocal microscope. MT network is a very complex 3D structure spanning through all the cytoplasm. It has been shown that the structure of tubules underneath the sarcolemma is different from that in the middle core of the cytoplasm (Robison et al. 2016). Therefore, to see if there is a change in MT amount following different treatments image analysis were divided into two groups: subsarcolemma (Fig.53) and cell center (Fig.54). No changes in the polymerized β-tubulin are present at all tested conditions. After Ang II treatment detyrosinated MT show a tendency towards decline in the subsarcolemmal area, but in the cell centre detyrosinated MT are significantly reduced. This effect is reversed by taxol or SB-431542 treatment in both locations after Ang II. Similarly, acetylated MT pool is markedly reduced at both locations and this effect is lost after taxol or SB-431542 incubation.
1) subsarcolemma

Figure 5.3. Alfa-, beta-, detyrosinated and acetylated polymerized tubulin protein level analysis at the submembrane area in control and treated cardiomyocytes. A) Representative immunofluorescent micrographs, B-tubulin(grey), detyrosinated MT (green), acetylated MT (yellow). B) Number of measured samples: 5-6 cells / 3 isolations. Data were analysed using parametric ANOVA test ** p<0.01, *** p<0.001.
2) cell centre

Figure 5.4. Alfa-, beta-, detyrosinated and acetylated polymerized tubulin protein level analysis at the cell center in control and treated cardiomyocytes. A) Representative immunofluorescent micrographs, β-tubulin (grey), detyrosinated MT (green), acetylated MT (yellow). B) Number of measured samples: 8 cells / 3 isolations. Data were analysed using parametric ANOVA test ** p<0.01, *** p<0.001.

5.3.4.5 Angiotensin II long-term treatment exerts different effect on cardiac mechanics

Ang II is known as a common hypertrophy inducer (Hernández et al. 2014; Sadoshima and Izumo 1993). Following a short-term beneficial effect, we sought to investigate Ang II effect
on cell mechanics after 24-hour treatment. Adult cardiomyocytes were incubated with conditioned M199 media with or without 20 µM Ang II and placed in a 37°C and 5% CO₂ incubator for 24 hours. The next day cells were scanned and YM was examined. Obtained data show that cells exposed to a longer Ang II treatment are no longer softer (Fig.5). On the contrary, an opposite effect is observed. Cell size analysis also show that cells treated with Ang II for 24-hours are bigger than control cells (p<0.05), which suggests that the hypertrophic signaling pathway has been switched on.

5.3.4.6 Angiotensin II doesn’t inhibit MT in a long-term treatment
Analysis of MT at the cell center, based on the immunofluorescent staining, demonstrates that culture supplemented with Ang II for 24 hours does not reduce the amount of β-tubulin, acetylated and detyrosinated tubulin as previously observed for the short-term treatment (Fig.56).

![Figure 5. Scanning Ion Conductance Microscopy measurement in 24-hour treated and not treated cardiomyocytes. A) 10µm x 10µm Young’s Modulus maps [kPa] and surface mas [µm]. B) Graph representing YM values in z-groove and crest domains. C) Cell size analysis in 2 hours and 24 hours cultured treated and non-treated cardiomyocytes. Data presented as mean ± SEM. Number of measured samples: 7 cells / 3 isolations. Data were analysed using non-parametric Student t- test, * p<0.05.]
Figure 5.6. Alfa- beta-, detyrosinated and acetylated polymerized tubulin protein level analysis at the cell center in 24-hour treated and non-treated cardiomyocytes. A) Representative immunofluorescent micrographs, β-tubulin (grey), detyrosinated MT (green), acetylated MT (yellow). B) Quantification graphs. Number of measured samples: 5-6 cells/3 isolations. Data were analyzed using parametric ANOVA test *p<0.05, **p<0.01, ***p<0.001.

5.3.4.7 Short-term angiotensin II treatment exerts the same effect on MI cells as on control

As already discussed before, cells during MI undergo several structural and functional changes by switching on and off several pathways. To test if short-term treatment with Ang II can act the same way in MI cells as in control samples, cells from the failing heart were incubated for 2 hours with 20uM Ang II. Interestingly, YM is decreased in MI cells following the treatment
respective to the untreated MI cells, and, importantly, the YM in these cells is similar to control + Ang II samples (MI Z-groove 3.5±0.2 kPa, crest 4.13±0.75 kPa vs Z-groove 2.285±0.08 kPa, crest 2.275±0.12 kPa, p<0.001) (Fig.57). This shows that despite pathological phenotype the Ang II-dependent YM reduction takes place in a similar way as in healthy cells. Also, Ang II supplementation reduces the amount of all tubulins: β-tubulin, acetylated and detyrosinated tubulin, as quantified based on the immunofluorescent staining in MI versus MI+ Ang II.
Figure 5.7. Ang II effect on cardiomyocytes isolated from hearts 16 weeks after MI. A) Scanning Ion Conductance Microscopy YM measurement in 24-hour treated and not treated samples. 10µm x 10µm Young’s Modulus [kPa] and surface maps [µm]. B) Graph summarizing YM values. C) Representative immunofluorescent micrographs, β-tubulin (grey), detyrosinated MT (green), acetylated MT (yellow). Scale bar 10 µm. D) Quantification graphs of C. Data presented as mean ± SEM. Number of measured samples: 6-8 cells / 3-4 isolations. Data were analysed using non-parametric Student t-test, * p<0.05, *** p<0.001.
5.4 Discussion
In this chapter I show that pathological states such as MI, Duchenne Muscular Dystrophy affect cell YM. Lower values are observed in Duchenne Muscular Dystrophy mouse and partially mechanically unloaded model; on the contrary, increased YM is seen in the Myocardial Infarction rat model. In the failing model, changes are observed in the mitochondria number as well as size. Interestingly, microtubules post-translational modifications are upregulated in failing cells. Additionally, a potential cross-talk might be present between acetylated and detyrosinated populations of microtubules. These results put microtubules forward as important regulators of cardiac mechanics. In the partial mechanical unloaded model, number and size of mitochondria drop compared to the MI rat. No changes are observed in the cardiac specific stretch complex proteins, however a decrease in microtubule post-translational modifications was reported. Lastly, Angiotensin II, a blood pressure regulator, exerts an effect on cell YM by decreasing it after a short-time (2 hours) treatment. This has been shown to potentially act though ATR1, TGF-β1, Rho kinase and the microtubular network, specifically detyrosinated and acetylated populations.

5.4.1 Regulation of mechanical stretch sensor complex mechanics in the Myocardial Infarction model
Failing cardiac myocytes are known to undergo structural changes. Findings from Lyon et al., (2009) show loss of the Z-groove structures based on the SICM-produced topographic surface representations. In our SICM study of failing heart rat model we observed increased whole cell Young’s modulus (Fig. 35). It is interesting to note that only in the failing cells a significantly higher YM increase is observed in crest as compared to the remaining Z-grooves. By contrast, in a failing heart mouse model Dague et al., (2014) reported a general increase of cell YM with no correlation in the surface topography, using Atomic Force Microscopy technique. My immunofluorescent data and supporting electron microscopy images identify mitochondria clusterization underneath the membrane as a possible explanation to the notable YM variation in different compartments (Fig. 36). This data corroborated with the reports on incorrect mitochondria fusion and fission regulation in heart failure (Knowlton, Chen, and Malik 2014). To understand other YM-related changes in the MI cells, further experiments were based on cell genome and proteome.
Z-groove is a highly regulated structure that is formed by aligned TT. These deep membrane invaginations contain several proteins including ion channels, among which L-type calcium channels play a major role in calcium handling during excitation-contraction coupling. Inside the cell, L-type calcium channels are coupled to ryanodine receptors that allow a synchronous calcium release and the regulation of Ca2+ induced Ca2+ release machinery (Bers, 2008). Therefore, proper TT formation and maintenance is necessary for cardiac performance. Tcap protein has been proposed as one of the binding TT partners that regulates their structure (Ibrahim, Siedlecka, et al. 2013). Additionally, this molecule is a part of the mechanosensitive stretch complex (Knöll et al. 2002). Elegant work from Chien group (2002) using different techniques has shown that Tcap is linked to MLP. Moreover, MLP is necessary for the Tcap-titin interaction. This triple-molecule complex is located at the Z-disc that is a mechanical hot spot in cardiac myocytes. Additionally, not only it has been proposed to be a part of mechanosensation and mechanotransduction pathways, but also it has been suggested to regulate cell YM (Lyon et al. 2015; Buyandelger, Miocic, Piotrowska, et al. 2011; Li, Lang, and Linke 2016). Therefore, in our failing heart model with an apparent increase in cell YM I aimed to investigate the stretch sensor complex. Pathological insult results in Tcap and MLP decrease at the mRNA level (Fig. 37). However, Western blot analysis show only a significant Tcap protein reduction (Fig. 38). Closer investigation of Tcap gene and its relation to heart failure has revealed mutations of this specific gene in HCM and DCM patients (Hayashi et al. 2004). Mechanical defects are observed in a model of limb-girdle muscular dystrophy type 2G that carries a Tcap mutation. In this work, extensor digitorum longus and the soleus muscles isolated from lower limbs of the Tcap deficient mice exhibit increased YM (Markert et al. 2010).

MLP is another stretch complex partner has been also shown to play a role in heart failure. Results presented in this thesis doesn’t show a MLP decline in failing myocytes (Fig. 38) as reported by others (Arber et al. 1997; Zolk, Caroni, and Böhm 2000). However, chamber dilation and contraction abnormalities have been found in MLP null mice partly as a result of attenuated cell and tissue tension (Arber et al. 1997). This confirms that Z-disc elements can provoke pathological signaling. Knoll et al., has further shown that MLP removal projects on the binding to Tcap, resulting in protein relocation to the cytoplasm (Knöll et al. 2002). Apart from its mechanical sensor function MLP has also been shown to be involved in cell differentiation, actin disassembly, calcium ions and contraction regulation as well as blood
vessel alterations (Buyandelger et al. 2011). Therefore, it’s multifunctionality is linked to different compartment localization. It can be found at the Z-disc, costameres, nucleus, cytoplasm and ID. To date, a clear role of MLP and the signaling pathways it operates through are still poorly understood. Results presented in this thesis doesn’t show a MLP decline in failing myocytes (Fig. 39) as reported by others (Arber et al. 1997; Zolk, Caroni, and Böhm 2000). Location variability, post-translational modifications and insufficient sample number could potentially contribute to difference between the results obtained here and literature findings.

The last element of the stretch sensor complex is titin. It is a half sarcomere length protein and this results in titin bonding with a variety of binding partners; that eventually makes titin a multifunctional protein. However, titin is well recognized for its function of tuning passive tension in the cardiomyocyte in different ways. Here, I focused on titin isoform alterations. At the RNA level we observe a reduction in the stiff isoform and an enhancement of the softer form (Fig. 36). A variability in titin expression pattern between animal species has been reported (Cazorla et al. 2000). Nevertheless, in healthy conditions a more stiffer form (N2B) of the protein is mostly present, rather than a softer one (N2BA) (Neagoe et al. 2003). Interestingly, in patients with diastolic dysfunction, two phenotypes of failing hearts are distinguished: preserved LV ejection fraction (HFpEF) or reduced ejection fraction (HFrEF). Both groups, exhibit notable YM increase, but differ between each other in titin isoforms levels. HFrEF group has been shown to express more softer form, N2BA, as opposed to HFpEF group with a higher level of the stiffer N2B (Makarenko et al. 2004; van Heerebeek et al. 2006). Titin-dependent YM can be regulated by protein kinases targeting different parts of the giant protein. Protein kinase A phosphorylates N2B in its spring domain and this reduces cardiomyocyte YM; similar effect is also exerted by protein kinase G (Krüger et al. 2009; Yamasaki et al. 2002; van Heerebeek et al. 2006). In contrast, protein kinase C related phosphorylation of titin changes YM in the opposite way (Hidalgo et al. 2009). Data from Kotter et al., (2016) show that cell YM was already increased as soon as 3 days after failing heart model is generated, either using an ischemia-reperfusion or left artery ligation procedure. It was shown to be due to titin PEVK (proline-glutamate-valine-lysine) hyperphosphorylation and N2B hypophosphorylation, but not due to the titin isoform change. Although no phosphorylation studies have been performed in this thesis, work form Kotter
shows that phosphorylation levels and the isoform change should be equally considered in terms of cellular mechanics examination and can be independent of each other.

As discussed above, titin passive stiffness is dependent on alternative splicing and post-translational modifications. Lately, RNA binding motif 20 (RBM20) gene has been shown to regulate titin introns removal in the splicing process. Guo et al., (2012) RBM20 null rat and a cardiomyopathy patient with a RBM20 point mutation have shown a significant decline in the N2B titin isoform (Fig. 37). RT-PCR data in this work also show decline in the RBM20 expression in the failing heart samples when a drop in N2B isoform was observed. Whereas, in the unloaded MI model the mRNA level of N2B titin as well as RBM20 go up (Fig. 46).

5.4.2 Regulation of generic cytomechanics in the Myocardial Infarction model

Apart from the mechanical stretch sensor complex, cardiac specialized machinery includes other proteins that are essential for cardiac performance. Actin-myosin cross bridge interaction is the main spot for force generation which involves a lot of conformational changes that contribute to myocyte YM. As presented in Chapter 4, inhibiting actin polymerization in healthy cells reduces cell YM. Same effect is observed in the diseased model. In both cases the YM values from treated samples are 32-35% lower (Fig. 41).

Study from Yoshikawa et al., (2013) tested the effect of actin-myosin cross-bridging in isoproterenol-induced hypertrophy Wistar rats on cellular YM assessed by Atomic Force Microscopy. Findings from this group showed reduced YM following the blebbistatin treatment, component that binds to myosin making it unable to couple with actin. Our results obtained from the failing heart using SICM corroborated with the data from Yoshikawa et. al., (2013) proving that in pathological condition there is still a pool of sedimentary actin-myosin connections possibly due to a defect in myosin motors (Fig. 39).

One of the main structural cardiomyocyte components is F-actin. Despite different isoforms present in cardiac myocytes, 99% is taken over by α-actin, which is a part of the acto-myosin complex (Sequeira et al. 2014). Besides it’s contractile function, actin is also responsible for cell shape, motility and division (Stricker, Falzone, and Gardel 2010). This range of mechanical functions fulfilled by generic actin cytoskeleton demonstrates that the cellular components could play a role in determining cell YM. Using α- and β-actin specific antibodies Balasubramanian et al. have shown a significant increase of the cytoplasmic β-actin, in a feline right ventricle overload pressure hypertrophy model. However, this effect can’t be noticed by
using a generic actin antibody because of the abundance of α-actin, the level of which is not altered during hypertrophy. GFP-β-actin overexpression has shown it is localized at the z-disk and costamere regions (Balasubramanian et al. 2010). Future work will need to address the contribution of different actin isoforms to cell YM. Following a pathological insult (MI), changes in the myocardium enter an adaptive phase to maintain the structural and functional balance. However, these prolonged alterations eventually lead to a maladaptive state. Comparison between physiological and pathological hypertrophy show densification of microtubular network, one of the cardiac cytoskeletal elements, in the pathological state (Cooper et al. 1985). In the previous chapter, experimental findings have shown that cardiomyocyte YM highly depends on the microtubular network in healthy cells. In this chapter results from the MI rat model identified a similar effect. Extensive studies carried out by Cooper’s group have proven that an excess of MT in the pressure-overloaded hypertrophy model leads to abnormal contraction, but could be reversed by MT-specific inhibitor (Zile et al., 1999). To get a broad view on the MT mechanics, early and failing pathology stages from left and right ventricles in different species were included in the studies (Cooper, 2006). Also, other MT manipulation methods such as temperature-induced depolymerization or hyperpolymerization caused by taxol were used to confirm that manipulating MT can replicate the disease state (Tsutsui et al. 1994). Upregulation of tubulin has been also reported in DCM patients (Hein et al. 2000). Observed effect on cardiac contractility due to excessive load led to a hypothesis that MT could play a substantial role in cardiac mechanics. Harris et al., (2002) looked at the biomechanics of the pressure-overloaded feline models and identified increased YM. Study of LV pressure-overloaded tissue has reported an increased YM that was due to collagen stability (Yarbrough et al. 2012). Despite such a comprehensive work inconsistent results on the role of MT in cardiac mechanics were reported from other groups (Granzier and Irving 1995; Nishimura et al. 2006; Zile et al. 1998). Therefore, these discrepancies indicate a need to clarify the function of MT. To follow up on the idea of microtubules as resistant elements to cardiac contractility (Tsutsui, Ishihara, and Cooper 1993) new literature data show MTs PTMs as key players in the cell mechanobiology (Janke and Bulinski 2012). Based on these findings, I explored the role of the two microtubule post-translational modifications, detyrosination and acetylation, in rat failing heart cardiac myocytes. From the Western blot results we observe an increase in the protein level of the two modified MT populations and also an intermediate filament, desmin (Fig. 41). Robison et
al., (2016) using super-resolution microscopy demonstrated that MT acquire a sinusoidal shape during the contractile state and then flatten at rest. Interestingly, this buckled form meets the length of the sarcomere suggesting a link between the two structures. Intermediate filament, desmin has been revealed as the anchoring molecule for MTs. Interestingly, the buckling effect has been shown to be dependent on the MT detyrosination. Genetic and pharmacological manipulation of post-translational modifications resulted in a drop in both longitudinal and transverse YM (Robison et al., 2016). Further work from Prosser group has shown that inhibiting detyrosinated MT pool in human failing heart myocytes led to cell softening and improved contractility (Chen et al. 2018). Here, I used a pharmacological approach to modulate the the microtubule posttranslational modifications. Treatment of failing cells with PTL, a specific detyrosination inhibitor, shows a significant YM reduction. Furthermore, Tubastatin A (HDAC6 inhibitor), a pharmacological compound that promotes MT acetylation, also reduces cell YM (Fig. 42). This indicates that less detyrosinated MT or more acetylated MTs exert a softening effect in the MI cells. These results on acMT corroborate with the findings from Portran et al. (2017) and Yan et al., (2018) which proposed that acMT provide more resilience to the MT network. To date, it has been reported that both, deMT and acMT contribute to cell mechanics. Interestingly, upregulation of acMT using Tubastatin A resulted in increased deMT level in the human breast cancer cells, but no effect on deMT was observed in madin-darby canine kidney cells treated with the same compound (Asthana et al. 2013; Quinones et al. 2011). This data suggests a possible cross-talk between these two MT populations. I sought to test if such interaction is present in cardiac myocytes using immunofluorescence. To address this question, I treated cardiac myocytes from an MI model with PTL, which is a deMT blocker, but no effect on acMT was observed. On the other hand, upregulation of acMT, using Tubastatin A, caused deMT to drop (Fig. 43), a similar result to the work of Quinones et al. (2011). Both PTMs are present on the α-tubulin in the tubulin dimer; however, acetylation takes place inside the lumen whereas, detyrosination on the outside. Future work is needed to resolve in detail the MT PTMs signaling interplay.

5.4.3 Reducing workload in the failing heart
5.4.3.1 YM examination
One of the life-saving therapies for advanced heart failure is the implantation of a Left Ventricle Assist Device (LVAD) that helps to pump the blood in the weakened heart. According
to clinical data there is a certain number of patients that do not recover after the LVAD therapy and still require a heart transplant (Pham and Chaparro 2018). The difference in the patient outcome is due to post-surgery infections, possible assisting comorbidities and the different stages of heart failure at the moment of implantation. A limitation of the human studies is often the lack of proper sample controls, for example tissue samples before and after the LVAD implantation, limited number of patients or high variability in the type of sample. Therefore, using an animal model mimicking the role of LVAD, would be a way to conduct such studies by eliminating the number of variables between individuals, despite the species difference. Here, the same parameters as in the MI model were tested in healthy and MI load-deficient rats. Removing the load notably decreases cell YM in both, control and MI unloaded hearts (Fig. 44).

Clinical studies on sarcomeric and non-sarcomeric proteins in human patients with LVADs show variable results (Aquila et al. 2004; de Jonge et al. 2002; Birks et al. 2005). Recently, our and other groups using the partial mechanical unloading rat model have shown that the mechanical device implantation can bring only a short-term improvement. With this model Ibrahim et al., have reported a change in cell structure, specifically TT disruption, alterations in calcium handling and overall cell atrophy. A follow up to this research, has been conducted by Wright et al, showing that mechanical unloading also results in suppression of β1 adrenergic receptors and L-type calcium channels, key elements in the cardiomyocyte physiology (Wright et al. 2018; Ibrahim et al. 2010).

5.4.3.2 Mitochondria analysis

Mitochondria are the main energy suppliers. LVAD transplantation eliminates the need of LV to beat which eventually leads to lower energy consumption. This is confirmed by the data in the load-deficient healthy cardiomyocytes showing that the mitochondria are reduced (Fig. 45). In the pathological state, mitochondria form clusters right underneath the membrane this could explain higher YM values in the MI crest domains versus Z-grooves.

A significant reduction in organelle number (unloaded MI) is also seen as compared to failing cells, which is accompanied by a drop in structured area. According to electron microscopy mitochondria in the load-deficient MI model are more organized, which resembles control samples. This is reflected in the mitochondria area quantification graph and also, could project
on a notable difference in the cell YM between crest and z-groove in the unloaded MI samples. This could mean that some degree of structural complexity is regained by unloading.

### 5.4.3.3 Changes in the sarcomeric protein

To follow up on the protein arrangement in unloaded failing cardiac myocytes, mechanical stretch sensor molecules were investigated. Obtained findings show no improvement in the Tcap and MLP mRNA or protein level compared to already severely reduced expression in the MI cells (Fig. 46). However, RT-PCR data show an improvement in the titin mRNA expression equal to control conditions.

As shown by Wright et al., (2018) mechanical unloading of healthy cells modifies the association between major proteins that take part in the TT maintenance and calcium influx. Following load removal, the interplay between the JPH2 and Cav3 decreased and interacting protein clusters were observed. A drop in Cav1.2/Cav3 association was noticed as well. Also, preliminary data on the Tcap density show a decline in unloading healthy cells compared to control (Wright, unpublished). These results show that mechanical load itself can affect the cell structure. On the other hand, De Jonge et al., reported an increased but not recovered protein amount of actin, tropomyosin, troponin C,T and titin, based on the immunohistochemical staining in the end stage heart failure, suggesting modifications at the post translational level (de Jonge et al. 2002). Data on chronic obstructive pulmonary disease (COPD) patients shed more light in this matter. In this disease respiratory muscles such as diaphragm, weaken notably due to the loss of workload. Ottenheijm et al., reported that in the diaphragm fibers, titin amount doesn’t change, but the protein undergoes alternative splicing in the elastic PEVK segment which correlates with decreased YM (Ottenheijm et al. 2006).

To date, the role of MT in the load-deprived MI model biomechanics has not been investigated. Therefore, this work for the first time demonstrates that unloading the failing cells brings down the MT PTMs, detyrosinated and acetylated pools, but doesn’t show much change in desmin protein (Fig. 47).

To summarize, presented evidence suggest that removing mechanical load from a failing heart does not fully rescue the failing phenotype. This goes in agreement with previously published studies that investigated other cardiomyocyte properties (Drakos et al. 2012; Ibrahim, Kukadia, et al. 2012; Wright, Sanchez-Alonso, et al. 2018).
5.4.4 Angiotensin II participation in cardiac mechanical regulation

5.4.4.1 New role of Ang II

Presented findings display a new role of Ang II in the regulation of cardiomyocyte YM that can have a potential link to the Ang II-dependent cardiovascular pathologies. Available literature presents a variety of studies that employ different Ang II concentrations (Zhang et al. 2015; Perbellini et al. 2018; Hernández et al. 2014). In order to establish the change in cardiac mechanics, a dose response curve was generated (Fig. 48).

Angiotensin II is a part of RAAS system that strongly controls systemic blood pressure and salt-water homeostasis. Its role in the cardiovascular system has been also indicated in numerous studies in the past decade. There is strong evidence showing that Ang II is involved in the pathophysiology in the cardiac diseases, largely known as a hypertrophy inducer (Sadoshima and Izumo 1993; Hernández et al. 2014). This demonstrates that Ang II plays a great role in both, control and disease states. As Ang II have been shown to act on cardiac myocytes, (Egger and Domenighetti 2010) conducted a comparison study of how Ang II effects on cardiomyocyte contractility in short- and long-term could offer interesting insights in cardiac mechanics. In my work, focus was placed on a 2-hour, short-term Ang II treatment to distinguish changes in cardiac myocyte biomechanics at the very onset, later extending this work to observe if the same changes occur after a 24-hour pharmacological intervention. Cell mechanical machinery is very dynamic and if changes are likely to happen, these two time points will allow to dissect the modifications.

Following the observed YM decrease, further work has shown that Ang II-induced process is dependent on the AT1 receptor activation (Fig. 49), by using a specific receptor blocker. Subsequently, TGF-β1 is activated (Fig. 50) which has been confirmed by SB-431542 inhibitor treatment. Ang II-induced TGF-β1 activation has been demonstrated to be a leading agent of the hypertrophy initiated by the hormone peptide that targets not only cardiac myocytes but also fibroblasts (Siddesha et al. 2013). TGF-β1 is widely recognized as the fibroblast-myofibroblast transition initiator and a promoter of fibrosis (Ma et al. 2003). Findings in this thesis broaden the spectrum of TGF-β1 functions, demonstrating an indirect effect of Ang II on cardiomyocyte YM via TGF-β1 receptor.

Countless studies have reported Ang II as a molecule with multiple functions. Wesselman and De Mey (2002) presented a timeline of Ang II effects on the vascular remodeling with cytoskeletal proteins as the participants undergoing changes within a minute range that can
be regulated via Rho kinase. Cellular cytoskeleton is composed of three main elements actin, intermediate filaments and microtubules that are fully regulated to maintain their function. Apart from well know roles such as cell division, adhesion, migration, generating contractile forces or keeping cell organelles in place, it has been revealed that it also contributes to cell YM (Lee et al. 2010).

Therefore, next I’ve tested the effect of ROCK (rho kinase) using the Y-27632 specific inhibitor, which has shown a potential role of Rho kinase in this signaling pathway (Fig. 50) that ultimately effected in MT drop, the Young modulus regulators in this incidence. The final outcome, which is the Ang II-induced reduction in cell YM corroborates with the result from Leite-Moreira. Although, data presented in that study does not clarify the full signaling pathway, only suggest a potential role of PKC and Na+/H+ exchanger in this process (Leite-Moreira et al. 2009). Patients that displayed symptoms of glomerulosclerosis, a kidney disease, were recognized to have upregulated Ang II level. Marked actin derangements have been shown in isolated kidney cells from these patients. However, it has been demonstrated that Ang II is also targeting other cytoarchitecture elements. In the work from Hashimoto-Komatsu it was shown that MT network is rearranged following the octapeptide treatment in the aortic epithelial cells (Hashimoto-Komatsu et al. 2011). Based on previous work (chapter 4 and 5) and delineating the role of MT post-translational modifications in cardiac mechanics, detyrosinated and acetylated tubulin populations have been investigated. Ang II-dependent effect was firstly confirmed by targeting the generic MT network. Using taxol, a stabilizing MT agent, we eliminated the effect caused by the octapeptide. Whereas, by vinblastine treatment that is a MT polymerization blocker, no further drop in cell YM was observed, which confirmed the MT as the main effectors in the YM attenuation of Ang II (Fig. 51). Robison et al., (2016) reported that specific MT PTMs such as detyrosination reinforces the mesh-like structure to buckle and bear load in the cardiomyocyte contractile state. On the other hand, another MT modification, acetylation acts to prevent the breakage of the stiff tubules during constant work. According to our results, Ang II is also targeting the post-translational modification populations. Decrease in both pools, detyrosinated and acetylated microtubules was confirmed by Western blot and immunofluorescence (Fig. 52-54).
5.4.4.2 Possible role of Rho kinase

Rho protein family constitutes a collection of different signaling molecules that coordinate cell dynamics where actin is greatly tuned by this group of proteins. So far, it has been shown to phosphorylate myosin light chain, LIM kinase and MLC phosphatase as the primary subjects of actin skeleton (Zeidan, Javadov, and Karmazyn 2006). Interestingly, MT network has been proposed as a new target of the Rho family (Wen et al. 2004; Gundersen et al. 2005). However, a possible mutual interplay exists between the aforementioned molecules and the structural network spanning all over the cell that can regulate G proteins (Enomoto 1996; Wojnacki et al. 2014; Takesono et al. 2010). MT acetylation and detyrosination has been shown to increase following the Rho kinase blockage in T cells (Takesono et al. 2010; Enomoto 1996) due to an interaction between histone deacetylase six and tubulin polymerization promoting protein1, which prevents from MT deacetylation (Schofield, Steel, and Bernard 2012). Studies in this thesis show how Rho kinase can regulate the MT network (Fig. 5.4.1-5.4.4). By using a specific Rho kinase inhibitor (Y-27632), Ang II-dependent effect was diminished, suggesting that ROCK is a part of the signaling cascade. However, a possible MT-based regulation of Rho kinase cannot be excluded here.

Han et al., (2007) has shown that actin cytoarchitecture is controlled by extracellular signal-regulated kinase (ERK 1/2). On the other hand, vascular smooth muscle cells and cardiac myocytes exposed to Ang II, which progressed to the hypertrophy phenotype does not show activation of ERK 1/2 (Yamakawa et al. 2000; Funakoshi et al. 2001; Takeda et al. 2001). These observations suggest that Ang II adopts a different signaling cascade than ERK-regulated actin polymerization, possibly modifying the microtubular network.

5.4.4.3 Ang II activation pathways and effect on cell contraction

It is well established that not only hemodynamic overload but also Ang II is a hypertrophy-inducer. Interestingly, Ang II secretion can be also stimulated by myocardium stretching. Malhotra et al., reported an increase in angiotensinogen, ACE, renin, and AT1R, RAAS signaling pathway components at the mRNA and protein levels following uniaxial mechanical stretch of neonatal cardiomyocytes (Malhotra et al. 1999). Furthermore, specific AT1R blocking doesn’t diminish the stretch-induced result, suggesting the activation of a different signaling cascade than in this thesis potentially related to the ERK protein (Nyui et al. 1997; Yamazaki et al.
1995). On the other hand, preincubation with AT1 receptor blocker and subjecting the sample to cyclic stretch eliminates all changes caused by the mechanical extension (Shyu et al. 1995). The impact of Ang II on cardiomyocyte contractility is of great importance, therefore this topic had been studied by different groups. Obtained results following the acute treatment from various models such as rat, guinea pig and human, show differing results. Palomeque et al., (2006) reported a decrease in the contractile function, whereas Lefroy (1996) notified no direct effect of Ang II on cardiomyocyte contraction. The result discrepancy possibly come from difference in study models and experimental design. Most recent work from Zhang et al., demonstrates a beneficial role on cardiac contractility following a short-term Ang II treatment where increase in contraction is noted in vitro and in vivo models (Zhang et al. 2015). In order to check the short-term Ang II incubation effect on the diseased samples, MI cells were subjected to 2-hour treatment. Unexpectedly, despite several structural and functional modification following a pathological insult, MI cells were still able to give the same response as control (Fig. 57).

By contrast, extending the time of octapeptide treatment, drastically changes the cellular phenotype. Cardiac myocytes become hypertrophic with contractility disturbances (Domenighetti et al. 2005). 48-hour cell incubation with Ang II is a widely used in vitro hypertrophy study model (Wang et al. 2016; Jaimes, Galceran, and Raij 1998). Performed studies in this thesis show that a 24-hour pharmacological treatment is sufficient to switch on the hypertrophy pathway, as cells become bigger and stiffer (Fig. 55, 56). This suggests that the beneficial Ang II effect is lasting only a short time, which is further overpowered by a harmful cascade of events.

5.4.4.4 Summary and limitations

On the whole, Ang II do not only target the renal system as several local RAAS systems were found in different tissues, which projects on the multitude of direct and indirect octapeptide effects. Here, a new Ang II role in cell mechanobiology have been introduced. Beneficial, short-term effect reduces cell YM, but already 24-hour compound treatment exerts an opposing effect. Interestingly, despite pathological condition, MI cells are still able to respond to the favorable Ang II impact by reducing the amount of MT and further decreasing cell YM. After treatment, control cells display a 27% decline in cell YM, whereas MI model a 36% that brings them to a level comparable with healthy cells. Elegant work from Zile et al., present
significant upregulation of MT network in the feline hypertrophy model. Incubating isolated cardiac myocytes from this model with MT depolymerizing agent, brings back the correct contraction parameters (Zile et al. 1999). Recent findings from Chen et al., show the same result but in human failing cells, which were pharmacologically manipulated to reduce the deMT population (Chen et al. 2018). Data presented here, show a decline in the generic pool of β-tubulin but also post-translational modifications, which are comparable to values from healthy cells. Based on the current literature, cell contractility in the diseased model can be improved by aiming at the MT network. Acute Ang II incubation is a potential method to improve the function of the failing cells. Overall, based on the Ang II experimental studies I propose a signaling pathways through which Ang II act on cardiomyocyte YM (Fig. 59).

![Figure 58. Angiotensin II pathway regulation of cardiomyocyte Youngs modulus. Ang II binds to AT1 receptor that activates TGFβ1 receptor, leading to further Rho kinase – MT interaction. Effectively, MT PTMs are the main structures that regulate cardiomyocyte treated YM.](image)

The reason for the selection of the pharmacological-only approach is the time of Ang II analysis. Pharmacological compounds concentrations and incubation time were chosen based on the previously published data (Tambelline et al. 2012; Dhamodharan et al. 1995; Prahl et
al. 2018; Whipple et al. 2013; G. Wang et al. 2012; Halder, Beauchamp, and Datta 2005; Leite-Moreira et al. 2006; C. Y. Chen et al. 2018; Nishimura et al. 2006). Future work will require obtaining dose response curves to confirm the observed effect. For instance, the use of siRNA/shRNA is an established method of silencing the expression to study the role of proteins. However, transfection protocols are usually at least 24 hours long, which is too long, given that the first changes in the YM are seen as soon as few hours in culture. Additionally, silencing RNA is not always a fully effective method of eliminating a protein. Nevertheless, as there is no gold standard technique to study the role of proteins, a number of complementary methods can be used. Therefore, despite the number of experiments showing the role of Ang II in cell mechanics and a potential signaling pathway, this result should be confirmed in a series of experiments that encompasses a genetic approach.
Chapter 6

Investigating cell interface mechanics in a cell-cell cardiomyocyte model
6.1 Introduction

Data included in chapter 7 shed more light on the mechanical and structural coupling of cardiomyocytes (CM) homocellular pairs and cardiomyocyte-fibroblasts (myofibroblasts) (CM-MFB) heterocellular pairs.

Cardiac cytoarchitecture is a complex make up of myriad proteins, which allows to carry out heart muscle cell function. Cardiomyocyte-specific cytoskeleton named sarcomere is known to be indispensable as a basic contractile unit. However, appropriate heart function would not be possible without the communication between the other cells and with the extracellular environment. Electrical signal is propagated between heart muscle cells owing to the end-to-end coupling via intercalated discs (ID) (Kleber and Saffitz 2014). These structures provide functional and structural continuum, by enabling electrochemical coupling and mechanical interaction between cells. Cell-cell interface is composed of three main structures: gap junctions (GJ), adherens junctions (AJ) and desmosomes (Sheikh, Ross, and Chen 2009). Six connexin molecules form a connexon (hemichannel) that with a connexon from an opposing cell make a gap junction. Such structure formation allows for ions and small compounds to be exchanged among cells. This is also the main gating structure that allows for an efficient electrical signal transmission in the whole heart with connexin 43 (Cx43) isoform as the most prevalent in the left ventricle (Rohr 2004). AJ provide the cellular integrity at the cell edges but also bind to the actin cytoskeleton inside the cell (Mezzano and Sheikh 2012). The mechanical coupling in cardiac myocytes is found to be highly dependent on N-cadherin as the principle fascia adherens molecule (Radice 2013), but the highest mechanical stress resistance is secured by desmosomes that are present only in tissues experiencing a high level of stretch (Mezzano and Sheikh 2012; Patel and Green 2014). It is known that the pathophysiological conditions lead to severe structural and functional alterations at the IDs that could be due to genetic mutations or binding to other cell types (Vermij, Abriel, and van Veen 2017; Kleber and Saffitz 2014; Li and Radice 2010). Therefore, close study of the changes at the contacting membrane should be performed.

During a cardiac disease, myocytes undergo acute or gradual death (Whelan 2010). As the heart has none or very little capability of regeneration, the space devoid of cardiomyocytes is ‘repaired’ by infiltration of other cell types. Fibroblasts are one of the most abundant cell types, right after epithelial cells, populating the infarcted heart, changing its composition and
functionality (Pinto et al. 2016; Shinde and Frangogiannis 2014). Due to the flurry of new cells, cardiomyocyte expand their spectrum of cell interaction and form heterocellular connections such as cardiomyocyte-fibroblast, which are not present in control conditions. Moreover, infiltrating fibroblasts are exposed to a new surrounding with altered mechanical cues and cytokines such as growth factors and interleukins (Driesen et al. 2007). This elicits a phenotypic fibroblast-to-myofibroblast (FTM) transformation (Santiago et al. 2010). Myofibroblasts (MFB) are known to express higher degrees of α-SMA stress fibers which contributes to increased cellular YM and potentially induce mechanical changes at the cell-cell interface (Goffin et al. 2006; Thompson et al. 2011; Shinde and Frangogiannis 2014).

Following the pathological insult, cardiomyocyte and (myo)fibroblast coupling has been shown to exert several adverse effects on the whole heart function electrophysiology such as conduction slowing and increase in cardiomyocyte resting potential, assisted by the gap junctional complexes (Czubryt 2012). Interestingly, our recent data show that the gap junctional protein, Cx43 could play a new mechanical role (Schultz, Swiatlowska, Alvarez-Laviada et al., FASEB accepted). Using three cell pairs models CM-CM, CM-MFB, MFB-MFB we have reported that the amount of Cx43 correlated with the speed of movement between cell pairs, demonstrating that higher mobility with less Cx43. Further protein modulation such as up- or down-regulation followed the predicted pattern.

Protein level and arrangement at the ID is a major part of the heart failure studies. However, one of the prevalent caveats of investigating the junctional protein composition is the lack of optimal study tools, thus the quantification process is usually performed in a commonly used software, ImageJ (Downie et al. 2016; Lu et al. 2018; Terryn et al. 2013; Wang et al. 2019). Currently we are facing limited availability of the interface characterization parameters in the commonly used software or the protein analysis are based on a time-consuming manual indication and further quantification (Terry et al. 2013; Lu et al. 2018; Downie et al. 2016; Wang et al. 2019).

This chapter will test the hypotheses:

- Mechanical properties of cell-cell junctions depend on whether these junctions are homocellular (CM-CM) or heterocellular (CM-MFB) and this impacts on the heart, in particular junctional mechanics change in different diseased models.
• Dynamism of heterocellular junctions is regulated by Cx43 and partially regulated by actin filaments.

Aims:
• To measure YM at the CM-CM, CM-FB and CM-MFB junctions.
• To quantify composition of homo- and heterocellular junctions in healthy and diseased models following immunostaining, using novel Junctional Mapper software.
• To investigate the novel role of Cx43 in regulating junction dynamics using SICM, study the functionality of the formed junctions using parachute assay and test the role of actin in this process by specific inhibitor application.

6.2 Materials and Methods

6.2.1 Neonatal rat cardiomyocyte and fibroblast isolation
To investigate junctional structural composition, YM and dynamism neonatal rat cardiomyocyte model was used for these experiments. 1-3 day old Sprague-Dawley rat pups were collected from the animal house. Following 5 % isoflurane sedation and a cervical dislocation, as a secondary Schedule 1 method confirmation, hearts were harvested. Cardiac fibroblasts and myocytes were isolated using the Miltenyi Biotec Neonatal Heart Dissociation Kit. Detailed procedure is described in the Materials and Method chapter. Cell cultures were plated on the 13-mm glass cover slips or glass MatTek dishes and cultured in the M199 media (Sigma) enriched with 10% calf serum (NCS, Biosera), 1% Vitamin B12 (Sigma), 1% L-glutamine (Sigma) and 1% antibiotics (Sigma). For the experiment cardiomyocytes and fibroblasts were used on the isolation day, whereas myofibroblasts were converted from fibroblasts following a 7-day culture.

6.2.2 Adult control and MI fibroblast isolation
Adult male Sprague-Dawley rats were used to generate the MI model as described in chapter 2.1.3 (A. R. Lyon et al. 2009). During the adult cardiomyocyte isolation, supernatant is obtained as a byproduct, which is further used for the fibroblast isolation. Received supernatant was centrifuged 10 min, RT, 1000 x g. Obtained fibroblast pellet was washed 3 times with PBS. After the final wash cells were suspended in DMEM culture media supplemented with 10% fetal bovine serum (FBS), 1% antibiotic and 1% L-glutamine and plated in T-25 culture flasks.
6.2.3 Contact Young’s modulus determination
Neonatal cardiomyocytes (130,000 per cover slip) and fibroblasts (60,000 per cover slip) were plated at the same time and cultured together. Three types of co-cultures were analyzed:
   a) CM (1 d.o.) + FB (1 d.o.)
   b) CM (7 d.o.) + MFB (7 d.o.)
   c) CM (7 d.o.) + MFB (14 d.o.)

Following the respective culture time samples were scanned and analyzed YM at the junction as described in chapter 4.2.2.1.
Culture media was removed and substituted with the scanning physiological solution.

6.2.4 Cell-cell dynamism quantification
SICM was used to study cell mobility. 60 μm x 60 μm images of the cell co-cultures were acquired. Next, a 15 μm x 15 μm high resolution image of the cell contacts were generated. In order to examine the contact dynamism a ‘scan loop mode’ was used, where the same sample area was continuously scanned for 45-60 minutes. Images were processed in the CellTrack software (Sacan, Ferhatosmanoglu, and Coskun 2008). Software randomly allocates indication points within the cell junction area. Using the software, pixel per frame information for each point was obtained. Next, based on the time at which each image was acquired and the image pixel size (512 x 512 pixels), an average of the cell-cell movement (μm/min) was possible to calculate (Fig.59). Cell samples were kept in the scanning buffer for the whole time of the experiment.

![Figure 59. Schematic of the CM-MFB dynamism calculation using CellTrack software (Schultz, Swiatlowska, Alvarez-Laviada et al., FASEB accepted).](image)

6.2.5 Cell treatment
4-phenyl butyrate (4-PB) has been shown to upregulate the amount of Cx43, thus was used as the junctional modulator in the dynamism experiments (Jia et al. 2012). (Myo)fibroblasts were incubated with 1mM 4-PB at 37°C, 1% CO₂ for 48 hours. Cx43 down-regulation was obtained by using Cx43-specific siRNA or eGFP Cx43-shRNA lentiviral construct.
Neonatal cardiomyocytes were incubated with 10μm phenylephrine at 37°C, 1% CO₂ for 48 hours to induce in vitro hypertrophy model.
6.2.6 Junctional proteins visualization

Cells were either fixed for 10 minutes in 4% PFA or for 5 minutes in ice cold methanol. Permeabilized in for 15 minutes with 0.5 % Triton X-100 and then blocked for 1 hour at RT (10% horse/calf serum diluted in PBS). Samples were incubated with primary antibodies diluted in the blocking buffer for 2 hours at RT, washed once in PBS and incubated with secondary antibodies for 1 hour at RT. DAPI was used as a nuclear staining. Samples were washed in PBS as well as water and mounted using ProLong Gold (Thermo Fisher) or Mowiol (Sigma). Samples were imaged using Zeiss Laser Scanning Microscope 780 Confocal inverted (Carl Zeiss, Germany) Plan Apochromat 63x/1.4 oil immersion at RT. Acquired images were analyzed using the Junctional Mapper software (Brezovjakova et al., eLife in revision).

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
<th>Species</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-vimentin</td>
<td>1:3000</td>
<td>chicken</td>
<td>Thermo Fisher</td>
</tr>
<tr>
<td>anti-β catenin</td>
<td>1:50</td>
<td>rabbit</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>anti-Cx43</td>
<td>1:1000</td>
<td>mouse</td>
<td>Merck Milipore</td>
</tr>
<tr>
<td>anti-Cx43</td>
<td>1:1000</td>
<td>rabbit</td>
<td>Sigma</td>
</tr>
<tr>
<td>anti-α SMA</td>
<td>1:1000</td>
<td>mouse</td>
<td>DAKO</td>
</tr>
<tr>
<td>anti-T cadherin</td>
<td>1:100</td>
<td>mouse</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>anti-desmoplakin</td>
<td>1:10</td>
<td>mouse</td>
<td>Gifted from D.Garrod, University of Manchester</td>
</tr>
<tr>
<td>anti-desmin</td>
<td>1:100</td>
<td>mouse</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>DAPI</td>
<td>1:1000</td>
<td>x</td>
<td>Thermo Fisher</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>1:1000</td>
<td>donkey anti-mouse</td>
<td>Thermo Fisher</td>
</tr>
<tr>
<td>Alexa Fluor 546</td>
<td>1:1000</td>
<td>donkey anti-rabbit</td>
<td>Thermo Fisher</td>
</tr>
<tr>
<td>Alexa Fluor 647</td>
<td>1:1000</td>
<td>donkey anti-chicken</td>
<td>Thermo Fisher</td>
</tr>
</tbody>
</table>

Table 11. Primary and secondary immunocytochemistry antibody list.

6.2.7 Contact functionality assessment

Parachute assay was performed in order to examine the functionality of established contacts in different cell co-cultures. Following the respective treatment, fibroblasts were incubated with 5 μM Cell Tracker Orange (CTO)/FarRed (FR) and 1 μM Calcein/Calcein Orange-AM for 30 minutes at 37°C, 1% CO₂. Cell Tracker is a permeable dye that acknowledges the formed contact between cells, whereas Calcein-AM is a non-transferable dye used for cell labeling. Next, cells were trypsinized and added to cardiomyocyte culture to form cell contacts. Cell co-cultures were kept for 24 hours that was sufficient to establish connections (Fig.60). Live cell imaging was performed using Zeiss Laser Scanning Microscope 780 Confocal inverted (Carl Zeiss, Germany) Plan Apochromat 20x. Acquired images were then manually counted. In each image, number of dually labelled fibroblasts and attached to them Calcein-AM-positive cardiomyocytes were counted. Data were presented as a change from control.
6.2.8 Cellular junction protein quantification

A new junctional protein analysis software Junction Mapper (Brezovakova et al., eLife, in revision) was used to study the coupling between different cell types. Semi-automated program employed in this work allows for a detailed examination of cell-cell interface based on a number of defined parameters. In this study the hypertrophy effect on the level and distribution of β-catenin, desmoplakin and Cx43 were analyzed in the CM cultures. Further work focused only on Cx43 measurement in control and hypertrophy CM-MFB co-cultures. Cell co-cultures were fixed, after specific pharmacologic manipulation. Acquired immunofluorescent images were uploaded and a skeletal map was generated that indicates cell membrane edges based on a reference protein membrane stain. As CM-MFB not always form straight junctions, those were manually adjusted where necessary. Cell corners were defined to indicate single junctions. Next, junction edges were dilated to cover the entire junctional staining. Lastly, generated raw values were used to calculate specific morphometric characteristics.

Here, three parameters were analyzed for each protein staining (Fig.61).

1) Percent of interface occupancy [%], measures the area of the protein of interest in the contacting membranes between two neighboring cells.

2) Intensity per interface area [A.U./pixel²], calculates the intensity of the protein of interest in the contacting membranes between two neighboring cells.

3) Cluster density [A.U./pixel²], demonstrates the junctional protein intensity within the total area of the staining of the junctional marker.

The first two parameters show a general picture of how much of the contacting interface is covered by the junctional marker and the intensity of the staining at the cell-cell contact. Whereas, the third factor represents more a functional property of the protein as it characterizes how dense is the protein distribution within a given area, and thus reports the relative spatial organization of the protein. In total 15 images from 3 independent isolations were analysed for each model.
6.3 Results

6.3.1 Cardiomyocyte coupling to fibroblasts reduces contact YM, but myofibroblast coupling has an opposing effect

In the cardiac pathophysiology a vast number of changes takes place trying to restore the disrupted homeostasis. Alterations in the mechanical properties are part of the occurring cardiac modifications as it was shown in the chapters 4 and 5. To fill the gap caused by the cell death, cardiomyocytes form connections with the infiltrating fibroblasts. To date, the mechanical changes at the cell-cell interface have been obscure. In order to get more insight into the biomechanics at the lateral cell edges, SICM scanning and the pressure-based YM analysis were used as previously described (chapter 3). Junctions between cardiomyocyte-cardiomyocyte (CM-CM), cardiomyocyte-fibroblasts (CM-FB) and cardiomyocyte-myofibroblasts (CM-MFB) were established and examined. Freshly isolated cardiomyocytes, following a 1 day in culture exhibit higher contact YM as compared to homocellular cultures after 7 days (1 day CM-CM 3.2±0.28 kPa vs 7d CM-CM 2.06±0.2 kPa, p<0.01) (Fig.62). An opposite effect is seen at the heterocellular junctions. One day old cardiomyocyte coupled to...
1 day old fibroblasts display lower interface YM (1 day CM - 1 day FB 1.89±0.12 kPa vs 1 day CM-CM 3.2±0.28 kPa, p<0.05). However, co-culture of 1 day old cardiomyocyte with 7 days old myofibroblasts significantly increases the YM at the junction as compared to co-culture with fibroblasts (1 day CM - 7 day MFB 3.73±0.39 kPa vs 1 day CM - 1 day FB 1.89±0.12 kPa, p<0.05). Prolonged culture does not change the contact YM between cells (7 day CM - 14 day MFB 3.8±0.4 kPa vs 1 day CM - 7 day MFB 3.73±0.39 kPa, p>0.05).

Figure 62. Scanning Ion Conductance Microscopy YM measurement at the cell homo- and heterocellular junctions in CM-CM, CM-FB, CM-MFB pairs. A) 10µm x 10µm Young’s Modulus maps [kPa] and surface topographical [µm] maps. B) Graph representing YM values at the cell contacts. Data represented as mean ± SEM. Number of measured samples: 3-6 cell pairs / 3-4 isolations. Data were analysed using non-parametric ANOVA test, * p<0.05, ** p<0.01, *** p<0.001.

6.3.2 Junctional Mapper demonstrates more meticulous changes at the ID during disease

Previous paragraph shows that CM-FB and CM-MFB coupling differ from the homocellular pairing between cardiomyocytes. Next, I aimed to investigate the difference in the protein composition at the contacting membranes. The electrical and mechanical cell-cell coupling at the ID have been implicated in the heart disease. In the arrhythmogenic right ventricular
cardiomyopathy α-catenin mutations have been found that link actin cytoskeleton and the AJ cadherin (Wickline et al. 2016) and genetic alterations have been reported in desmoplakin located at the desmosomes (Rampazzo et al. 2002). On the other hand, a rat hypertrophy model exhibits upregulation in specific cadherin and catenin isoforms (Craig et al. 2010). In order to study in more details changes of the structural composition at the ID, a novel Junctional Mapper software have been used. As more findings indicate that IDs behave as a joint complex rather than three separate structures, I sought to investigate proteins from AJ, GJ and desmosomes in the model of neonatal cardiomyocytes coupled to myofibroblasts. Three parameters have been analyzed: interface occupancy, intensity per interface area and cluster density (Fig.63). Beta-catenin is a member of AJ that connects all the molecules in the complex. Quantification shows that in the hypertrophy model intensity per interface area (hypertrophy 27.16±0.6 A.U./pixel² vs control 17.87±0.37 A.U./pixel², p<0.001) and interface occupancy (47.62±0.74 % vs control 35.15±0.7 %, p<0.001) are significantly higher compared to control cells. However, no difference is seen in the cluster density (hypertrophy 54.15±0.62 A.U./pixel² vs control 56.52±0.6 A.U./pixel², p>0.05).
Chapter 6 Investigating cell interface mechanics in a cell-cardiomyocyte model

Figure 63. Beta-catenin level and distribution at the cell-cell junctions in CM-CM and hypertrophy CM-CM models. A) Representative immunofluorescent micrographs; nuclear staining DAPI (blue), β-catenin (green). Scale bar 25 µm. B) Graph representing analysis of Junctional Mapper software parameters. Data represented as mean ± SEM. Number of measured samples: 30-35 junctions/3 isolations. Data were analysed using Student t-test or Mann-Whitney test, *** p<0.001.

Next, the terminal protein in the desmosomal complex that binds with the intermediate filaments, desmoplakin was analyzed. Significant increase is observed in the intensity per interface area (hypertrophy 20.98±0.57 A.U./pixel² vs control 17.71±0.4 A.U./pixel², p<0.001) (Fig. 64). On the other hand, no change in the interface occupancy (hypertrophy 34.93±0.77 % vs control 39.01±1.17 %, p>0.05) and cluster density (hypertrophy 59.24±0.72 A.U./pixel² vs control 60.35±1.12 A.U./pixel², p>0.05) is noted.
Chapter 6 Investigating cell interface mechanics in a cell-cardiomyocyte model

Figure 6.4. Desmoplakin levels and distribution at the cell-cell junctions in CM-CM and hypertrophy CM-CM models. A) Representative immunofluorescent micrographs; nuclear staining DAPI (blue), desmoplakin (green). Scale bar 25 µm. B) Graph representing analysis of Junctional Mapper software parameters. Data represented as mean ± SEM. Number of measured samples: 36-40 junctions/3 isolations. Data were analysed using Student t-test or Mann-Whitney test, *** p<0.001.

Lastly, Cx43 have been analyzed as the main participant in the gap junction panel. Observed changes demonstrate a severe change at the cell-cell adhesion. Hypertrophy model exhibit a remarkable enhancement in all of the analyzed parameters, intensity per interface area (hypertrophy 10.85±1.43 A.U./pixel² vs control 4.42±0.54 A.U./pixel², p<0.001), interface occupancy (hypertrophy 13.14±1.5 % vs control 6.25±0.62 %, p<0.01) and cluster density (hypertrophy 72.12±2.3 A.U./pixel² vs control 64±2.02 A.U./pixel², p<0.05) (Fig. 6.5).
Figure 6.5. Cx43 levels and distribution at the cell-cell junctions in CM-CM and hypertrophy CM-CM models. A) Representative immunofluorescent micrographs; nuclear staining DAPI (blue), Cx43 (green). Scale bar 25 µm. B) Graph representing analysis of Junctional Mapper software parameters. Data represented as mean ± SEM. Number of measured samples: 40-45 junctions/3 isolations. Data were analysed using Student t-test or Mann-Whitney test, * p<0.05, ** p<0.01, *** p<0.001.

6.3.3 Myofibroblast coupling reduces junctional Cx43 area and intensity but has a different effect on cluster density that is hypertrophy-dependent

Due to the major Cx43 alterations observed in figure 63 I sought to investigate the level and distribution of this molecule in a control (CM) and hypertrophic cardiomyocyte (hCM) model coupled to myofibroblasts where such interactions are present in the failing heart. The results have shown a striking difference in Cx43 distribution at the heterocellular junctions (Fig.66). Intensity per interface area significantly dropped compared to respective homotypic controls (CM-MFB 1.4±0.33 A.U./pixel² vs CM-CM 4.42±0.54 A.U./pixel², p<0.01; hCM-MFB 1.05±0.32
A.U./pixel² vs hCM-hCM 10.85±1.43 A.U./pixel², p<0.001). CM-MFB contacts exhibited similar Cx43 intensity levels, irrespective of the induction of hypertrophy. Identical result is seen when the interface occupancy was quantified, with a marked and significant decline observed in heterotypic contacts. Myofibroblast models values declined (CM-MFB 1.95±0.4 % vs CM-CM 6.26±0.62 %, p<0.01; hCM-MFB 3.14±0.78 % vs hCM-hCM 13.14±1.5 A.U./pixel²; p<0.001).

Interestingly, cluster density did not change in the CM-MFB co-culture compared to pure CM-CM culture (CM-MFB 63.9±4 A.U./pixel² vs CM-CM 63.98±2 A.U./pixel², p>0.05). However, inducing hypertrophy in cardiac myocytes demonstrates a more complex situation. Hypertrophic homocellular pairs exhibit higher cluster density compared to control cardiomyocyte-cardiomyocyte (hCM-CM 72.12±2 A.U./pixel² vs CM-CM 64±2 A.U./pixel², p<0.05). On the other hand, when hypertrophic cardiomyocyte couple to myofibroblast the density goes significantly down as compared to cardiomyocyte-myofibroblast (hCM-MFB 32±2 A.U./pixel² vs CM-MFB 63.9±4 A.U./pixel², p<0.001).
Figure 6.6. Cx43 levels and distribution at the cell-cell junctions in CM-CM, hCM-hCM, CM-MFB and hCM-MFB models. A) Representative immunofluorescent micrographs; nuclear staining DAPI (blue), Cx43 (green). Scale bar 10 µm. B) Graph representing analysis of Junctional Mapper software parameters. Data represented as mean ± SEM. Number of measured samples: 20-22 junctions/3 isolations. Data were analysed using Student t-test or Mann-Whitney test, * p<0.05, ** p<0.01, *** p<0.001.
6.3.4 CM-MFB coupled pairs form functional GJ contacts.
In the first paragraphs of this chapter, CM-MFB heterocellular coupling has altered mechanical coupling at the cell-cell interface. Further investigation has also shown changes in the protein distribution and levels in the hypertrophy, fibroblast-coupled or combined models. Recent findings from our group show that, apart from its main role in electrical signal transfer, Cx43 is also important for the mechanical coupling. To build up on the previous findings, this section will examine the functionality of the established contacts. Downregulation of Cx43 in myofibroblasts has shown to reduce cell-cell dynamism (Schultz 2015). Myofibroblasts were dually labelled with a permeable (Calcein-AM) and non-permeable dye (Cell Tracker Orange). Performed parachute assay has shown that using Cx43 siRNA or shRNA diminishes by 50% the gap junction dye transfer. On the other hand, treating both cell types CM-MFB or just MFB with 4-PB (Cx43 enhancement), remarkably upregulates the dye uptake by 60% compared to control (Schultz, Swiatlowska, Alvarez-Laviada et al., 2019) (Fig.67).
6.3.5 Disrupting actin filaments slows down the contact movement and reduces dye transfer in neonatal CM-MFB co-cultures.

Cx43 is delivered to the cell membrane via a network of spread actin filaments and microtubules. However, using Latrunculin B an actin-disrupting drug, inhibition of protein supply to the cell surface is observed (Smyth et al. 2012). Also, disturbing α-smooth muscle actin in MFB have been shown to suppress the adverse effect that these cells exert on CM (Rosker et al. 2011). Here, cell dynamism was investigated following the actin polymerization inhibitor, Latrunculin B treatment in neonatal rat CM-MFB model (Fig.6.8). Results show that disrupting α-SMA reduced cell motility (CM-MFB Lat-B 0.017±0.01 μm/min vs control...
0.149±0.03 μm/min, p<0.001). As a consequence of the fibers disruption and declined Cx43 delivery to the membrane due to the Lat-B treatment, Calcein-AM dye transfer dropped to 25%.

Figure 6.8. Effect of Latrunculin B on contact dynamism and functionality. A) Representative SICM neonatal CM-MFB contact scans B) Cells speed movement graphs. C) Parachute assay immunofluorescent images; Calcein-AM (green), Cell Tracker Orange (red). Dually labelled MFB are indicated with a black arrow, whereas blue show the Calcein-AM-positive cardiomyocytes. Scale bar 100 μm. D) Parachute assay quantification graphs. Data represented as mean ± SEM. Number of measured samples: 5 images/3 isolations. Data were analysed using Student t-test, *** p<0.001
6.3.6 Inhibiting actin polymerization slows down the contact movement and reduces dye transfer in adult MFB.

Next, cell dynamism was tested in adult myofibroblasts pairs from control and MI rat models. Latrunculin B treatment exerted the same effect on the dynamism of adult cells as on neonatal (Fig.69). Disrupting actin fibers significantly decreased dynamism of control MFB and MI MFB (control: Lat-B MFB-MFB 0.23±0.05 μm/min vs untreated MFB 0.12±0.02 μm/min, p<0.05; MI: Lat-B MFB-MFB 0.03±0.004 μm/min vs untreated MFB 0.07±0.01 μm/min, p<0.01). Functional consequence is also observed in the dye transfer assay. Latrunculin B treatment markedly decreased Calcein-AM transfer in both models. Control MFB demonstrate a 42% decrease in the dye movement and a more pronounced difference is seen in the MFB from an MI model displaying 74% drop.
Figure 6.9. Latrunculin B reduces dynamism and dye transfer in control and MI MFB cultures. Representative SICM adult rat control (A) and MI (C) MFB-MFB contact scans. Cells speed movement graphs of control (B) and MI (D). Parachute assay immunofluorescent images of control (E) and MI (G) MFB cultures; Calcein-AM (green), Cell Tracker Orange (red). Dually labelled MFB are indicated with a black arrow, whereas blue show the Calcein-AM-positive cells. F) and G) Parachute assay quantification graphs of control (F) and MI (H). Scale bar 100 µm. Data represented as mean ± SEM. Number of measured samples: 5 images/3 isolations. Data were analysed using Student t-test, p<0.05*, p<0.01**, *** p<0.001.
6.3.7 Latrunculin B treatment reduces Cx43 functionality

Using the Junctional Mapper software Cx43 levels at the CM-MFB interface were analyzed (Fig. 70). No difference was seen in the interface occupancy (Lat-B 1.98±0.52 % vs control 1.86±0.39 %, p>0.05), as well as in the intensity per interface area (Lat-B 1.04±0.27 A.U./pixel² vs control 1.28±0.3 A.U./pixel²; p> 0.05), in the two analyzed groups. On the other hand, cluster density has shown a significant reduction following the α-SMA disruption (Lat-B 49.24±3.59 A.U./pixel² vs control 63.89±4 A.U./pixel², p<0.01).

![Figure 70](image_url)

**Figure 70.** Effect of Latrunculin B on Cx43 level and distribution. A) Control and MFB-only Lat-B treated co-cultures stained for Cx43(green), α-SMA (red), vimentin(magenta) and DAPI (blue). Scale bar 10 µm. B) Data represented as as mean ± SEM. Number of measured samples: 5 images/3 isolations. Data were analysed using Student t-test, ** p<0.01.
6.4 Discussion
Chapter 6 focuses on the mechanics in homo- and heterocellular junctional cell pair models. Junctional YM in the CM-CM pairs decreases with time, however if the CM is coupled to MFB the values is significantly increased, being constant over time. New junctional mapper software has shown significant changes in the junctional protein level and distribution in control versus hypertrophy CM-CM. The biggest alterations were observed in the gap junctional protein, Cx43, which was next shown to be decreased in the hypertrophic CM – MFB pairs. Lastly, I have shown that CM and MFB form a functional contact via Cx43 which serves not only as electrical signal transducer but also as regulator of cell-cell junction dynamism. Additionally, disrupting actin fibres in CM-MFB and MFB-MFB models significantly reduces contact dynamics and functionality.

6.4.1 Junctional mapper as a tool to precisely delineate changes at the ID in control and disease
The contractile heart performance is only possible by fixed organ organization. Billions of cardiomyocytes, as the basic functional unit in the heart, work in a coordinate manner in order to transmit electrical signals. This shows that cell-cell integrity is of great importance. Located at the lateral ends, cardiac myocyte IDs are made up from three main complexes: AJ, GJ and desmosomes, which have been shown to be dysregulated in cardiac diseases leading to hypertrophy (Sepp, Severs, and Gourdie 1996; Tansey et al. 2006). Here, a commonly used in vitro hypertrophy-inducible model was generated to investigate the protein patterns at the ID complexes using different Junctional Mapper parameters (Miragoli et al. 2011). Firstly, I analyzed the β-catenin as an AJ anchoring element between N-cadherin and cytoskeletal actin. The hypertrophy stimulus shows increase in β-catenin protein area and intensity at the cell-cell interface. However, no significant change is seen at the β-catenin density, which suggests that clusters of newly delivered β-catenin are found in the same organization and density as in control samples (Fig. 62). Data from Zhang et al., 2009 show that knocking out β-catenin from the TCF/LEF1 signaling pathway restricts the cells from growth, whereas adenoviral β-catenin overexpression guides cardiomyocytes to hypertrophy phenotype (Lee 2017), therefore looking at this protein in our model is a good starting point. The result on the β-catenin pattern corroborated with genetic removal of the protein as no functional changes
has been shown to the whole heart most likely via compensation by other catenin isoform (Zhou et al. 2007).

Even stronger adhesion between cardiac myocytes is provided from the desmosomal junctions. Data presented in this work show that inducing hypertrophy with phenylephrine increases the intensity levels of desmplakin along the contacting interface of cardiomyocytes but does not cause any change in other parameters. Desmplakin is the linking protein between the intermediate filament desmin and the desmosomal complex. Its importance has been shown in different cardiac pathologies where desmplakin is found mutated. For example, Carvajal syndrome is characterized by left ventricular cardiomyopathy due to desmplakin point mutation (Norgett et al. 2000). Rare cases of juvenile cardiomyopathies show cardiac-specific desmplakin I mutation leading to heart failure at an early age (Williams et al. 2011; Uzumcu et al. 2006). In addition, arrhythmia and sudden cardiac death are known to be present in arrhythmogenic right ventricular dysplasia (ARVD) that is caused by desmplakin missense mutation (Yang et al. 2006; Alcalai et al. 2003). Closer investigation of ARVD has shown that in some cases the left ventricle is also affected as a first target, often wrongly diagnosed as myocarditis, heart inflammatory disease, or dilated cardiomyopathy (Lopez-Ayala et al. 2015). A small population study has found desmplakin mutation that leads to protein truncation (85% length) at the N-terminal where it binds to desmosomal plakophilin and plakoglobin leading to tissue hypertrophy. Unfortunately, much less is known on the desmplakin function in cardiac hypertrophy. Desmosomal complex plays a major role in balancing and tuning the force at the ID between contacting cells. Whereas, desmplakin is a linker between the ID composite and the cytoskeleton, specifically IF. As no mutation was introduced in our model, we could speculate that this direct binding is of paramount importance, hence is preserved in the early stage of hypertrophy. Here, the hypertrophic changes were assessed following a short-term stimulation. Future work should examine if long-term hypertrophy affects cardiac desmplakin level and distribution.

Lastly, in our 48-hour in vitro hypertrophy model I’ve examined also changes in the Cx-43, gap junctional protein. Results have shown a notable increase of all protein parameters (% interface occupancy, intensity per interface area, cluster density) (Fig. 64), resembling the early stage of hypertrophy onset. This alteration could partially contribute to the majority of sudden cardiac deaths occurring at the very beginning of the heart failure (O’Mahony and Elliott 2014). Current data show that Cx43 level is variable in different pathologies; it is down-
regulated in dilated cardiomyopathy (DCM) and ischemic cardiomyopathy (ICM). Whereas, in rat and human models Cx43 is upregulated in the early stage of disease and later declines as the pathology progresses (Kostin et al. 2004; Formigli et al. 2003). As a future direction, it will be interesting to investigate how the protein localization changes with the disease development?

Intercalated discs are proposed to act as a functional syncytium that is supported by the available data. More in-depth work shows that components of the complexes coordinating mechanical cell-cell coupling are able to mix together. Franke et al., has introduced for the first time the “hybrid junction” term as a name for the entire ID structure (Franke et al. 2009). Immunoelectron microscopy study has shown desmoplakin and plakophilin in both, desmosome- and adherens-like junctions (Al-Jassar et al. 2013). Interestingly, this mixed junction panel is only found in the hearts of higher vertebrates suggesting a greater need to maintain the integrity of a four chamber heart (Li and Radice 2010). Also, N-cadherin removal reduces desmosome level in intercalated discs but not the other way around, which means that the AJ is the first complex to be established at the cell-cell edges (Yin and Green 2004; Vermij, Abriel, and van Veen 2017). Moreover, AJ and desmosomes also interact with GJ. N-cadherin KO mouse has reduced number of Cx43 in the ventricle leading to sudden cardiac death (Li et al. 2005). Kam et al., (2018) has shown that desmosomes, specifically desmoplakin, regulate GJ through inhibition of Ras/MAPK and lysosomal Cx43 degradation. This shows that looking not only at a single protein but rather a complex of proteins by taking a holistic approach is necessary.

In recent years, imaging techniques has greatly improved and shed more light on the protein organization at the cellular level. However, the development of quantitative analysis of the imaged protein molecules have not changed that dramatically. Still, most of the quantification techniques are based on the use of ImageJ software that provides basic information. For instance, Terryn et al., (2013) used the intensity peak parameter as a simple way to quantify the junction structure, so did Downie et al., (2016). Similarly, Wang et al., (2019) utilized ImageJ but with a different approach where the automatic segmentation of pixel count in the region of area wasn’t that precise and re-segmentation after visual assessment was needed. Whereas, Lu et al., (2018) proposed a new software that allows image classification and morphology analysis that is relatively time consuming.
Chapter 6 Investigating cell interface mechanics in a cell-cell cardiomyocyte model

In this thesis, a novel, straightforward Junctional Mapper software has been used to validate the cardiomyocyte ID proteins distribution and level using a variety of parameters. As the AJ, GJ and desmosomes slowly emerge as a functional continuum, using this program would be an excellent approach to investigate the changes in all three complexes at the same time in order to get more insights into their functional interaction in health and disease. Next, due to a marked Cx43 change in the diseased model and significance of the GJ in the heart functionality, further work is focused on this protein.

6.4.2 Heterocellular junction Cx43 distribution

Facing the ambiguities on the Cx43-dependent heterotypic communication, I aimed to look into the localization and distribution of Cx43 at junctions as a reporter of gap junction assembly and function. Cardiomyocyte binding to myofibroblast significantly reduced the interface occupancy and intensity per interface area, but demonstrated no change in Cx43 cluster density, which describes how densely spread is the protein within a specified region. This suggests that the leftover Cx43 between CM-MFB is functionally equal to the CM-CM GJ protein. Myofibroblast coupling to hypertrophic cardiomyocytes display a similar level of decrease in the interface occupancy and intensity per interface area as compared to CM-MFB, indicating no further change in Cx43 area and intensity at the junction following hypertrophy induction. One of the potential reasons for Cx43 decline in both models is the protein redistribution in the cell edge (Fontes et al. 2012). Interestingly, a notable reduction in cluster density is seen in the hCM-MFB co-culture. The cause of the observed result is not known, but possibly a major modification of the ID complexes during hypertrophy impacts on the Cx43 functionality.

In the failing heart a vast number of adaptive events is triggered in order to restore the functional balance (Coats 2000; Lyon et al. 2009). However, such prolonged adjusting state is converted to maladaptive actions. At the cardiac injury site, loss of cardiomyocytes is substituted by the infiltrating fibroblasts that form a scar tissue. Due to a new environment such as entering cytokines and mechanical stimuli, fibroblasts undergo phenotypic change to myofibroblasts (Kendall and Feghali-Bostwick 2014). This cell type is characterized by higher level of α-smooth muscle actin (α-SMA) and soluble factors release, such as TGFβ-1 that is proposed to induce cardiac hypertrophy and further promote fibroblast proliferation (Popova et al. 2010; Schultz et al. 2002). The formed scar tissue alters the heart function, because it is
not capable of performing the same work as native myocardium (Driesen et al. 2007; Shinde and Frangogiannis 2014). However, on the border of the scar, viable cardiomyocyte form heterocellular contacts with (myo)fibroblasts. The current concept confirms the presence of low number of electrotonic connections between myocyte-active regions in the heart and (myo)fibroblasts \textit{in vitro} (P. Zhang, Su, and Mende 2012). However, \textit{in vivo} such connection is not that obvious, and in the last 30 years only few groups managed to present reliable data. Only recently more findings prove the presence of such interaction (Quinn et al. 2016; Mahoney et al. 2016; Camelliti, Borg, and Kohl 2005). A novel study from Roell et al., (2018) shows a direct injection of Cx43 lentiviral construct to the non-cardiomyocytes at the acute infarction site, eventually improving conduction velocity and showing a Cx43-dependent heterocellular functional interplay.

6.4.3 Cx43-dependent dynamism
To obtain further insight in the interaction at the heterocellular cell pairs, mechanical properties, specifically contact YM was examined. Alfa-SMA negative cells, for example CM or FB, do not affect the interface YM to such a degree as seen in the MFB (Fig.63). This confirms that CM-MFB coupling not only disrupts functional but also mechanical properties. It is known that (myo)fibroblast connections slow down cardiomyocytes conduction velocity, but the low number of GJ electrical connections may not be sufficient to cause this change. Differentiated myofibroblasts are characterized by the presence of α-SMA stress fibers that provide contractile properties, which are different than those observed in cardiac myocytes (Hinz et al. 2004). This force generated feature is especially exploited by myofibroblasts during the wound closure. A positive correlation has been shown between increased number of α-SMA stress fibers and upregulated contractile work. Findings from Thompson et al., show that silencing the CM-MFB mechanical junction component, N-cadherin, can improve conduction velocity (Thompson et al. 2011). As ID junctions can possibly function as a one complex, we cannot rule out that other junctional proteins could participate in this mechanical interaction. And so, results from our lab demonstrated a non-canonical mechanical function of Cx43, when studying cell-cell border movement. MFB have been shown to be very motile as well as the contacts formed between each other. In contrast, CM make up stable cardiac interactions and do not present high dynamism. This has been presented to be dependent on the Cx43 protein as confirmed by protein modulation, downregulation and upregulation. Using a rabbit model,
in vivo, a dye transfer between CM and MFB convincingly demonstrate the functional coupling between these two cell types in the heart (Camelliti, Borg, and Kohl 2005). Our in vitro experiment using a parachute assay confirm that the CM-MFB junctions are active. Moreover, Cx43 down-regulation by silencing or internalization is reflected in lower dye transfer. However, when forcing Cx43 expression, the relation becomes more complex. 4-PB has been shown to act as Cx43 agonist and up-regulates protein level in cardiac myocytes (Jia et al. 2012). Treating the CM-MFB co-cultures has shown a significant decrease in contact motility simultaneously increasing the dye transfer. However, MFB only 4-PB incubation demonstrates no change in contacting membrane but an increased dye transfer. This result shows that the pharmacological compound acts differently on MFB when compared with CM. MFB tend to form weak junctions between each other, exhibiting a high degree of plasticity, but are capable to make a connection with CM even 300 µm away (Gaudesius et al. 2003; Camelliti, Borg, and Kohl 2005). Following the heterotypic junction formation, the binding is stable and strong enough to reduce MFB dynamism.

To date, vast number of studies has shown the negative effect that MFB exert on CM following heterotypic junction formation. Here, I have shown a significant increase in junction YM and Cx43 dependent modified cell-cell interface dynamism induced by MFB. Rosker et al., (2011) reported that α-SMA stress fibers markedly contributes to the arrhythmogenic effect and by targeting this structure using a specific actin polymerization blocker that effect can be diminished. To get more mechanistic insight, I tested the effect of Latrunculin B, actin polymerization inhibitor, on the heterotypic contact dynamism. It is known that actin also links to AJ, but recent study from Tang et al., (2012) present that the actin pools at AJ are resistant to Latrunculin, therefore our pharmacological intervention will not directly affect that structure. Alfa-SMA stress fibers are imperative to cell-cell coupling and functional interaction in both models, neonatal CM-MFB, adult control and MI MFB-MFB. In cardiac myocytes, it was believed that Cx43 movement to the membrane is only regulated by microtubules. New evidence show that actin network is an important player in the protein trafficking (Smyth et al. 2012). Further findings from this group also demonstrate that truncated Cx43 C terminus isoform, GJA1-20k, is responsible for actin organization, which in turn regulates microtubule spatial arrangement (Basheer et al. 2017). Therefore, modulating actin filaments can result in not full-length channel assembly. Using Junctional Mapper software we saw that the Cx43 number and intensity in the whole CM-MFB junction does not change. However, the
functionality is severely disrupted in the Lat B-treated samples, meaning that α-SMA is necessary to form functional GJ in MFB. Our data suggest that possibly a similar process of Cx43 delivery to the cell membrane is present in myofibroblasts and the lack of all isoforms can affect the GJ functionality.

Results in this chapter show that cell-cell interaction is a very dynamic and highly regulated process. Cardiomyocyte coupling to fibroblasts and myofibroblasts significantly changes contact YM, by reducing or increasing the values, respectively. Using novel junction analysis software I was able to precisely investigate changes in ID proteins in CM-CM hypertrophy model. Most significant changes were observed in Cx43 level and distribution, therefore I expanded studying this protein on the CM-MFB non-treated and PE-treated models. Both, MFB binding and hypertrophy resulted in Cx43 level and distribution downregulation. Moreover, a follow-up study on the Cx43 mechanical role shows that CM-MFB form functional GJ and MFB actin regulates Cx43-dependent contact dynamism.
Chapter 7

General discussion
7.1 Cardiac mechanical properties

Research in biology is mostly focused on biological and chemical factors that influence living systems, less attention is drawn towards physical factors. Physical properties of biological samples are routinely ignored, even though mechanical properties were first appreciated as equally important in biology at the end of the XIX century (Wolff 1986). In the following years biological methodology has moved significantly forward, new methods have been invented, such as genetic manipulation, sequencing, advanced microscopy etc., blunting even more the need to account for the physical (mechanical) properties of biological objects, therefore mechanobiology has been more and more neglected (Wang et al. 2014). However, not all biological phenomena can be explained by genomics and proteomics studies alone. Difference in shape and YM between different cells and tissues are left without explanation and purpose, if one only focusses on biological properties. In more recent years, after the genomics revolution gave its fruits and receded, mechanobiology came forward again. Now we are witnessing a renaissance in mechanobiology, as number of publications studying the role of physical forces in different biological samples increases steadily (Eyckmans et al. 2011). One of the organs that are inherently influenced by mechanics is the heart. It’s simply manifested by the need of a heart massage as an emergency first aid with a person experiencing a cardiac arrest. Manual application of mechanic stimulation forces the blood to leave the heart and also aims to bring back the heart rhythm. Looking into a smaller scale, heart mechanical properties are closely coupled with the electrophysiological properties (Takahashi et al. 2013). This phenomenon is known as Mechano-Electric Coupling. Active ion channels provide an intracellular gateway for calcium necessary for the contraction. Later the mechanical response acts in a feedback manner to propagate cardiac excitation. Electrical coupling and propagation in the heart have been studied extensively, whereas a more detailed studies of cardiomyocyte mechanics are needed.

Published literature shows that muscle fibers in the whole heart run in different directions and that the degree of orientation can vary from -70° up to +80° (Geerts et al. 2002; Arts et al. 2001). This structural variability allows for efficient performance of the mechanical and electrical functions of the heart. Thus, it shows that studying mechanical properties of myocytes is not as straightforward as horizontally aligned skin keratinocytes, since the multidirectional organization of the fibers should be taken into account. To date, several
studies investigated cardiomyocytes longitudinal YM along the cell axis, but not as many looked into the transverse direction (Borlaug et al. 2013; Yoshikawa et al. 2013; Borbély et al. 2005, 2009; Zile et al. 2015). In order to understand the mechanical function in the cardiac pathophysiology and designing new drug therapies basic knowledge is needed. Therefore, this thesis is focused on examining cell mechanics, in particular focusing on the role of transverse YM in the models of isolated single myocytes and their couples. First result chapter shows the impact of intracellular components on cell YM at baseline. Obtained findings direct to investigation of altered cell mechanical properties in diseased models. Subsequent work examines mechanical properties at the cell edges in CM-CM and CM-MFB models also demonstrating a novel mechanism of GJ protein in cell-cell dynamism.

7.2 Cardiomyocyte mechanical regulation in physiological and pathophysiological conditions.

A thorough investigation of the myocyte transverse YM at the baseline provided information which cellular components regulate cardiomyocyte mechanics. We concentrated our attention on two cell surface nano-domains on the plasma membrane of myocytes: z-groove and crest, an area between grooves. Based on previous reports from polyploidal giant and endometrial cancer cells on the correlation between actin filaments and cell YM (Salker et al. 2016; Xuan et al. 2018), I aimed to look into cytoskeletal proteins in cardiac myocytes. In fact, following actin polymerization blockage using cytochalasin D, a significant drop in cell YM is observed.

Recently, role of microtubules (MT), another generic cytoskeleton element, in cell mechanics has been revised. Using a new, high-resolution imaging technique, Robison et al., (2016) have shown a geometrical change in shape of MT at a µm-range between contracting and resting cardiomyocytes. Inhibitor analysis with a specific MT blocker, vinblastine, in this thesis has demonstrated that cells become softer without MT.

Yet, in this work we uncovered that another regulator of YM in heart muscle cells is plasma membrane, and in particular lipid rafts composed of cholesterol. Removing cholesterol using cyclodextrin from the lipid bilayer leads to stiffening of myocytes, which was rather unexpected. This surprising increase in cell YM could be explained by the accumulation of actin binding proteins and the formation of new connections between the membrane and the actin cytoskeleton as proposed by de Oliviera Andrade (2016). Interestingly, in a mouse model
deficient for a main protein component of caveolae, caveolin 3, we didn’t see such a big change in myocyte YM as in the cyclodextrin-treated cells. This shows that mechanical properties of plasma membrane, rather than the caveolae structure itself may be important.

When we analyzed cardiomyocyte domains, crest and z-groove, they both presented increased YM, but significance was only obtained in the latter, which is probably due to a low number of analyzed animals. Nevertheless, these results suggest that caveolae can contribute to cell mechanoproperties.

In a model of heart failure in rats, myocytes were isolated 16 weeks after inducing MI; a significant increase of cell YMs was observed. Previous data from our group and others have shown that in failing myocytes mitochondria change shape and become disorganized (Miragoli et al. 2016; Dague et al. 2014). Miragoli et al., proposed that mitochondria and microtubular network can modify cell mechanical properties in the diseased model. Using a mitochondria specific staining, I have shown that in the HF cardiac mitochondria become bigger, but their number is reduced. Electron microscopy further demonstrated that clustering of mitochondria is taking place underneath the membrane in failing cells.

On the other hand, former studies observed that MT in the failing heart form a more dense network than in healthy (Cooper 2006; Miragoli et al. 2016; Robison et al. 2016). I have shown that in failing myocytes the numbers of both acetylated and detyrosinated MT are increased, as well as the intermediate filament desmin, a detyrosinated MT-sarcomere anchoring protein. The role of detyrosinated MT and desmin in cell mechanics has been previously proposed (Robison et al. 2016). Interestingly, my further investigation has shown that the while the level of acetylated MT in the MI model is increased, the level of detyrosinated MTs is markedly decreased. Future work will be needed to unravel this mystery. In this thesis, the microtubular network has been presented as a considerable participant and a promising target for future work on cell mechanics.

In (2002) Knoll et al. proposed that a complex of three proteins: titin, MLP, and Tcap, serves as stretch mechanosensor. It is located at the Z-disc that borders the basic force generating unit, sarcomere. Titin exists in two isoforms: softer (N2BA) and stiffer (N2B) (Cazorla et al. 2000). My expression analysis has shown that in failing myocytes softer N2BA isoform level is increased whereas N2B isoform expression is decreased respective to normal cells. Additionally, drop in N2B form is accompanied by the decrease in RBM20, titin splicing regulatory factor. At the same time, I found that Tcap expression is downregulated in disease,
which is consistent with data published previously (Knöll et al. 2011). However, I haven’t noted any change in the level of MLP in the MI myocytes, possibly this is due to a low number of animals.

To date, titin and collagen were considered as the main proteins which regulate the YM of the myocytes and cardiac tissue (Yarbrough et al. 2012; Linke 2003). Others reported alterations in titin isoforms and/or phosphorylation levels leading to change in cell mechanics (LeWinter and Granzier 2010). Zile et al. (2015) confirmed recently that titin and collagen both modify tissue mechanics in a cooperative manner.

HF is widely recognized as a pathology with increased muscle load. Patients with a failing heart can be subjected to LVAD transplantation procedure, which unloads the diseased organ, reducing the load to recover the heart function or working as a bridge before transplantation.

In the present work, using partially mechanically unloaded rat hearts, that mimics the role of LVAD in human patients, I studied the physiology of load-deficient cardiac myocytes. Indeed, cells became softer than the failing cells, reaching the level comparable to normal cells. Even more intriguing, lower number of mitochondria was noted in unloaded cells, which was accompanied with a fall in organelle size, as compared to the failing cells. A trend towards lower protein level of desmin and MT PTMs was seen. Looking at the Tcap and MLP proteins no change was noted when comparing to the HF. On the other hand, titin mRNA analysis has shown an isoform ratio similar to control samples. These results and recently published work from our laboratory (Wright et al. 2018) led us to conclude that LVAD use leads to a partial cardiomyocyte recovery in advanced stage of heart failure. This hypothesis is summarized in Figure 71.
The Role of Load on Cardiomyocyte Structure

Figure 7.1. Schematic demonstrating changes in myocyte mechanical properties driven by different load. Following an upregulation in mechanical load (after MI) the most striking difference in cell morphology is increased cell size. Also, nanoscale surface imaging shows rearrangements and loss of regularity of features such as Z-grooves and T-tubule openings. Further, YM is higher. Mitochondria number drops, whereas average size increases, with higher level of microtubule PTMs and desmin. At the same time, the ratio of titin isoforms N2B/N2BA decreases, expression of RBM20 and Tcap decreases, but no change in MLP protein level is observed. However, removing mechanical load does not bring full recovery as commonly claimed. Load-deficient cells exhibit YM values similar to control but become atrophic. The size of mitochondria is reduced and the number drops even further as compared to MI model. No change in Tcap and MLP proteins are found. Lower microtubule PTMs, desmin, titin isoform ratio and RBM20 levels are present.

7.3 Relationship between cardiac mechanical load and cardiomyocyte YM

Heart muscle tissue develops from different embryologic origins to be specialized to form a four-chambered organ in all mammals. Along with region-specific physiological roles, the thickness of the heart muscle is location-dependent. Higher pressure requires more muscle mass to provide enough resistance, which is observed in the ventricles. Atria are much thinner as they don’t need to cope with high load. The variability in the thickness is also distinguished between right and left side of the heart, as the left ventricle in particular is responsible for pumping out the blood from the heart to the aorta which further distributes it to all organs in the body. It is clear that the mechanical load is not equally spread around the heart. According to the Starling law, heart muscle is able to adapt to the acute load changes such as increase in
contractility following applied stretch (Jacob, Dierberger, and Kissling 1992). This was also confirmed by Derumeaux et al., (2002) in an aortic banding rat model, which increases the load to the left ventricle, leading to hypertrophy. Removing the band after 2 months recovers normal contractility and cell size in the left ventricle. Ellison et al. (Ellison et al. 2012) have shown that both, overloading and unloading initiate tissue remodeling in order to meet current demands. On the other hand, prolonged mechanical alterations may have detrimental effects, leading to maladaptive modifications. In the same study Derumeaux (Derumeaux et al. 2002) left the aortic band for 9 months that caused irreversible changes. In this thesis, I have analyzed the YM of adult rat heart muscle cells subjected to different mechanical load. Figure 72 shows a comparison of the YM values between cardiomyocytes isolated from different models, arranged by increasing YM from softer to stiffer.

![Figure 72. Scanning Ion Conductance Microscopy YM measurement in Sprague-Dawley (SD) and Lewis (L) rat models. Summary graph depicting cardiac myocytes YM following exposure to a different mechanical load. Graph representing YM values in Z-groove (no pattern) and crest (chess pattern) domains. Data represented as as mean ± SEM. Number of measured samples: 6-8 cells / 3-4 isolations.](image)

Left ventricle is the main heart pumping chamber, which represents the part of the organ that is highly loaded even in the control condition. On the contrary, right ventricle is pushing the blood only to the lungs. The difference in the function is reflected in cell mechanical properties. When cardiac myocytes are compared between RV, LV and LV after MI in cardiac myocytes isolated from a Sprague-Dawley rat, we found that cells isolated from less work-demanding RV have lower YM as compared to left counterpart, although not statistically
significant; cells from failing hearts exhibit a substantial increase in YM as it was shown by us and others (Miragoli et al. 2016; Dague et al. 2014; Borbély et al. 2005). This has been reported to cause diastolic abnormalities in HF normal ejection fraction patients exhibiting upregulated end diastolic pressure as well as reduced stroke volume over atrial pacing (Westermann et al. 2008). Heart muscle cells exhibiting a pathophysiological phenotype after MI were extensively studied. To date, multitude of adverse changes have been reported such as increase in size, loss of the regular Z-groove/crest surface structures, membrane receptor redistribution, modified ion channel activity, alteration in sarcomere and intercalated disc make-up (Lyon et al. 2009; Sanchez-Alonso et al. 2016; Miragoli et al. 2016; Vermij et al. 2017). These modifications contribute to the overall decrease in cardiac function after MI. Chronic pathology progression is usually caused by a pressure or volume overload that leads to heart failure. One of the commonly used treatments is an LVAD implantation, which effects in a mechanical unloading of LV. As mention before, to mimic the effect of LVAD on the failing heart in this thesis, I used a Partial Mechanical Unloaded Lewis rat model in control and failing hearts. Obtained results show that load-deficient cardiac myocytes significantly reduce YM values. Also, mechanical stress removal from the MI cells brings the YM values down, which are comparable to control cells. However, despite the mechanical parameter is regained, molecular biology studies do not show the same outcome as already discussed in the previous paragraph. These results show evidence why not all patients with LVAD implantation recover. A critical element in the effectiveness of assist devices is the stage of the heart failure (Takeda et al. 2014; Arnold et al. 2016). Here, male rats that underwent proximal coronary ligation in order to provoke a chronic myocardial infarction up to 16 weeks were used. Mimicking the role of LVADs does not recover the damaged heart at this stage. Nevertheless, this phenomenon could be patient specific and dependent on the other comorbidities. Disclosing the precise role of mechanoproperties in the cardiac myocytes function and putting them into context will allow us to form a fully functional composite resulting in more effective drug therapies.

7.4 Angiotensin II as a novel mechanics regulator
Angiotensin II hormone is a part of RAAS playing a major role in vasoconstriction and increasing blood pressure. As several local RAAS systems were found in different organs the spectrum of Ang II function has increased. Furthermore, it has been shown that the hormone
contributes to cardiac disease by inducing hypertrophy, hypertension and fibrosis. This work shows an interesting finding of a potential new Ang II role in cell mechanics. Treatment with Ang II for two hours decreases the YM of myocytes acting via the AT1 receptor. Blocking either TGF-β1 receptor or inhibiting Rho kinase abolishes this effect. This signifies that the effect seen after short-term incubation with Ang II may be indirect. First records of the mechanical regulation following a short-term hormone incubation were shown by Leite-Moreira, but the signaling pathway was not fully revealed (Leite-Moreira et al. 2009). Analysis of the cytoskeleton found that changes in cardiomyocyte YM are MT-dependent. The effect of Ang II on microtubules occurs via decreasing detyrosinated and acetylated microtubule populations. This further highlights MT network as an attractive future target in studying cell mechanics. Interestingly, short-term Ang II treatment also reduces cellular YM in failing cardiomyocytes, reducing the YM of failing cells to that seen in control cells. This suggests that despite a major structural and functional changes, failing cardiac myocytes are still able to trigger the same pathway in response to Ang II as control cells. Future work is needed to test if the same signaling cascade components are involved in this Ang II-dependent mechanical change in MI cells. These data suggest a potential beneficial role of Ang II on cardiac cell mechanics in the short term, as opposed to well-characterized detrimental long-term effects.

7.5 Cell-cell communication

To maintain the mechanical continuum in organs and tissues, fully functional and undisrupted cell-cell interactions are essential. This is granted by three major adhesion complexes found in the ID between two myocytes: AJ, desmosomes and GJ. AJ and desmosomes provide physical anchoring whereas the gap junctions provide also electrical coupling. In this work, I have investigated the mechanical interaction between two cardiac cells. Because of the fragmented and undulated nature of cardiomyocyte contacts (Wilson et al. 2014; Vermij, Abriel, and van Veen 2017), quantitative imaging tools specifically designed for intercalated discs have not been available or systematically used. In this thesis I carried out study of the level and distribution of the three relevant proteins (namely, β-catenin, desmoplakin and connexin43) in homocellular CM-CM IDs of control and hypertrophic myocytes. Taking advantage of the multiple parameter software (Junction Mapper), I found significant difference in the hypertrophic CM-CM junctions as compared to control. Whereas control cardiomyocytes have clusters of cadherin receptors in IDs that are far apart. The level
of β-catenin staining in IDs potently increases after hypertrophic stimulus, consistent with what reported in hamster and human hypertrophic hearts (Masuelli et al., 2003). Additionally, I found clusters of reduced β-catenin density and increased spreading along junctions. Connexin 43 is found in cardiomyocyte IDs and its total protein and mRNA levels are augmented by hypertrophic signals (Salameh et al., 2008; Stanbouly et al., 2008) or in human hearts with compensated left-ventricular hypertrophy by pressure-overloading (Kostin et al., 2004). At IDs of hypertrophic rat cardiomyocytes, Junction Mapper detected increased levels and clustering of connexin 43. This confirms that the broadening of IDs and higher number of gap junctions is there to compensate increasing load, as seen in the hypertrophic hearts in human patients (Kostin et al., 2004). The profiling obtained with Junction Mapper suggests that, although both β-catenin and connexin 43 levels are increased at intercalated discs after hypertrophy stimulation, they are regulated in distinct ways: β-catenin cluster density is decreased leading to a more continuous distribution while connexin 43 is localized in clusters of higher density. Cardiomyocytes may also couple with other cell types such as fibroblasts (myofibroblasts in failing and damaged hearts), which leads to several changes in cardiomyocyte function. This type of interaction is believed to be more prominent in heart failure, rather than in normal heart (Pellman, Zhang, and Sheikh 2016). Despite interest in the junctions, their YM has never been investigated. In this thesis I studied CM-MFB coupling and I was able to show a significant increase at the YM at the cell-cell interface. The presence of Cx43 in CM-MFB contacts has been a matter of debate for years, as just few groups have shown the presence of Cx43 between CM and MFB in vivo (Camelliti, Borg, and Kohl 2005). Most recent work using an optogenetic approach has confirmed Cx43 presence in the CM-MFB junctions (Kostecki et al. 2019). Therefore, I looked into this protein further in the rat and human heterocellular CM-MFB couples. I noticed a decrease in the area and intensity of Cx43 at the whole contacting membrane. Moreover, I was found a new role of Cx43, apart from the widely known function of making GJ, showing that Cx43 regulates CM-MFB junction mobility.

7.6 Study limitations

a) Human samples

While working on animals models can provide answers to many questions, it is imperative to perform experiments on the human samples in order to identify potential species-dependent
differences and determine how good is the animal model. Unfortunately, during my study I had only limited success in obtaining samples from human donor hearts. Only cells from 2 samples were available to be used in this study. Due to a big variability between individual human myocytes, statistical significance has not been reached in many cases; obtaining stronger conclusions will require more cells from different donated hearts.

b) Titin protein Western blotting

One of the aims in this thesis was to investigate mRNA and protein levels of several mechanical stretch sensor proteins in control and load-variable rat models. This task was accomplished for Tcap and MLP proteins, but for titin it has only been possible to study the mRNA level. Titin is one of the biggest proteins known so far (~3 MDa). Therefore, standard WB protocols are not able to separate this big molecule. Despite much effort taken by me to implement a titin-specific WB protocol, prepared samples of isolated myocytes and fibroblasts showed bands at different molecular weights than expected, indicating protein truncation during the cell isolation.

c) Pipette restrictions

Cardiomyocyte surface structure is characterized by regular composition of crest areas and T-tubules that are arranged in Z-grooves. Smooth crest areas are easily accessible by the scanning nanopipette, on the contrary to deep, tubular membrane invaginations (T-tubules). The average T-tubule opening is 300-400 nm, however, it becomes more and more narrow as it gets deeper in the cell. Therefore, the scanning probe that is used to deliver a pressure pulse to the surface is not able to reach the bottom of the T-tubule and the YM measurements in the T-tubules were rather an indication of how deep the pipette can go.

d) Single approach

In this work, the role of Ang II in cardiomyocyte mechanics and the signaling cascade have been shown only using pharmacological approach based on the treatment protocols reported in the literature. In order to confirm this result, another more robust technique such as genetic manipulation using knock-out animals or gene silencing, should be used to corroborate the outcome.
7.7 Future work

This work has shown a new adaptation of the Scanning Ion Conductance Microscopy to measure YM in transverse direction (including a new modification of scanning software). By measuring different locations in cardiomyocytes and fibroblasts, I have proven that it is a reliable technique that can be used as a standard tool. It’s high resolution and non-contact scanning mode emerges as an excellent method to study live biological samples. Due to its reduced risk of damaging the specimen it outperforms the commonly used Atomic Force Microscopy. Here, different cellular elements have been investigated, however a number of other factors are still awaiting to be studied as shown in the Figure 4 in chapter 1. Future work will focus on using this technique to study a broader spectrum of intracellular factors contributing to cellular mechanics.

Additionally, direct contact with other cells such as fibroblasts and myofibroblasts strikingly modifies cell lateral edge YM. Myocytes do also form communication with postganglionic efferent neurons, which regulate the myocardial function (Bang et al. 2015). In future work it would be crucial to examine also the mechanobiological interaction between these two cell types at the baseline and in the pathophysiological state.

The use of Junctional Mapper in this work has proven to be a handy, reliable, semi-automated tool to precisely investigate the ID make-up. Future work should address further proteins located at the cell contacting membrane both, between cardiomyocytes and other cell types, such as neurons, (myo)fibroblasts.

On the other hand, apart from the cellular studies in the vitro cultures, cardiovascular research is carried out using induced-pluripotent stem cells, cardiac slices, tissue wedges and Langendorff-perfused whole hearts (Karakikes et al. 2015; Chen et al. 2006; Watson et al. 2017; Bell, Mocanu, and Yellon 2011). A model of cardiac slices is a cost effective promising new study platform. It preserves native connection between cells and ECM with the presence of different cell types. Recently, a new, improved technique of preparing 300 µm thick slices has been published, which provides a 60% cell viability in the rat samples (Watson et al. 2017). This study and others have characterized the slice model in terms of pharmacology and electrophysiology as well as molecular biology (Watson, Terracciano, and Perbellini 2019; Perbellini et al. 2018; Watson et al. 2017). The next step, but still challenging, will be to use...
the SICM technique to investigate the YM of different cell types in the slices in their native environment.

Currently, an interdisciplinary approach is widely incorporated in the scientific research to tackle most urgent problems. The main goal has now shifted to studying complex systems. The application of mathematical and computational models is on the rise; this will eventually help to predict what will happen in the real biological specimens. These models are already commonly used in the biological sciences. Recent study from our group employed a mathematical model of the L-type calcium channel distribution and function in heart failure; it has been recently further extended to a 3-D model of the left ventricle (Sanchez-Alonso et al. 2016). The recurrence in the interest in mechanobiology is still quite recent, therefore not many models are available due to the complexity of the study area and variety of mechanical properties of the biological samples. Future work in this area will greatly benefit from a computational approach that will address changes in YM at multiple levels: cellular, tissue and organ.
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