Tampere University

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MECHANOBIOLOGY OF CARDIAC TISSUE IN PHYSIOLOGY AND DISEASE

Faculty of Medicine and Health Technology
Bachelor's thesis
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ABSTRACT

Paavo Virtanen: Mechanobiology of cardiac tissue in physiology and disease
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Mechanobiology is a relatively new interdisciplinary field combining mechanics, engineering, and cell biology, based on physics, biology, and chemistry. My bachelor's thesis is focused on cardiac mechanobiology, specifically to the junction interface between myocardial cells and cell-extracellular matrix. Intercalated discs are one of the basic structures on these interfaces. They connect single, neighbouring cardiomyocytes into bigger functional units with cell junctions which is why abnormalities in them cause serious diseases. Deviations in cardiac tissue's normal mechanobiology affect the behavior, development and differentiation of heart cells, impacting ultimately the whole heart by leading to the development of heart diseases.

This bachelor's thesis is a literature review, which aims to summarize previous studies' main observations about cardial junctions' structure and possible changes in them, especially the alterations leading to dilated cardiomyopathy, from a mechanobiological perspective. In addition, this thesis explains shortly some of the methods used to study the cell junctions. These methods have also been used to analyse the changes in cells which are related to heart diseases.

Cell junction studies utilize various methods. This thesis explains a few of them: how they work and how they are used in heart studies. Methods discussed have been used to study, for example, intercalated discs, cell junctions and blood flow. They have had a great impact on understanding the cause and effect of cardiomyopathies.

Cardiomyopathies are a group of heart diseases that affect the heart, reducing its pumping efficiency, which leads into chronic fatigue, recurrent loss of consciousness, and chest pain. Cardiomyopathies cause variations in size and structure of the heart, enlarging the heart and making the muscle walls thicker and inflexible. In some rare cases, myocardial tissue may even start to convert into scar tissue. These symptoms are a result of changes in the mechanobiology of the heart, i.e., in cell interactions and their attachments to each other. Dilated cardiomyopathy is most often caused by mutations in desmosomal proteins with overexpression or complete lack of expression of N-cadherin.

The overall function of the heart is well known, but there are still many unknown mechanisms at the molecular and cellular level to do research on. For example, the impact of various protein changes on the development of heart diseases is not completely known yet. Furthermore, understanding of cellular signals and their effect on gene expression in pathogenesis is deficient. New types of research methods such as computational models, may reveal information on the mechanobiology of the heart in the future.

Keywords: Mechanobiology, heart, cardiomyocyte, intercalated disc, electron microscopy, fluorescence microscopy, cell staining, AFM-SCFS, DPA, PIV heart disease, dilated cardiomyopathy

TIIVISTELMÄ

Paavo Virtanen: Sydänkudoksen mekanobiologia normaalifysiologiassa sekä laajentavassa kardiomyopatiassa
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Mekanobiologia on melko uusi mekaniikkaa, insinööritieteitä ja solubiologiaa yhdistelevä poikkitieteellinen ala, joka pohjautuu fysiikkaan, biologiaan ja kemiaan. Kandidaatintyöni keskittyy sydämen mekanobiologiaan, erityisesti sydänlihassolujen sekä solu-soluväliainerajapinnan välisiin kiinnityksiin. Interkaloidut levyt ovat yksi kyseisen rajapinnan perusrakenteista. Ne yhdistävät yksittäiset, vierekkäiset sydänlihassolut suuremmiksi toiminnallisiksi yksiköiksi solu-soluliitosten avulla, minkä vuoksi niiden rakennepoikkeamat aiheuttavat vakavia sairauksia. Poikkeamat sydänkudoksen tavallisessa mekanobiologiassa vaikuttavat sydänsolujen käytökseen, kehitykseen, erilaistumiseen ja lopulta myös koko sydämeen, johtaen sydäntautien syntyyn.

Tämä kandidaatintyö on kirjallisuuskatsaus, joka pyrkii tiivistämään aikaisempien tutkimuksien keskeisiä tuloksia sydänsolujen liitosten rakenteesta ja mahdollisista muutoksista, selvittäen erityisesti laajentavaan kardiomyopatiaan johtavat muutokset soluissa mekanobiologisesta näkökulmasta. Lisäksi työ käsittelee soluliitostutkimuksen menetelmiä, joiden avulla pystytään analysoimaan myös sydäntauteihin johtavia liitosten muutoksia.

Soluliitostutkimuksessa käytetään lukuisia menetelmiä, joista muutamia käsitellään tässä työssä pintapuolisesti niiden toimintaperiaatteiden sekä sydänkudostutkimuksen sovellusten osalta. Menetelmiä on käytetty muun muassa interkaloitujen levyjen rakenteiden, soluliitoksien sekä verenkierron muutosten analysointiin. Tutkimusmenetelmillä on ollut suuri vaikutus myös kardiomyopatioiden vaikutusten ja syiden tutkimisessa.

Kardiomyopatiat ovat ryhmä sydänsairauksia, jotka vaikuttavat sydämeen vähentäen sen pumppaustehokkuutta, mikä johtaa muun muassa krooniseen väsymykseen, toistuviin tajunnanmenetyksiin sekä rintakipuihin. Kardiomyopatiat aiheuttavat muutoksia sydämen koossa ja rakenteessa, laajentaen sydäntä ja tehden lihasseinämistä paksummat ja joustamattomat. Joissakin harvinaisissa tapauksissa sydänlihaskudos saattaa jopa alkaa muuntua arpikudokseksi. Nämä ovat seurausta muutoksista sydämen mekanobiologiassa eli solujen vuorovaikutuksissa ja kiinnittymisessä toisiinsa. Laajentavaan kardiomyopatiaan johtavat useimmin desmosomaalisten proteiinien mutaatiot sekä N-kadheriinin liiallinen ekspressio tai ekspression puuttuminen kokonaan.

Sydämen toiminta kokonaisuutena tunnetaan melko hyvin, mutta molekyyli- ja solutasolla on vielä paljon tutkittavaa. Esimerkiksi erilaisten proteiinimuutosten vaikutus sydäntautien syntyyn ei ole vielä täysin tunnettu. Lisäksi ymmärrys solusignaaleista ja niiden voimakkuuksien vaikutuksista geenieksprессioon taudinsynnyssä on vajavaista. Uudenlaiset tutkimusmetodit, kuten laskennalliset mallit voivat kuitenkin tulevaisuudessa paljastaa lisää tietoa sydämen mekanobiologiasta.

Avainsanat: Mekanobiologia, sydän, kardiomyosyytti, interkaloitu levy, elektronimikroskopia, fluoresenssimikroskopia, soluvärjäys, AFM-SCFS, DPA, PIV sydäntauti, laajentava kardiomyopatia

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

AJ	Adherens junction
CAM	Cell adhesion molecule
CM	Cardiomyocyte
Cx43	Connexon-43
DCM	Dilated cardiomyopathy
Dsc	Desmocollin
Dsg	Desmoglein
Dsp	Desmoplakin
ECM	Extracellular matrix
EM	Electron microscope
GJ	Gap junction
ICD	Intercalated discs
IF	Intermediate filament
MEF	Mechano-electrical feedback
MGC	Mechanically gated channel
MSC	Mechanosensitive ion channel
N-cad	N-cadherin
PIV	Particle image velocimetry
Pkg	Plakoglobin
Pkp	Plakophilin
REM	Reflection electron microscope
SEM	Scanning electron microscope
STEM	Scanning transmission electron microscope
STM	Scanning tunnelling microscope
TEM	Transmission electron microscope
Zo-1	Zonula occludens-1

1. INTRODUCTION

Heart and vascular diseases are the most common causes of death around the world: about one third of all deaths are related to the cardiovascular system. Modern western lifestyle contributes greatly to these deaths, but some fatal diseases are caused by mutations in the genome. Particularly difficult are inherited mutations causing diseases, as they may affect generations of people, originating from a simple, unlucky mutation in a gamete.

By understanding the molecular mechanisms of the heart, it could be possible to prevent cardiovascular deaths or at least prolong people's lives with treatments. There are still less studied factors affecting the heart, especially the effects of long-term forces on heart tissue, and the mechanosensitivity generally in different timescales. Cell junctions and cell adhesion, ion channels and the mechanobiology of the heart have been studied a lot but mostly separately. To create a better overall understanding on how the different structures of cardiomyocytes (CMs) and heart tissue function together, it could help to also study their action together.

Mechanobiology is a relatively new area of study, combining knowledge of mechanics and cells, thus linking some of the most basic fields of science: engineering, biology, and physics [1]. In practice, this means the study of how different forces and cell's mechanical properties affect the cell's development and differentiation and contribute to diseases. This thesis focuses on cardiac mechanobiology and how the changes in cardiac cells' connections between each other and the extracellular matrix (ECM) alters the tissue's behaviour and the heart muscle.

2. MECHANOBIOLOGY IN NORMAL PHYSIOLOGY OF CARDIAC TISSUE

This main chapter explains the ideal function of cardiac tissue and cells, explaining the different junctions, mechanical and electrical properties of cells and the basic structures which connect CMs, making it possible for them to operate together.

2.1 Cellular mechanosensitivity

Cells respond to environmental stimuli. This includes mechanical stimuli, and the cell's response to it is called mechanosensitivity. Mechanosensitivity affects cells physiological and biochemical processes such as opening of certain ion channels on the cell's plasma membrane. This then affects its processes in molecular level and as a consequence the cellular level and even the tissue [2]. The phenomenon where a mechanical stimulus is converted into electrical or chemical signals, is called mechanotransduction. In mesenchymal stem cells, mechanical forces and the mechanosensitivity also regulate the cells' functions such as differentiation [3]. Therefore, we can conclude that mechanosensitivity plays an important role in cell's physiology. Some mechanical stimuli systems also have a combined effect, called crosstalk, between different mechano-induced signalling pathways. Stretch also reinforces junctions with cadherins (adherens junctions (AJs) and desmosomes). [2] Cells experience different amounts and different kinds of stress such as stretch, shear, bending and compression which all may trigger different signalling pathways, affecting the cellular function and cell-to-cell interaction differently.

In tissue, conduction velocity also slows down as the capacitance and membrane surface increase through stretch. [4] Stretch also increases the affinity of Ca2+-binding troponin C, which enhances activation of muscle contraction via complex regulatory protein mechanism, exposing myosin-binding site to actin [5].

2.2 Cardiac cell response to mechanical stress

The heart is under constant stretch, compression, and twisting. These mechanical factors affect its electrical and mechanical properties, as demonstrated in Figure 1. Heart's responses to mechanical stress is based in mechanotransduction: heart's mechanosen-

sitive ion channels (MSCs) work as mechanoreceptors, giving mechano-electrical feedback (MEF) to the regulation system of the heart. In the heart, short-term mechanical forces affect electrophysiological conditions and the long-term mechanical changes may even affect gene expression. Gene expression in turn affects. tissue and cell remodelling using mechanically gated channels (MGCs) located on the cell membrane. MGCs, work either as effectors or sensors in MEF system, reacting to environment's mechanical stimulus and converting it to molecular and electrical signals which then regulate the cell's different processes, both short and long-term (including those mentioned earlier.) [6]

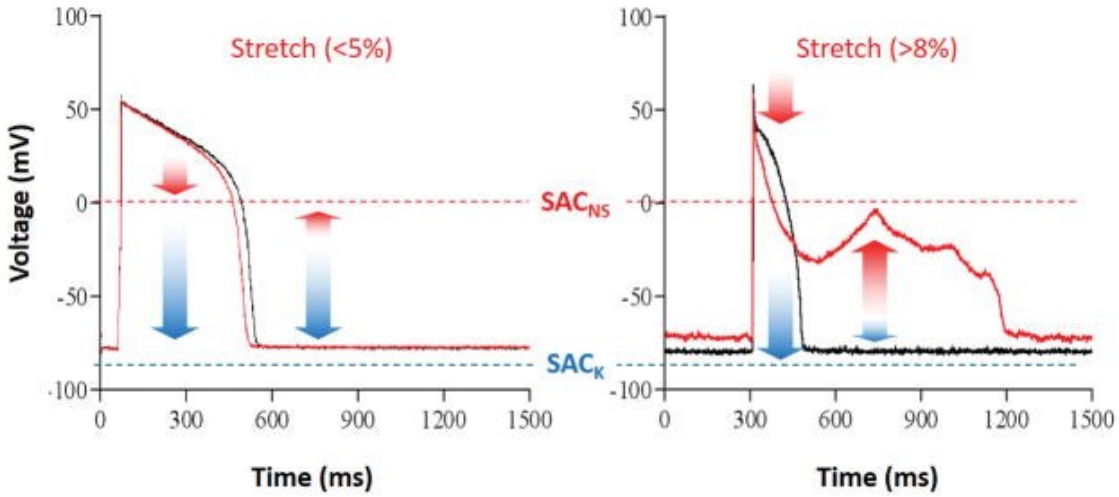

Figure 1: Stretch of cardiomyocytes affect their action potential through activation of mechano-sensitive ion channels. The first graph demonstrates voltage as time's function with under 5 % muscle cell stretch (compared to normal cell length), and the second graph demonstrates voltage as time's function with over 8 % muscle cell stretch (compared to normal cell length.) [6]

As cell's charge is constantly changing, the voltage differs between extracellular and intracellular space. Besides mechanosensitive, extracellular, and intracellular ligand-gated and leakage ion channels, there are also voltage-gated ion channels. [7]

2.3 Cell-cell interactions

Cells are connected and interact in various ways by using signalling molecules, such as hormones, neurotransmitters, and cytokines by using intracrine, autocrine, juxtacrine, paracrine and endocrine cell signalling. Intercalated discs (ICDs) are specialized struc-

tures in cardiac muscle cells that connect them to each other, synchronizing their electrical and mechanical action, the whole function of the heart (Figure 2). ICDs consist of three different complexes: AJs, desmosomes and gap junctions (GJs) which together form “area composita”, a name given to the ICD interactions together (Figure 3). There is also a fourth type of junction: tight junction, although they don’t participate to the forming of ICD. GJs connect neighbouring cells’ cytoplasms which means that they are electrically and metabolically connected, making it possible for electrical stimulus to travel from CM to another. This allows the electrical impulse to travel through the whole heart, making the heart muscle contract. AJs and desmosomes give mechanical support to the CMs by anchoring adjacent cells to each other: AJs connect the actin cytoskeletons and desmosomes connect the intermediate filaments (IFs) between CMs. This makes the cells together (the myocardium) more stable and stronger. [7,8]

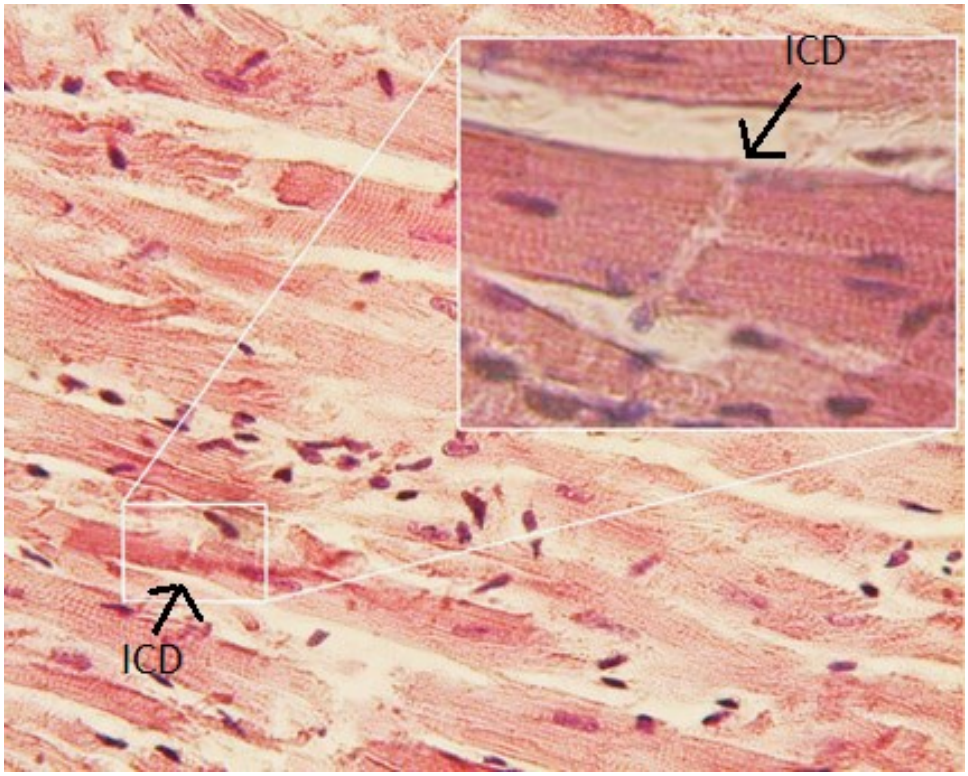

Figure 2: A macro view of the intercalated disc structure, joining two cardiomyocytes. Dark blots are nuclei. This picture has been edited for this thesis: added arrows and marked an intercalated disc (ICD). [9]

Cell adhesion is formed by numerous proteins, especially in ECM adhesions. ECM receptors (cell adhesion molecules, CAMs) are proteins on the cell's membrane, which participate in adhesion. CAMs make both cell-cell interactions and cell-ECM interactions possible. Selectins and cadherins are important CAMs with a similar task in cell-cell interactions: selectin forms heterophilic adhesion (adhesion between different kind of cells) while cadherins form homophilic adhesion (adhesion between two cells of same type). [10]

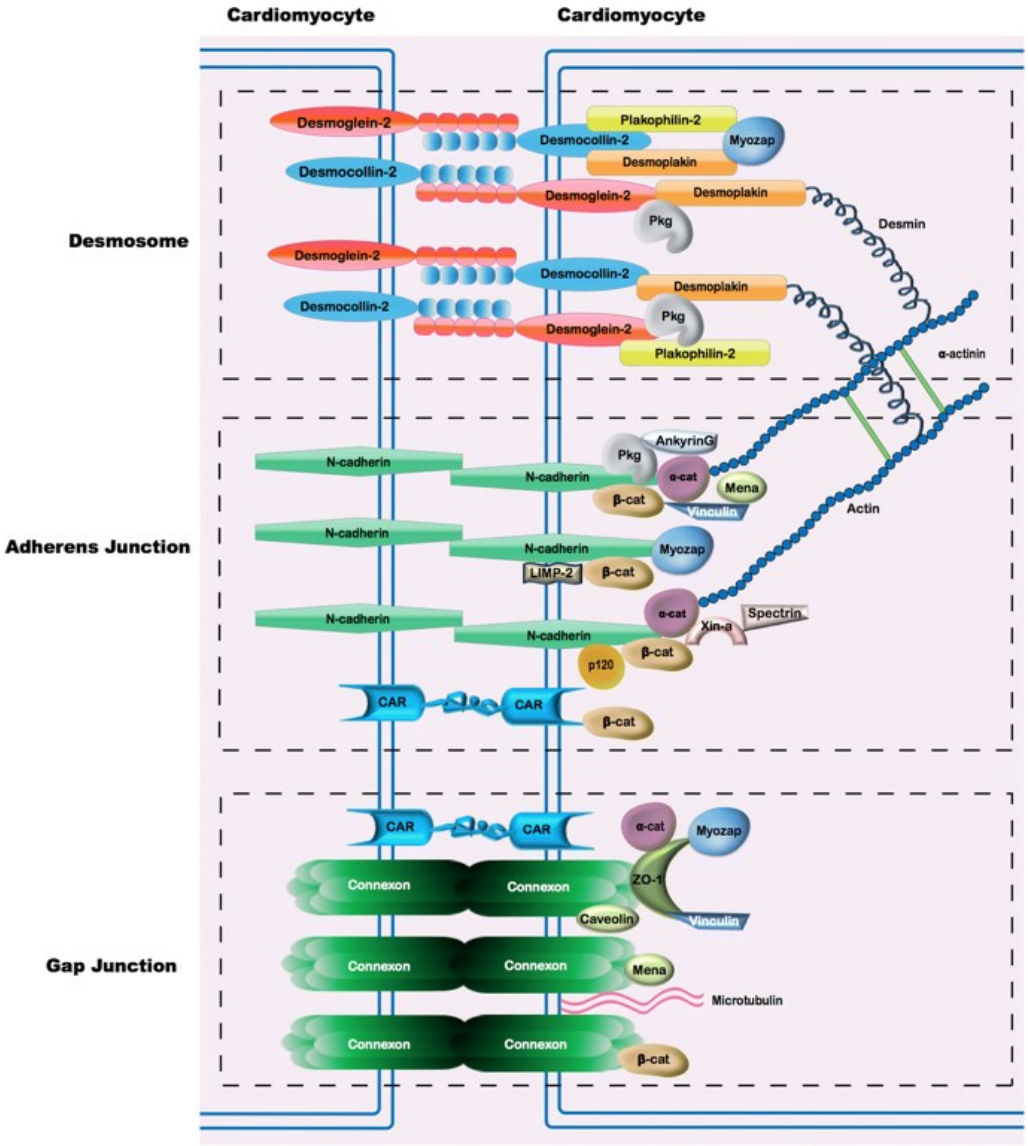

Figure 3: All three junctions of intercalated discs with their most important proteins visualized. PKG is an abbreviation of plakoglobin, β-cat is β-catenin, Mena is mammalian-enabled protein, LIMP-2 is lysosomal integral membrane protein-2, p120 is p120 catenin, α-cat means α-catenin, CAR is coxsackievirus and adenovirus receptor protein, ZO-1 is zonula occludens-1. [8]

AJs (Figure 3, junction on the middle) are responsible for connecting the cells' actin cytoskeletons to each other, stabilizing the tissue. Cadherin binds the separate actins to each other on the cell membrane. [10] Their main purpose is to provide strength to the cells. Cadherins are also particularly mechanosensitive. When junctions with cadherin experience mechanical forces such as stretch, signalling pathways are activated and cadherin contacts are reinforced. [8] CM's AJs are called fascia adherens and are similar structures as AJs, stabilizing non-epithelial tissues by anchoring cardiac muscle's actin as a part of ICD. Their main proteins are N-cadherin (N-cad), β-catenin along with few other catenins, CAR, LIMP-2, spectrin and Xin-α (see Figure 3 for visual demonstration of the proteins and their locations in relation to each other). N-cad is the only cadherin expressed in the heart, working as the primary anchor in myofibrils, connecting neighbouring cells' actin filaments. They perform this by forming homodimers with another cell's N-cad in ECM, resulting to a zipper-like structure. Catenins bind to N-cad and connect the AJs to the cell's actin cytoskeleton. β-catenin and p120-catenin both have important roles in signalling. For example, p120-catenin controls the cell's shape and adhesion via GTPase activation. Additionally, p120-catenin connects to microtubules with mediator proteins PLEKHA7 and Nezha, linking AJ and microtubule while α-catenins link N-cad to actin via actinin, β-catenin, γ-catenin or vinculin. α-catenin also has a role in GJs.

CAR (coxsackievirus and adenovirus receptor), LIMP-2, spectrin and Xin-α are all recently found proteins, so their role isn't completely clear. However, CAR has been noticed to be a part of the ICD, interacting with β-catenin and GJ's CX45 and ZO-1. LIMP2 affects the connections of N-cads and phosphorylated β-catenins. Spectrin creates tetramers to connect actins' distal ends, meaning that it is another N-cad's connection protein. Xin-α seems to have a role in N-cad and p120-catenin's signalling, actin assembly and cell-cell adhesion while Xin-β may have a role in hypertrophy via angiotensin II down-regulation signalling. [8]

Desmosomes (Figure 3, junction on the top of the picture) bind IFs with neighbouring cells, found in tissues which experience a lot of mechanical stress, such as the heart. [10] Their main function is to anchor the cells to each other. Desmosome consists of mainly six proteins: desmoglein (Dsg), desmocollin (Dsc), plakoglobin (Pkg, also called γ-catenin), plakophilin (Pkp), desmoplakin (Dsp) and myozap (see Figure 3 for visual demonstration of the proteins and their locations in relation to each other.). There are different protein variants (isoforms) of the Dsg, Dsc and Plk. The isoforms in the CMs are mainly Dsg-2, Dsc-2 and Plk-2. Dsg-2 and Dsc-2 make up the core of the desmosome by forming dimers with heterophilic interactions between each other. Sequence-

wise they are very similar, as about a third of their amino acid sequences are homologous. Pkg and Pkp-2 both participate on cadherins connecting to IFs. Pkg has a role in AJs, but its affinity is higher to Dsg-2, which contributes to its role as Dsg-2's main localization support. Pkp-2 has a lot of roles in desmosomes: it connects to Dsp, Dsc-2, actin, desmin, keratin, Pkg and even interacts with GJ's connexin 43. Pkp-2 also recruits Dsp in heart injuries and development when creating new desmosomes. Dsp connects desmin and desmosomes to each other. Beside connecting to Pkp-2, it connects to Pkg and therefore is one of the proteins with a very important role in desmosomes' composition. Myozap binds to Dsp and TJ's protein zonula occludens-1 (Zo-1). Its overexpression leads to oversensitivity of mechanical stress, and knockdown causes cardiomyopathy. [8]

GJs (Figure 3, on the bottom of the picture) are formed by two connexons, and one connexon is formed by six connexin proteins [11]. GJs allow molecules and ions to pass through from a cell to its neighbour [8]. This is particularly important in neurons and the heart, as GJs electrically and chemically couple cells, allowing their electrical stimuli to travel through the tissue. Most important protein of GJs is connexin-43 (Cx43). One GJ channel consists of 12 Cx43 monomers, although only 6 is needed to form one connexon. The connexon connects with another cell's connexon to create the whole channel. Other relevant proteins include caveolins, Mena, tubulins and Zo-1 which are all binding proteins for the GJ channels (see Figure 3 for visual demonstration of the proteins and their locations in relation to each other). Caveolins interact with the CX43's and act as the caveolae's main scaffold parts. Mena protein interacts with the Cx43 and vinculin, affecting the adhesion, cell movement and Cx43 remodelling. Microtubules are connected to the Cx43's, meaning that the GJs too are connected to cytoskeleton. Lastly, ZO-1 regulates GJ's size by interacting with Cx43's. In addition, ZO-1 guides Cx43's and keeps them at the ICD. [8]

Tight junctions are one of the primary adhering mechanisms but they are not found from the mature ICDs. They are expressed in developing heart and in some heart diseases their formation is induced by heart tissue remodelling [8]. Their major proteins include claudins, occludins and Zo-1, forming impermeable barrier which does not allow any liquids to flow through it. Claudin-5 is also found from lateral membranes of cardiac muscle in CMs, while Zo-1 is essential in GJs on guiding and keeping CX43's at ICDs. [7,12] Tight junctions may also contain channels for smaller ion molecules or water [7].

In conclusion, the three ICD junctions have a lot in common. Myozap is found from all of them, while α-catenin, β-catenin, CAR, Mena and vinculin is found from GJ's and AJ's. Pkg is found from desmosomes and AJ's.

2.4 Cell-extracellular matrix interactions

Cells adhere to their environment called ECM. ECM consists of different macromolecules, mostly different proteins such as enzymes and glycoproteins. Cells adhere to the matrix in interactions named focal adhesion and hemidesmosomes. Focal adhesion resembles AJ while hemidesmosome is similar with desmosome. The difference comes from the connecting junction protein: in ECM, cell's IFs (hemidesmosomes) and actin (focal adhesions) connect to integrin. Integrin is an ECM's example of CAM. It anchors cells to various ECM proteins or glycoproteins, such as collagen, laminin and fibronectin, sometimes even to multiple proteins. [9] Studies show that the cardiac ECM induces embryonic stem cells' differentiation to CMs [13]. McCain et al. [14] and some other studies have found indications that focal adhesions are very important on the early stages of CM development because they guide the cell's migration. As the ICD's mature and the amount of cell-cell attachments increase, the amount of focal adhesions decreases as they aren't as needed anymore, as demonstrated in Figure 4. However, if the environment's stiffness increases, the amount of load on the cell-cell junctions increases. This then induces focal adhesion formation and may cause cell-cell junction detachment to stabilize the cells and compensate the increased load. The phenomenon was studied by mimicking fibrosis, and McCain et al. concluded that longevity of ICD's requires constant mechanical stress. [14]

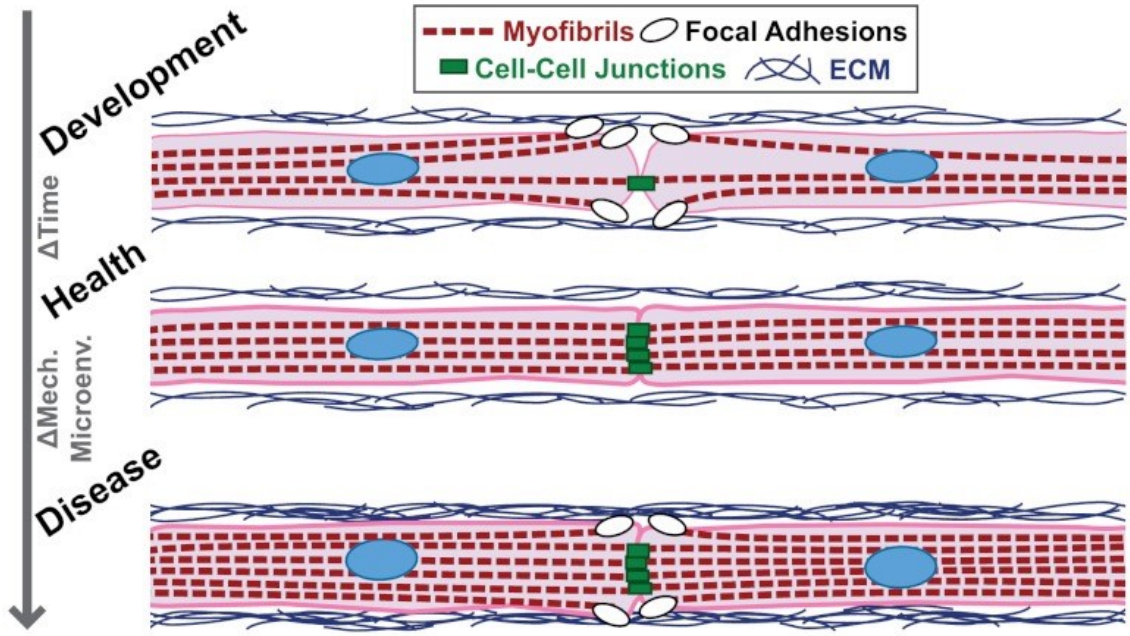

Figure 4: Focal adhesions' amount in CMs differ: developing CMs have focal adhesions before the ICD's completely form, while in fully developed healthy CMs there are none. Some cardiac diseases that cause increased stiffness (as seen on the bottom CMs titled "disease" with denser ECM) cause the neoformation of focal adhesions. [14]

There are also cell-ECM junctions called hemidesmosomes, but they are mainly only found in keratinocytes.

3. METHODS FOR STUDYING MECHANOBIOLOGY

Different methods for studying cell adhesion reveal different features from the cells. Common methods are varying appliances of electron microscopy (EM) such as transmission electron microscopy (TEM) and scanning electron microscopy (SEM), atomic force microscopy-based single cell force spectroscopy (AFM-SCFS), dual micropipette aspiration (DPA) and cell staining with fluorescence microscopy. As computing power has increased a lot over the years, many simulations and algorithms have been utilised on predicting and calculating how different mechanisms cooperate to make heart's pumping action possible.

3.1 Atomic force microscopy-based single cell force spectroscopy

Atomic force microscopy-based single cell force spectroscopy (AFM-SCFS) is a cell adhesion study method capable of measuring the adhesion strength between cells and even single junction's adhesion strength. The method is based on a liquid chamber with the studied cells within. Single cell is taken with a cantilever and then it is brought into a contact with a material or another cell. Adhesions are allowed to form for different time periods. Then, the cantilever with the cell is detached with a steady speed. The cantilever bends slightly and this bending can be measured using a laser. By measuring the bending, the adhesion strength can be measured as it is directly proportional to it. The contact time between the cell and material/another cell may be altered to influence the amount of forming adhesion junctions. [15] The method is visually demonstrated in Figure 5.

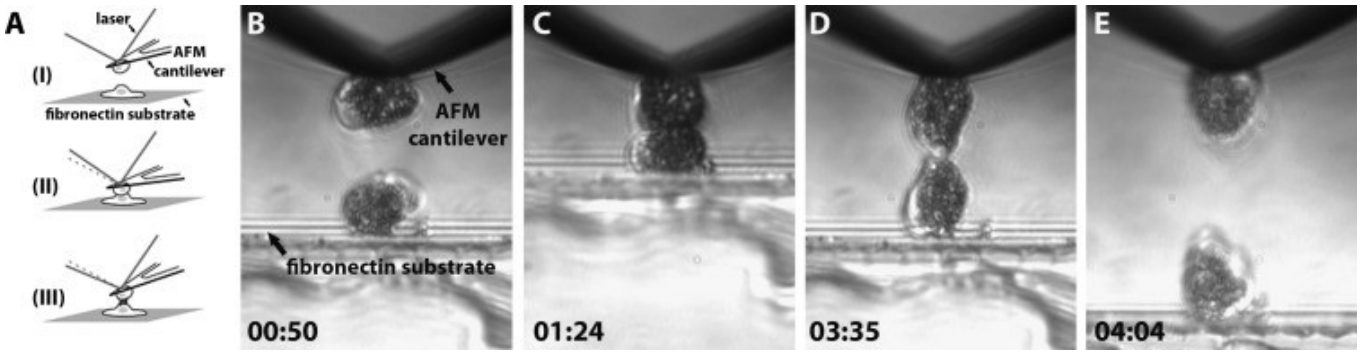

Figure 5: AFM-SCFS in use. Picture A demonstrates the laser's changed reflection after the cantilever bends. Pictures B-E are screenshots from a AFM-SCFS measurement, showing how the two cells first form adhesions and then the cell on the cantilever is pulled away. [10]

AFM-SCFS measurement gives a force-distance curve as a result, revealing the adhesion force on a certain distance between the cells. The measurement curve has jump-like deviations in it, called rupture steps. The rupture steps are a sign of an adhesion junction breaking. By analysing the rupture steps, adhesion junctions' amount and strength can be estimated. For example, E-cadherins break in a zipper-like fashion, one at a time. [11]

3.2 Dual micropipette aspiration

Dual micropipette aspiration (DPA) can be used to analyse cell-cell adhesion using two micropipettes. The cells are brought next to each other with the micropipettes' tips. The cells stay on the tip with a small aspiration on the pipette. As the cells are next to each other, they start to form junctions and adhere to each other. As with AFM-SCFS, the cells are allowed to interact for certain times for the adhesion to form. Then, by controlling the aspiration intensity, the adhesion strength may be measured: as the cells detach from each other, the measured aspiration pressure needed to do so corresponds to the adhesion strength. [10] The method is demonstrated in Figure 6.

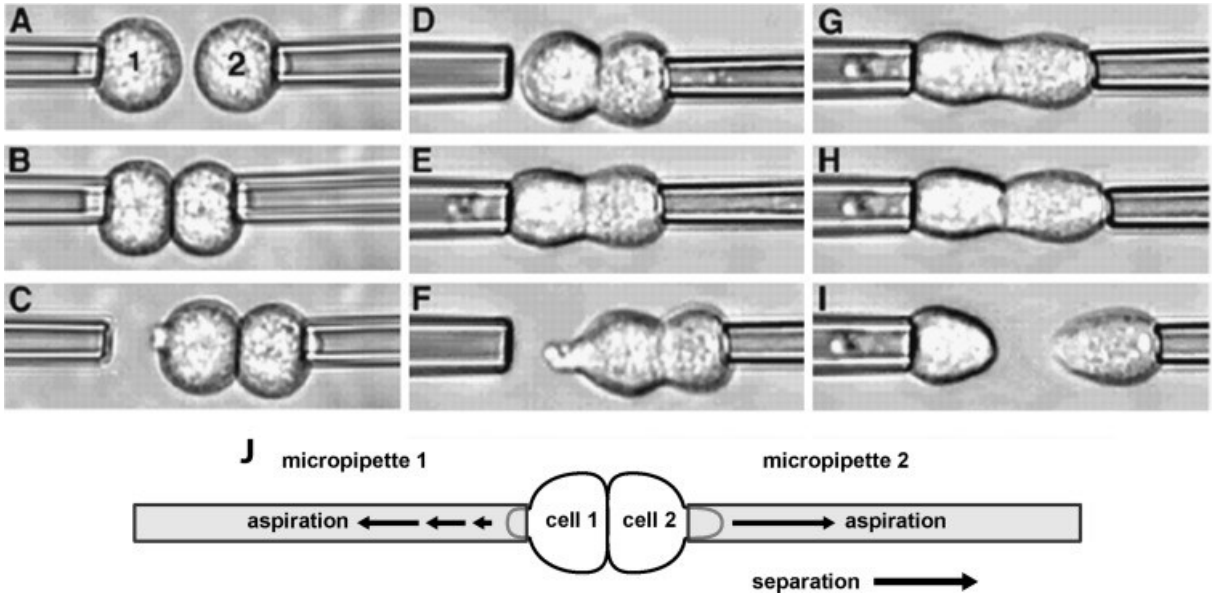

Figure 6: Picture collage visually demonstrating the DPA-method. Two cells are brought next to each other with micropipettes and allowed to form adhesions between each other. Then, by increasing aspiration on the micropipettes, the cells are detached from each other. Pressure needed to detach is directly proportional to the adhesion strength. [10]

The method is most useful in studying the cell contact time's effect on adhesion strength. Unlike in AFM-SCFS, DPA cannot be used to measure single junction's strength but rather the accurate sum of adhesion strength between two cells. DPA has had an important role on studying cadherin's importance in cell adhesion, and its use in developmental studies has increased: how individual's different developmental stages' cell adhesion differs. [10]

3.3 Electron microscopy

EM is a versatile method which can be used for different purposes with appropriate modifications. The basic principle is the same in all variations: a beam of electrons is accelerated towards the studied target, where collision results in illumination. As the electron's wavelength is much shorter than of light's, resulting pictures have much higher resolution which means much smaller objects such as tissues, cells and even cell structures may be studied in much higher detail. [16]

Two of the most common types of EM are SEM and TEM. SEM is used to produce high-detail overall pictures of cells and organisms, size estimations and determining the number of particles. It works with rebound secondary electrons, X-rays and photons emitted by the target, along with scattered electrons. TEM on the other hand is used to study thin

tissue sections, cell's interior, molecules' organization, protein's structures and cytoskeletal filaments. This method works by the target allowing parts of the electron beam through it and parts scattering away. [16] Figures 7 and 8 can be used to compare how SEM and TEM pictures differ.

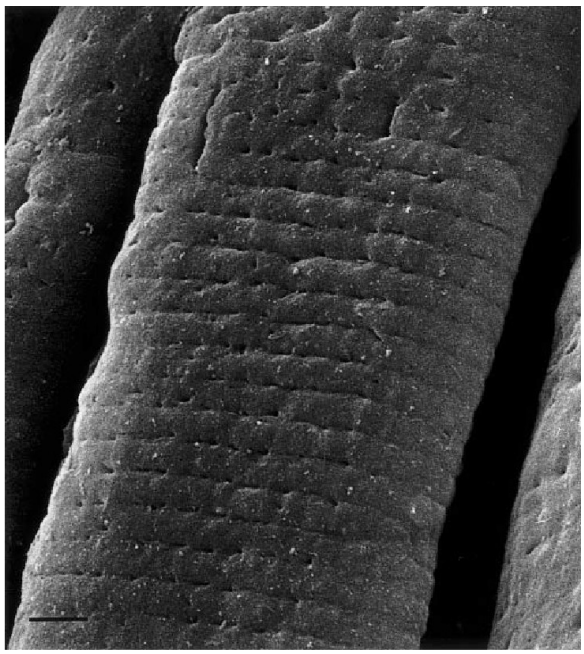

Figure 7: Picture of a monkey cardiomyocytes taken with a scanning electron microscope. Opened T-Tubules can be seen on the apertures of the cell surfaces. [17]

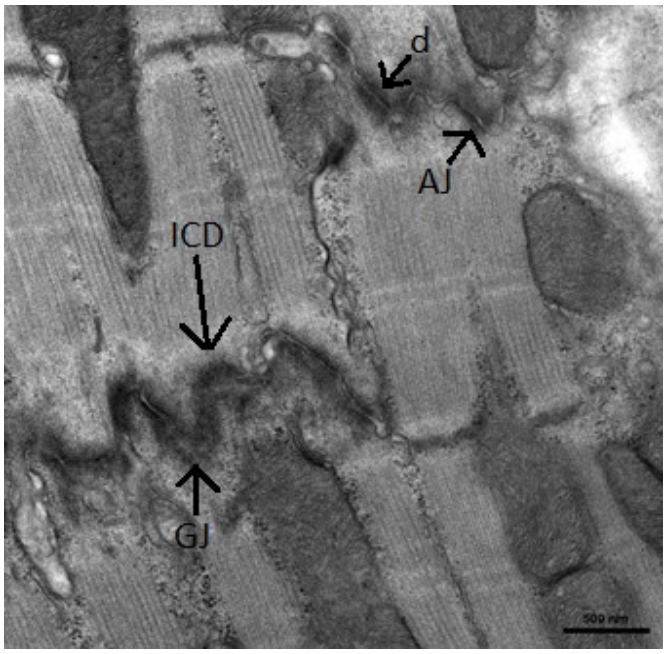

Figure 8: Picture of a rat's left ventricular muscle intercalated disc, taken with a transmission electron microscope. This picture has been edited for this thesis: intercalated disc (ICD), desmosome (d), adherens junction (AJ) and gap junction (GJ) have been marked to the picture with arrows. [18]

As Figure 7 and Figure 8 demonstrate, a high-detail picture may be taken with SEM while TEM gives a better overall view.

Other EM methods include scanning transmission electron microscope (STEM), reflection electron microscope (REM) and scanning tunnelling microscope (STM). STEM combines the TEM and SEM into a one method, which requires thin samples. Unlike in TEM, STEM may also use secondary electrons, scattered electrons, X-rays, and electron energy lost to calculate signals from the target, achieving higher resolution than with SEM. [19] REM utilizes elastically scattered electrons to reveal a detailed structure of a surface. and material's crystal structure [20]. STM is based on quantum tunnelling: probability of an electron "tunnelling" to the target from conductive, charged object's tip in function of distance. Like REM, it can be used to determine detailed surface conformation, even in

atom level. [21] Both REM and STM could be used in quality control of biomaterials or to study their suitability in different applications such as prosthetics.

3.4 Fluorescence microscopy and cell staining

When staining cell's cytoskeleton, certain adhesion proteins are made visible with fluorescent antibodies attached on them. One of the most common cytoskeleton proteins to be stained is actin. Actin staining is frequently used to analyse cell's adhesion as actin's location and amount gives a decent overview if there are adhesion junctions and how much junctions there are. When the cell's adhesion is made visible, it can be compared to usual adhesion of same cell type to see if there are any differences. Cell staining is relatively cheap and easy way to analyse cell adhesion, but some of the fluorescent dyes are toxic to cells which limits further use of cells. Staining also only gives a visual overview of the adhesion but it does not give any accurate approximates of adhesion strengths. [22] Figure 9 demonstrates different kind of stainings.

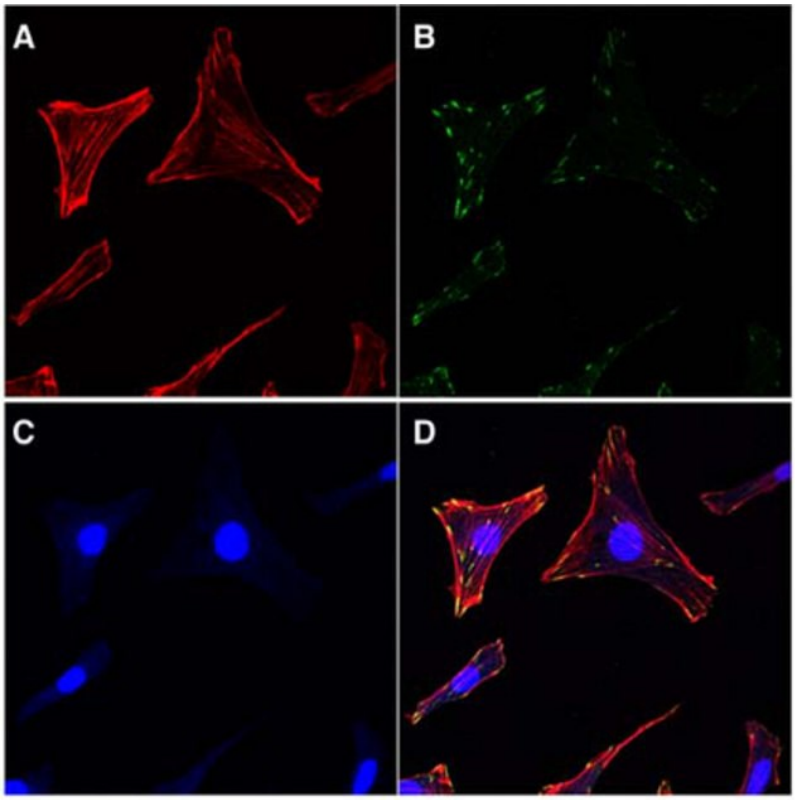

Figure 9: Different cell proteins stained. A: F-actin staining. B: vinculin staining. C: nuclear staining with DAPI. D: all three stainings stacked in one picture. [22]

A suitable staining method should be chosen according to needs: some of the staining methods are toxic which means some of the cells become unusable after the treatment. Some of the dyes may be washed away, meaning certain methods allow cells to be re-used after washing them.

Another method is vinculin staining. Vinculin connects integrin to actin cytoskeleton. Vinculin also affects focal adhesions and it is also present in some AJs where it has a role in F-actin's anchoring. It is one of the proteins which makes the cell's movement and attachment possible. Vinculin can be stained using immunofluorescencive vinculin antibody, revealing cytoskeleton's actin-integrin interaction. [22]

Another fluorescence microscopy method worth mentioning is expansion microscopy, although it does not use electrons. In expansion microscopy, the sample is physically expanded using polymer network which can be "swelled" using chemicals. As the polymer network expands, it forces the sample to expand with it. This makes it possible to analyse biological samples which would normally be too small to study without expensive research equipment such as EM, as a light microscope is enough. It also allows the use of staining on the samples, which EM does not. [23] This method could be used to expand CMs and other heart cells or to locate and identify cardial cell RNA.

3.5 Particle image velocimetry

Particle image velocimetry (PIV) is an optical flow visualization method used for velocity and direction measurements in fluids. It usually works by using traceable particles which flow in the studied chamber or piping and are being constantly monitored using a camera with a connected laser and lenses which turn the laser into a laser thin sheet, although the laser and the tracking particles aren't always necessary. If the tracking particles are used, they should be as spherical as possible, and their density should be close to the analysed fluid particles' density. In ideal conditions, they have the same density.

A synchronizer is also needed to sync the camera and the laser pulse when laser is utilized. Laser light scattered from the tracking particles are recorded in sequence of frames captured with the camera. After the PIV recording is made, algorithms can be deployed to evaluate and analyse the flow of the fluid. Figure 10 shows an example of PIV recording, analysed with PIVLab open source tool. [24]

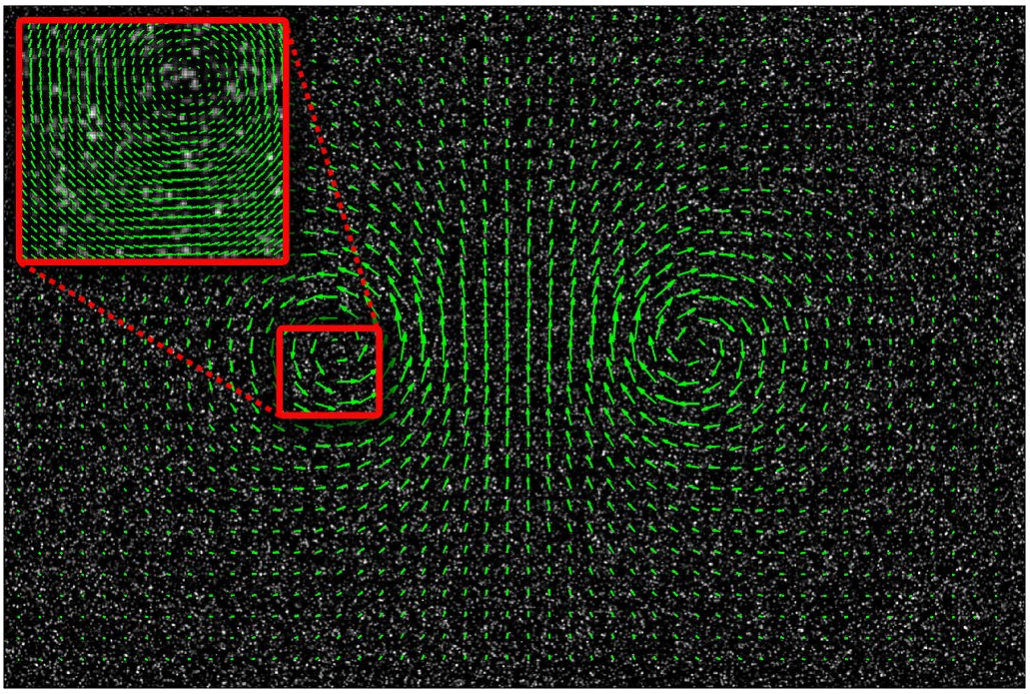

Figure 10: A modern particle image velocimetry recording of flow in fluids. The white particles on the black background are the tracking particles, which are used to measure the velocity vectors (green arrows.) [25]

As can be seen from the Figure 10, velocity vectors are computed using the speed and direction of the tracking particles. Main advantage of this method is that the method is very accurate and the tracking particles, if chosen right, will not disrupt the natural flow of the studied fluid. On the other hand, if the tracking particles are not well suitable for certain measurement, they may not follow the flow of the studied fluid. [24] A more modern method to study muscle cells for example, is to record videos of them with a microscope. This has been used to study the beating of CMs: the contraction movement between frames can be found out by comparing the frames using minimum quadratic difference, a subtype of digital image correlation. [26] This frame comparison with suitable computational methods (correlation) can be used to calculate the velocimetry.

One application of PIV has been used to analyse the left ventricular fluid dynamics in dilated cardiomyopathy (DCM). PIV could be used to study altered blood flow in DCM and other heart diseases, both in heart and blood vessels.

3.6 Summary of the methods

Methods discussed have contributed to the study of heart and cells. They have provided insight on cell connections, cell and heart tissue structure and the blood flow changes in heart. Table 1 sums up these methods and presents their applications.

Table 1: Discussed methods and their applications.

Method	Based on	Used for	Application in heart studies	Advantages	Disadvantages
Atomic force microscopy-based single cell force spectroscopy (AFM-SCFS)	Laser measuring of cantilever bending as cells attach to each other.	Cell-cell and cell-ECM studies: accurate force measurement for even single junctions	CM junction strength studies, CM and ECM protein connection studies	Accurate, allows measuring of single junction's adhering strength	Expensive, requires trained personnel to use
Dual micropipette aspiration (DPA)	Measuring, how much pressure is needed to separate cells	Cell-cell studies: measuring sum of forces between cells	CM junction strength studies, ICD studies	Cheaper compared to AFM-SCFS	Allows only the measurement of total adhering strength
Electron microscopy (EM)	Electron beam with target collision illumination	Cell and tissue structure studies: normal structure and changes in the structure	Heart tissue studies, especially ICD structure studies	Depends on EM subtype. Detailed overview pictures of cell structures	Compared to light microscope, very expensive
Cell staining and fluorescence microscopy	Fluorescent-tagged molecules attaching to studied molecule	Cell junction alteration studies: an overall view	Comparison of protein cytoskeleton structures between normal and diseased heart cells	Relatively cheap, good overall picture	Resolution and details are limited, as visible light's wavelength sets limitations
Particle image velocimetry (PIV)	Measuring difference between two frames of video or two pictures, with a laser	Fluid flow, cell contraction studies	Alterations in the blood flow (especially in the heart), CM contraction	Very accurate	If tracking particles are used, choosing the most suitable is important for accurate measurement.

These are only a fraction of the methods which have been used to study the heart in the last century. Still, they have provided valuable insight to heart and myocardial cell structure, and blood flow.

4. MECHANOBIOLOGY IN DILATED CARDIOMYOPATHY

Cardiomyopathies are diseases related to the heart. They cause the heart muscle to undergo changes which slowly make the muscle thicker, inflexible and/or enlarged, and decrease the efficiency of heart's ability to pump blood. In some cases, the heart muscle tissue may even start to transform into scar tissue, although this is rare [27].

DCM is a cardiac condition in which the ventricular chamber becomes enlarged, ventricular wall becomes stiffer and thinner and parts of the tissue suffer from interstitial fibrosis and inflammation. At the cellular level, CMs have a higher risk of going into apoptosis and their nuclei become enlarged. [28] The heart becomes weaker and the patient may suffer heart failure, irregular heart rate or a heart valve disease [29]. DCM's symptoms are severe and usually require medical care. Treatments include blood-thinning medication, angiotensin-converting inhibitors, angiotensin II receptor blockers, beta blockers etc., sometimes even a heart transplant if no other treatments prove effective [28]. If the non-invasive methods don't work alone, irregular heart rate can be treated with a pacemaker which may include a defibrillator feature to start the heart in case of a heart failure. What makes DCM an interesting disease is that sometimes if it has occurred suddenly, it may also go away on its own [29].

According to Weintraub et al. [30], the frequency of DCM is 1 in 2 500. DCM is among the many diseases caused by mutations in ICD protein genes [6]. It has been shown that factors such as some autoimmune diseases, toxins (alcohol, cobalt) and viral infections (hepatitis, HIV) may cause or are predisposing factors for DCM, although the mechanisms aren't still completely known. [31,32]. Genetic mutations may also either predispose or even cause the disease. Most common genetic factors are mutated dominant autosomal alleles and recessive X-chromosome alleles. [31]

4.1 Cell-cell interactions in DCM

Mutations in desmosome proteins are the major reason for DCM. Dsc-2, Dsg-2, Dsp and Pkp-2's mutations are more often the cause of the DCM, compared to the rest of the desmosomal proteins' mutations. [8,33]

AJ mutations also cause DCM: changes in genes of α-catenin, β-catenin, N-cad and Xin-α. [32] The AJ mutations include overexpression of N-cad and misexpression of E-cadherin. J. Li et al. [33] speculated that if there is too much cadherin or catenin compared to myofibrils, the force transduction of cadherin-myofibril connection becomes less efficient due to changed contractile dynamics. N-cad's overexpression also causes intracardiac thrombus and cardiac calcification. Their study revealed that when N-cad gene is completely knocked out in mice with completely developed myocardium, desmosomes, AJs and the whole intercalated structures are lost, and DCM occurs along with cardiac dysfunction. This led to sudden cardiac death within 2 months after the removal of N-cad. Another outcome was that sarcomeres shortened, and Z-lines of cardiac muscles thickened. [34]

Mutations in GJs may induce DCM. Connexins are delivered to GJ's in dephosphorylated state, but active connexons require phosphorylated connexons. Dephosphorylation of GJ's connexins cause dysfunction of the junction's normal behaviour. Particularly Cx43's increased dephosphorylation has been noticed in DCM, as phosphorylation of CX43 causes its displacement on the CM, leading to slower conduction velocity. [34] Metavinculin mutations such as missense mutations have also been noticed to cause DCM, as metavinculin affects the actin interaction of the cell through changes in actin filament organization [35].

4.2 Cell-extracellular matrix interactions in DCM

There are no clear junction structures between healthy, fully matured CMs and ECM but the cells are connected to the ECM with collagen fibres by fibronectin and laminin. DCM alters these connections. J. Bishop et al. [36] found out that the concentration of collagen may be 2-fold, and D. Unverferth et al. [37] claimed the concentration could be even 5-fold in DCM. Unrestricted growth of the collagen network leads to enlarged ECM which may cause overload on CMs. Changes in collagen network also contribute to weakened transmitting of forces between myofibrils and collagen which decreases heart pressure and dilates the heart. Increased amount of collagen is also related to fibrosis of the heart. [38] Increased collagen concentration could explain the stiffening of the heart walls, while enlarged ECM causes the heart to enlarge.

5. CONCLUSIONS

Heart is a complex structure where almost everything is somehow connected to everything. As Figure 3 demonstrated, the junctions have some same proteins and are connected to form ICDs, bigger functional units of cardiac tissue. ICD structure makes the tissue congruent by fusion of neighbouring cells. Forming GJs connect the neighbouring cells' cytoplasms, allowing free flow of ions between cells while AJs and desmosomes keeps the disc structure intact and mechanically stable. Mechanically sensitive ion channels are the key to MEF system, which responds to different phases of heart beating. As mechanosensitive structures such as cadherin-linking junctions are reinforced through increased stretch, it is clear how exercise is good for heart by making it stronger and more durable.

Studying the effects of forces on different time scales, particularly long-term forces' impact on the heart cells, could reveal more about the onset of heart diseases. It may possible that forces of certain strength combined with certain time could trigger cell signalling pathways which would lead to abnormal protein expression: too much or too little of certain proteins, wrong regulation of phosphorylation, etc. Abnormal protein expression may affect adversely ICD structures, changing their proteins, modifying AJs, GJs and desmosomes. At the level of the whole organ, those structural alterations can lead to diseases such as DCM.

Various methods of study are used to observe the function of the heart and cardiomyocytes. Atomic force microscopy-based single cell force spectroscopy, dual pipette aspiration, electron microscopy and particle image velocimetry are just few examples of methods which have helped cardiac research. They all have their advantages and disadvantages which is why it is useful to use them together. These methods have also contributed to DCM research.

DCM is a serious disease which may be caused by many different mutations in ICD structures. Mutations may change the mechanosensitive structures, or the mechanical stability of CMs. DCM's symptoms may be treated to the point where it does not affect much the patient's everyday life, but its root cause is hard to eliminate. In the future, it may be possible to treat DCM with gene therapy or engineered heart tissue made with induced pluripotent stem cells, grown to a mould.

REFERENCES

[1] C. Jacobs, H. Hayden, R. Kwon. “Introduction to Cell Mechanics and Mechanobiology”. Garland Science. November 2012.

[2] A. Kamkin & I. Kiseleva. ”Mechanosensitivity in Cells and Tissues”. Academia Publishing House Ltd. 2005.

[3] R. Delaine-Smith & G. Reilly. “The effects of mechanical loading on mesenchymal stem cell differentiation and matrix production”. Vitamins and hormones. 2011; Vol. 87. pp. 417-480.

[4] B. de Oliveira, E. Pfeiffer, J. Sundnes J, S. Wall, A. McCulloch. “Increased cell membrane capacitance is the dominant mechanism of stretch-dependent conduction slowing in the rabbit heart: a computational study”. Cellular and Molecular Bioengineering. June 2015; Vol. 8 (2). pp. 237-246.

[5] P. Tavi, C. Han, M. Weckström. “Mechanisms of Stretch-Induced Changes in $[Ca2+]_i$ in Rat Atrial Myocytes”. Circulation Research. 1998; Vol. 83 (11). pp. 1165-1177.

[6] R. Peyronnet, J. Nerbonne, P. Kohl. ” Cardiac Mechano-Gated Ion Channels and Arrhythmias”. Circulation Research. January 2016; Vol. 118(2). pp. 311–329.

[7] B. Alberts, A. Johnson, J. Lewis, M. Raff, K. Roberts, P. Walter. “Molecular Biology of the Cell”. 4th edition. New York: Garland Science, 2014. Print.

[8] G. Zhao, Y. Qiu, Z. Huifang, D. Yang. ”Intercalated discs: cellular adhesion and signalling in heart health and diseases”. Heart Failure Review. January 2019; Vol. 24. pp. 115-132.

[9] Wikipedia. ”Glanzstreifen im Myokard” by Dr. S. Girod and Anton Becker. March 2006.

[10] J. Kashef, F. Clemens. “Quantitative methods for analyzing cell–cell adhesion in development”, Developmental Biology. May 2015; Vol. 401(1). pp. 165-174.

[11] G. Cheung, O. Chever, N. Rouach. "Connexons and Pannexons: Newcomers in Neurophysiology". Frontiers in Cellular Neuroscience. November 2014; Vol. 8. pp. 348.

[12] U. Lisewski, y. Shi, U. Wrackmeyer, R. Fischer, C. Chen, A. Schirdewan, R. Jüttner, F. Rathjen, W. Poller, M. H. Radke, M. Gotthardt. “The tight junction protein CAR regulates cardiac conduction and cell-cell communication”. The Journal of experimental medicine. September 2008; Vol. 205(10). pp. 2369–2379.

[13] S. Higuchi, Q. Lin, J. Wang, T. Lim, S. Joshi, G. Anand, M. Chung, M. Sheetz, H. Fujita. “Heart extracellular matrix supports cardiomyocyte differentiation of mouse embryonic stem cells”. Journal of Bioscience and Bioengineering. March 2013; Vol. 115 (3). pp. 320-325.

[14] M. McCain, H. Lee, Y. Aratyn-Schaus, A. Kléber, KK. Parker. “Cooperative coupling of cell-matrix and cell-cell adhesions in cardiac muscle”. Proceedings of the National Academy of Sciences of the United States of America. June 2012; Vol. 109 (25): pp. 9881-9886.

[15] J. Helenius, C.-P. Heisenberg, H. Gaub, D. Muller. ”Single-cell force spectroscopy”. Journal of Cell Science. 2008; Vol. 121: pp. 1785-1791.

[16] University of Massachusetts Medical School. “What is Electron Microscopy?” Available online: https://www.umassmed.edu/cemf/whatisem/

[17] S. Kostin, D. Scholz, T. Shimada, Y. Maeno, H. Mollnau, S. Hein, J.Schaper. “The internal and external protein scaffold of the T-tubular system in cardiomyocytes“. Cell and Tissue Research. 1999; Vol. 294: pp. 449-460.

[18] ”Rat left ventricular muscle intercalated disc”. University of Connecticut, Bioscience Electron Microscopy Laboratory.

[19] Thermo Fisher Scientific. “An Introduction to Electron Microscopy: The Scanning Transmission Electron Microscope”. Available online: https://www.fei.com/introduction-to-electron-microscopy/stem/#gsc.tab=0

[20] J. Eades. “Reflection Electron Microscopy and Reflection Electron Diffraction in the Electron Microscope”. Surface Science. 1992; Vol. 62: pp. 99-103.

[21] Nanoscience Instruments. “Scanning Tunneling Microscopy”. Available online: https://www.nanoscience.com/techniques/scanning-tunneling-microscopy/

[22] Sigma-Aldrich. ”Actin Cytoskeleton / Focal Adhesion Staining Kit”. Available online: https://www.sigmaaldrich.com/catalog/product/mm/fak100?lang=fi&region=FI

[23] F. Chen, P. Tillberg, E. Boyden. “Expansion microscopy”. Science. Vol. 347(6221). pp. 543-548.

[24] M. Raffel, C. Willert, S. Wereley, J. Kompenhans. “Particle Image Velocimetry A Practical Guide”. Second Edition. Berlin, Heidelberg: Springer Berlin Heidelberg; 2007.

[25] Wikipedia. ”Particle Image Velocimetry (PIV) analysis of a Hamel-Oseen vortex pair” by user Willa. November 2011.

[26] A. Ahola., A. Kiviaho, K. Larsson, M. Honkanen, K. Aalto-Setälä, J. Hyttinen. ”Video image-based analysis of single human induced pluripotent stem cell derived cardiomyocyte beating dynamics using digital image correlation”. Biomedical engineering online. April 2014; Vol. 13(39).

[27] National Heart, Lung, and Blood Institute. “Cardiomyopathy.” Available online: https://www.nhlbi.nih.gov/health-topics/cardiomyopathy

[28] P. Harvey, L. Leinwand. “Cellular mechanisms of cardiomyopathy”. Journal of Cell Biology. August 2011; Vol. 194(3): pp. 355-365.

[29] Mayo Clinic. “Dilated cardiomyopathy”. March 2018. Available online: https://www.mayoclinic.org/diseases-conditions/dilated-cardiomyopathy/diagnosis-treatment/drc-20353155

[30] R. Weintraub et al. “Dilated cardiomyopathy.” The Lancet. 2017; Vol. 390: pp. 400-414.

[31] European Society of Cardiology. T. Rasmussen, T. Gadgaard, J. Mogensen. ”Desmosomal Mutations in Dilated Cardiomyopathy”. March 2011. https://www.escardio.org/Working-groups/Working-Group-on-Myocardial-and-Pericardial-Diseases/Publications/Paper-of-the-Month/Desmosomal-Mutations-in-Dilated-Cardiomyopathy

[32] American Heart Association. ”Dilated Cardiomyopathy (DCM)”. March 2016. https://www.heart.org/en/health-topics/cardiomyopathy/what-is-cardiomyopathy-in-adults/dilated-cardiomyopathy-dcm

[33] S. Vermij, H. Abriel, T. van Veen. “Refining the molecular organization of the cardiac intercalated disc”. Cardiovascular Research. March 2017; Vol. 113(3): pp. 259–275.

[34] L. Jifen, V. Patel, G. Radice. ”Dysregulation of Cell Adhesion Proteins and Cardiac Arrhythmogenesis”. Clinical Medicine & Research. March 2006; 4(1): pp. 42-52.

[35] V. Vasile, M. Will, S. Ommen, W. Edwards, T. Olson, M. Ackerman. “Identification of a metavinculin missense mutation, R975W, associated with both hypertrophic and dilated cardiomyopathy”. Molecular genetics and metabolism. February 2006; 87(2): pp. 169-174.

[36] J. Bishop, R. Greenbaum, D. Gibson, M. Yacoub, G. Laurent. “Enhanced deposition of predominantly type I collagen in myocardial disease”. Journal of molecular and cellular cardiology. 1990; Vol. 22: pp. 1157–65

[37] D. Unverferth, P. Baker, S. Swift, R. Chaffee, J. Fetters, B. Uretsky, M. Thompson, C. Leier. “Extent of myocardial fibrosis and cellular hypertrophy in dilated

cardiomyopathy". The American journal of cardiology. 1986; Vol. 57: pp. 816–820.

[38] V. Kapelko. "Extracellular matrix alterations in cardiomyopathy: The possible crucial role in the dilative form". Experimental & Clinical Cardiology. 2001; Vol. 6(1): pp. 41-49.