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# CELL SHAPE DETERMINES GENE EXPRESSION IN CARDIOMYOCYTES

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# Cell Shape Determines Gene Expression in Cardiomyocytes

# Thesis for Doctoral Degree (Ph.D.)

By

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# Abstract

The fundamental biological processes involve sensing biophysical stress, strain and forces along with conversion of these stimuli into chemical signals. These processes are linked to the atrophic and hypertrophic responses. Deficiencies in these biological processes are associated with different diseases, particularly in the circulation system. Although cardiomyocytes are exposed to significant hemodynamic stimuli that alter their shapes, it was not known until recently whether changes in cardiomyocyte shape impact gene expression. However, recent progress in single-cell RNA sequencing have enabled the profiling of transcriptomes of individual cardiomyocytes with engineered geometries, which are specific to normal or pathological conditions such as preload or afterload.

Cardiomyocytes undergo considerable changes in cell morphology, either due to mutations, causing various cardiomyopathies such as hypertrophic cardiomyopathy or dilated cardiomyopathy or via changes in hemodynamic conditions. Moreover, because of various patterns of contraction-relaxation cycles, the membrane of cardiomyocytes is dynamically reshaped in each beating cycle. The overall aim of this thesis was to investigate the effects of cardiomyocyte geometry on gene expression and signaling.

In study I, we engineered a novel platform to study cardiomyocyte morphology. In this article, we presented a single-cardiomyocyte trapping strategy, consisting of a method for growing neonatal rat cardiomyocytes with different aspect ratios. The study also proposes a protocol to sort patterned cardiomyocytes based on their acquired geometrical aspect ratios and pick up these adherent cells from their pattern. The described approach paved the way to profile the transcriptome of single cardiomyocytes with specific geometric aspect ratio.

In study II, we employed single-cell RNA sequencing to investigate impacts of cardiomyocyte aspect ratio on its transcriptome, using the approach proposed in study I. We observed that distinct morphotypic cardiomyocytes had noticeably varied gene expression patterns, implying that the shape of a cardiomyocyte plays a role in gene expression. This was apparent from the separate cluster of cells, detected in unsupervised clustering analyses.

In study III, we proposed a mathematical model of a sarcomere to examine whether and how signaling activity at the membrane of cardiomyocyte depends on its beating rate. Based on this model, a multiphysics program was designed to simulate the cardiomyocyte dynamic geometry throughout the contraction and relaxation phases. The main finding of this study was that an increase in the rate of cardiomyocyte contraction leads to an increase in the concentration of activated Src kinase, especially underneath the costameres. Since hypertrophy of cardiomyocyte modifies the ratio of surface to volume at the plane of membrane, the finding of this study suggests that hypertrophy might be considered as part of a feedback, equilibrating membrane-mediated signaling cascades.

These studies identify the shape of the cardiomyocyte as a significant determinant of its gene expression and signaling. Our findings illustrate a novel and important observation, with potentially far-reaching impacts in medicine and biology.

# List of scientific papers

I. **Payam Haftbaradaran Esfahani**, Ralph Knöll, An Approach to Study Shape-Dependent Transcriptomics at a Single Cell Level. *J Vis Exp*, (2020).

II. **Payam Haftbaradaran Esfahani**, Zaher Elbeck, Sven Sagasser, Xidan Li, Mohammad Bakhtiar Hossain, Ahammad Talukdar, Rickard Sandberg, Ralph Knöll, Cell shape determines gene expression: cardiomyocyte morphotypic transcriptomes. *Basic Res Cardiol* 115, 7 (2019).

III. **Payam Haftbaradaran Esfahani**, Jan Westergren, Lennart Lindfors, Ralph Knöll, Frequency-dependent signaling in cardiac myocytes. *Front Physiol* 13, 926422 (2022).

# List of scientific papers (not included in the thesis)

I. **Payam Haftbaradaran Esfahani**, Ralph Knöll, Cell shape: effects on gene expression and signaling. *Biophys Rev* **12**, 895-901 (2020).

# Contents

# List of abbreviations

| | |
|---|---|
| AR | Aspect Ratio |
| cDNA | Complementary Deoxyribonucleic Acid |
| CM | Cardiomyocyte |
| DCM | Dilated Cardiomyopathy |
| DEG | Differentially Expressed Genes |
| ECFP | Enhanced Cyan Fluorescent Protein |
| ECM | Extracellular Matrix |
| ERCC | External RNA Control Consortium |
| FRET | Fluorescence Resonance Energy Transfer |
| HCM | Hypertrophic Cardiomyopathy |
| HF | Heart Failure |
| hiPS-CM | Human Induced Pluripotent Stem cell-derived Cardiomyocyte |
| hMSC | Human Mesenchymal Stem Cell |
| hPSC | Human Pluripotent Stem Cell |
| LV | Left Ventricle |
| MAPK | Mitogen-Activated Protein Kinase |
| MOI | Multiplicity Of Infection |
| NRCM | Neonatal Rat Cardiomyocyte |
| PCA | Principal Component Analysis |
| PCR | Polymerase Chain Reaction |
| PDGF | Platelet-Derived Growth Factor |
| PDGFR | Platelet-Derived Growth Factor Receptor |
| SD | Sarcomeric Disc |
| scRNA-seq | Single Cell RNA Sequencing |
| T-Tubule | Transverse Tubule |
| YPet | An improved version of Yellow Fluorescent Protein |

# 1 Introduction

## 1.1 Mechanotransduction

Mechanotransduction is a crucial biological process in which cells detect mechanical stimuli, process them, and transform them into biochemical signals [1]. Abnormal mechanotransduction has been linked to a variety of diseases in a wide range of different in fields such as hearing, skin perception, cardiovascular sciences including cardiology. Abnormal mechanotransduction can occur when any of the components involved in sensing and converting mechanical forces, such as cell surface receptors, the cell cytoskeleton, the extracellular matrix (ECM) and various signaling molecules in the cytoplasm, experience alterations or malfunctions. Many different types of cells experience mechanical stress, particularly through changes in the ECM.

Hoffman et al. [2] divided the mechanotransduction process into three stages: mechanotransmission, mechanosensing, and mechanoresponse. Mechanotransmitters are structural components that transfer force from the ECM to the cytoskeleton, where it impinges on a mechanosensor. Mechanosensors that are subjected to force undergo a conformational change, resulting in a functional alteration. The ensuing signaling cascade is referred to as the mechanoresponse.

Cells are continuously exposed to mechanical forces due to a variety of factors, including organ motion, extracellular cell-to-matrix and cell-to-cell interactions, blood flow and intracellular traction. These mechanical factors have been demonstrated to impact a wide range of cellular processes, including differentiation, proliferation, gene expression and transcriptional responses.

Cells use a fundamental biological process called mechanotransduction to sense mechanical stress, transform them into biochemical signals, and produce downstream responses. Since biomechanical stimuli can be transmitted quickly via microtubules, cytoskeleton and stress fibers, cells can respond promptly to their fluctuating environment.

Recent research indicates that various cytoskeletal components and intracellular organelles such as the nucleus are significant mechanosensing units [3, 4] that can be affected by mechanical forces exerted on its envelope. This can lead to changes in chromatin structure, nuclear membrane organization, and gene expression, triggering downstream responses [5, 6]. The LINC complex is a crucial component that directly spreads mechanical stimuli from the cytoskeleton to the nucleus envelope, influencing nuclear shape, position, and chromatin positioning [7, 8]. Additionally, the nuclear lamina links the nuclear envelope to the chromatin, and both interplay in the nuclear response to stress [9, 10].

## 1.2 Mechanotransduction in heart diseases

The heart has the ability to adapt its rate and force of contraction to meet the body's needs, as it is a mechanosensitive organ [11]. The mechanical stimuli experienced by cardiac cells include shear stress from blood flow, torque, and the cyclical contraction and relaxation of the heart [12].

The World Health Organization states that cardiovascular diseases are the primary reason for illness and death on a global scale. The impact of these diseases on society's economic and social well-being is considerable, and they cause a drastic reduction in the quality of life for those who suffer from them [13].

Major diseases of the heart muscle, known as cardiomyopathies, such as hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM), are significant contributors to heart failure (HF) and are responsible for a high rate of morbidity and mortality [14, 15]. Cardiomyopathies may arise from both environmental factors such as exposure to drugs or toxins [16], along with genetic causes such as mutations. Alterations in genetic structure affecting ECM molecules, integrin receptors, or cytoskeletal proteins are thought to be possible causes of mechanosensory disorders and various types of heart disease [17].

The primary characteristic of DCM is an enlargement of the left ventricle (and sometimes the right ventricle) without a clear explanation, typically with a more pronounced effect on the interventricular septum [18]. HCM is additionally marked by diastolic dysfunction, as well as fibrosis and disarray and of cardiomyocytes (CMs). In most instances, mutations in sarcomeric proteins impact the contractile machinery of the heart, resulting in heightened contractility of CMs. DCM is identified by the enlargement of one or both ventricles and has a familial origin in 30% to 50% of cases. It impairs a diverse series of cellular functions, leading to compromised contraction of CMs, fibrotic repair and cell death. CMs experience significant alterations in their shape as a result of mutations that cause HCM or DCM, or due to changes in hemodynamic conditions [19]. However, it is unclear at present whether changes in cell shape alone have an impact on gene expression and signaling.

The heart's dynamic nature implies that mechanotransduction deficiencies are deemed to be critical contributors to heart diseases. The malfunctioning of any of the components involved in mechanotransduction can trigger HF, such as ECM proteins, focal adhesion proteins [20], integrins, and molecules responsible for the structure of the CM cytoskeleton [19, 21]. CM cytoskeletons comprise repetitive contractile units referred to as sarcomeres, with two adjacent sarcomeres interacting through a region known as the Z-disc. Telethonin (also called T-cap or Titin-cap) and Muscle LIM protein (MLP) are two proteins that contribute to the Z-disc architecture. Telethonin was recently discovered to participate in the regulation of apoptosis, establishing a connection between mechanosensing deficiencies and HF [22]. Titin is a spring-like molecule situated in the I-band region of the sarcomere, serving as a stretch sensor. Studies on MLP-deficient mice demonstrated that they developed DCM and displayed a severely distorted cytoarchitecture of the CMs [21]. Additionally, a prevalent mutation in MLP was linked to cardiomyopathies [23]. According to Knöll, Masahiko et al., Titin, T-cap and MLP are constituents of a mechanosensing complex, and impairments in this complex are associated with DCM and HF [19].

### 1.3 Cellular processes are influenced by global geometry of cells

The signaling activity at the cell membranes is influenced by various global geometry parameters, such as membrane surface area, size [24], the cellular aspect ratio (AR) [25], and the curvature of the membrane [26]. Previous studies have highlighted the significance of cell shape and geometry on cellular plasticity, including signaling, differentiation and metabolic activity. Several studies have reported plating of various cell types on patterned surfaces affect their cytoskeleton structure and dynamics [27-32], which consequently modulate cell signaling [33]. One notable study by Rangamani et al. [33] proposed a mechanism of curvature-dependent boost of transient receptor activity, which could enhance activity of kinases in signaling cascades through increased binding of ligands in curved membrane regions of elliptic cells. This study demonstrated that cell shape contains information that can impact level of MAPK (Mitogen-activated protein kinase) phosphorylation in the nucleus. Lateral diffusion can facilitate the exchange of molecules between regions of low and high curvature at the

2

plane of membrane, which gradually balances the inhomogeneity of receptor activity caused by diverse ligand binding. Nevertheless, if there are regions of membrane fission with high curvature, the exchange of matter may not occur. This lack of exchange could result in the reinforcement of the interaction between cytoplasmic effectors and the membrane boundary of endocytosed vesicle.

The commitment of embryonic stem cells to a certain lineage may be influenced by their shape. According to a study by McBeath et al. [34], the shape of human mesenchymal stem cells (hMSCs) can determine whether they will become osteoblasts or adipocytes. This is achieved by controlling the RhoA activity and its impact on the cytoskeletal tension that is mediated by ROCK. Spread cells were found to have higher levels of ROCK activity compared to round cells. It has been observed that hMSCs that spread out tend to differentiate into osteocytes, whereas those that remain round tend to develop into adipocytes.

During the early specification stage, induced pluripotent stem cells (iPSCs) are affected by mechanical factors. Ribeiro et al. [35] have successfully enhanced myofibril alignment and mechanical capacity using tunable substrates and defined adhesion geometry. The research demonstrated that hPSC-CMs were directed to acquire a shape with an AR of 7:1 and these cells imitate fully developed CMs concerning their electrical properties and the development of their t-tubules. Furthermore, hPSC-CMs with AR of 7 exhibited superior sarcomere function and alignment. Similarly, neonatal rat CMs (NRCMs) plated on substrates that had a specific length:width AR similar to that of healthy adult hearts had better contractile function than those with ARs similar to failing hearts [36]. In hypertrophy, cells become wider in the early stages, resulting in an AR of 1:1. However, in later stages, HF develops, and the cells become longer to an AR of 11. Thus, chronic hypertrophy models *in vivo* have declared an increase in left ventricular myocyte length of about 30%, whereas adult CMs treated extremely with hypertrophic stimulant *in vitro* have indicated similar increases in cell width [37, 38]. Kuo et al. [36] showed that sarcomeres aligned with the main axis of elongated NRCMs when cultured on micropatterned plates. Moreover, durations of calcium transient were extended when CMs had a shape with AR 11 rather than a normal AR, indicating that cell shape played a role in calcium metabolism. Concentric hypertrophy occurs when CMs thicken but remain in their normal length, while eccentric hypertrophy occurs when the left ventricle (LV) dilates and CMs elongate due to the sequential addition of sarcomeres. The type of hypertrophy that progresses depends on the cause and development of contractile dysfunction. For instance, an increase in afterload, as in hypertensive heart disease and valve stenosis, often leads to concentric hypertrophy, whereas eccentric hypertrophy is usually seen in ventricular volume overload, as in valve regurgitation, shunts, or as an overdue response to myocardial infarction.

## 1.4 Cell shape regulates the response to extracellular signals

Cell shape not only plays a direct role in intracellular signaling, but it also regulates the transcriptional responses of cells to signals from their microenvironment. The response of cells to external biochemical signals like cytokines and growth factors is influenced by their geometry. For instance, research has revealed that the cellular response to pro-inflammatory cytokine TNFα is dependent on cell geometry. In a study, fibroblasts plated on circular substrates showed distinct nuclear translocation of the effector molecule of TNFα signaling, which is the NFκB p65 subunit [39].

The way a cell responds to external biomechanical signals, such as compressive and external tensile forces, is influenced by the shape of the cell. When a cell experiences compressive force, its chromatin condenses, causing a decrease in transcriptional activity. As the arrangement of the genome and the

location of transcription factors vary among cells with different shapes, the effects of compressive forces on transcriptional responses are also dependent on the cell's geometry [40]. The studies conducted by Damodaran et al. [40] and Mitra et al. [39] compared rectangular and circular cells to investigate how cell geometry affects responses to external biochemical and biomechanical signals. However, it is uncertain whether the observed differences between the two shapes were solely due to their geometry. This is because the size of the cells was not consistent between the two morphotypes, which could have been a confounding factor. Another study found that genes related to actin, such as Zyxin, RhoA, and Actin-γ3, were expressed differently based on cell size [41].

## 1.5  Cell shape modifies the chromatin and epigenetic machinery

Recent studies, including those by Alisafaei et al. [42] and Dreger et al. [43], have focused on the connection between mechanotransduction and epigenetic regulators. Pereira et al. found that SMYD3 lysine methyltransferase, an epigenetic modifier, was influenced by cell shape and AR in murine myoblasts, affecting its nuclear and cytoplasmic localization. TAZ, an important mechanotransduction effector, was also regulated by cell size and AR. Other transcription factors, such as YAP and NFκB, showed nucleocytoplasmic shuttling that was modified by cell spreading. In three-dimensional microniches with different geometrical features, Bao et al. [44] observed that cell contractility, nuclear shape, and YAP/TAZ distribution were affected by cell volume. Interestingly, the orientation of chromosomes within the nucleus, influenced by the global geometry of the cell, also played a role in the mechanosensitivity of chromosomes.

# 2 Research Aims

CMs undergo considerable changes in cell morphology, either due to mutations, causing various cardiomyopathies such as HCM or DCM or via changes in hemodynamic conditions. Moreover, because of various patterns of contraction-relaxation cycles, the membrane of CMs is dynamically reshaped in each beating cycle. The overall aim of this study was to investigate the effects of CM geometry on gene expression and signaling.

**Study 1**

This study aimed to engineer a novel platform to study CM morphology. Therefore, we designed a single-CM trapping strategy, consisting of a method for growing NRCMs with different ARs and sorting these adherent live cells based on their AR at a single-cell level. The study also proposes a protocol to pick up single cells from their pattern, using a semi-automated micro-pipetting cell picker.

**Study 2**

This study aimed to systematically analyze the effects of CM AR on gene expression, using single-cell RNA sequencing (scRNA-seq).

**Study 3**

This study aimed to design a mathematical model of a sarcomere to examine whether and how signaling activity at the membrane of CM depends on its beating rate.

# 3 Materials and Methods

## 3.1 Study I: An approach to study shape-dependent transcriptomics at a single cell level

This study aimed to devise an innovative platform to study CM morphology. In this article, we designed a single-CM trapping strategy, consists of a method for growing NRCMs with different ARs and sorting these adhering live cells based on their AR at a single-cell level. The paper also proposes a protocol to pick up single CMs from their patches by a semi-automated micropipetting cell picker. The methods protocol has been described in the paper in detail; however, the main points are summarized here.

### Single-CM isolation

The hearts of 2-day-old Sprague-Dawley rats were quickly extracted, and tissue specimen were taken from the dorsal of the tip of the left ventricle [45, 46]. The Neonatal Heart Dissociation Kit (Miltenyi Biotec) was used to isolate single CMs from the tissue using the gentleMACS Octo Dissociator (Miltenyi Biotec) with heaters set at 37°C. The isolated cells were suspended with plating medium and left to settle in an uncoated cell culture flask for 2 hours. This allowed non-CMs to attach to the flask's surface, while the CMs remained in suspension. Non-adhering cells were then labeled magnetically with MicroBead-conjugated antibodies (Miltenyi Biotec) specific for non-CMs and removed by magnetic sorting. The resulting enriched NRCMs were diluted to the concentration of $3 \times 10^4$ cells/ml. 4 ml of the CM suspension were added onto a CYTOOchip, which was placed at the bottom of a 35 mm Petri Dish. This allowed CMs to attach to the fibronectin patches on the chips and form the defined patterns. The chip was immersed in the plating medium for 72 hours during the culture period.

### Micropatterned chip

To grow CMs in predetermined rectangular shapes, we tailored a CYTOOchip consisting of a square glass coverslip with 3000 fibronectin micropatterns arranged spatially, enclosed by cytophobic area. The chip was split into three zones, each containing 1000 rectangular patches with particular AR of 11 (elongated), 7 (rectangular), and 1 (square), all having a consistent pattern area of 2200 μm2.

### Single-CM sorting

We employed a semi-automated micropipetting cell picker (CellSorter, Budapest, Hungary) to pick individual CMs from the chip immersed in the medium [47]. Following a 72-hour culture time, the media was substituted with 1:1000 Vibrant Dye Cycle (Invitrogen, Carlsbad, CA, USA) in PBS-4:1 TryplE (Thermo Fisher Scientific) to enable visualization of the nuclei of live CMs. The cell picker program recorded the spatial coordinate of the selected cells for the succeeding picking. The selected CMs were then individually collected using a microcapillary pipette, which crossed to the saved location of each selected CM and exerted a predetermined vacuum to pick up that specific CM. The picked single CMs were instantly lysed in individual polymerase chain reaction (PCR) tubes containing 2 μl of lysis buffer and were subsequently processed using the Smart-Seq2 protocol for complementary deoxyribonucleic acid (cDNA) synthesis and scRNA-seq library preparation [48]. The whole process, starting from media removal, was accomplished within 40 minutes. 19 PCR pre-amplification cycles were applied to generate 15 μL of 1 ng/μL purified yield of cDNA.

## 3.2 Study II: Cell shape determines gene expression: cardiomyocyte morphotypic transcriptomes

To examine the impacts of CM ARs on its gene expression, we patterned single NRCMs, by directing them to grow on the fibronectin patches with defined ARs and named them morphotypes AR1, AR7, and AR11. CMs which take AR7 morphotype resemble the morphology of normal CM in a healthy heart. The detailed approaches for growing the study morphotypes and for sorting the qualified cells based on their morphotype were reported in the corresponding article of study I.

### *Single-CM mRNA sequencing and gene expression analysis*

Since the picking process of all selected single CMs were monitored through microscope, we did not prepare cDNA for those cells which were not picked properly. In addition, the Smart-Seq2 library was not generated for those cells with poor cDNA quality. Ultimately, 213 single CMs were subjected to scRNA-seq using the Illumina HiSeq2500 with 56 bps single-end sequencing. The raw reads in the FASTQ format were processed using the Genome Analyzer Analysis Pipeline (Illumina). Tophat2 was applied to align the reads to the RGSC Rnor 6.0, in combination with Bowtie2 [49, 50]. The mapped reads belonging to each gene were counted using FeatureCounts software, and uniquely mapped reads were extracted for further analysis [51]. We assessed the quality of individual cells by analyzing the total count and the proportion of spike-in transcripts. The expression of well-known CM-specific transcript markers like Tnni3, Tnnt2 and Myh6 confirmed the cellular cardiac identity of the single cells. Out of 213 cells, 178 were qualified the quality control criteria. We selected genes with an average count of $> 1$ across all cells for further analysis. To normalize the data, we utilized the external RNA control consortium (ERCC) spike-ins. Since the same number of ERCC RNAs was injected to each sample, differences in the content of ERCC spike-in RNA were presumed as technical bias. Therefore, we considered the size factor for each library as the total number of spike-in counts in that cell. To normalize the data, we scaled the size factors such that the mean of all size factors was one. To obtain the normalized expression values, we divided the counts of each cell by its corresponding size factor. The scran Bioconductor package's computeSpikeFactors function was used to compute size factors of ERCC spike-ins. Next, we normalized the expression values of genes by adjusting the sum of the ERCC counts across the libraries, applying the normalize function of the scater package [52]. We obtained log-normalized values by addition of one to the normalized count and then applying a log2 transformation. To identify differentially expressed genes (DEGs) between different morphotypes, we exploited the Seurat package [53] in R (The R Foundation, Vienna, Austria). We carried out differential gene expression analysis and selected DEGs based on an adjusted p-value of less than 0.05 for multiple-testing correction, utilizing the Benjamini-Hochberg approach.

### *Ingenuity pathway analysis (IPA)*

We identified significant DEGs by comparing pairwise morphotypes. We employed the IPA software (QIAGEN, Hilden, Germany) to analyze these DEGs and identify enriched canonical pathways, diseases, and biological functions [54]. We imported 3 sets of DEGs into the IPA with their corresponding regulation directions and magnitudes. To determine the gene set enrichment p-values of a specific canonical pathway, we used the right-tailed Fisher's exact test. The calculated enrichment p-values explains how likely the similarity between the list of DEGs and a specified pathway or biological function was accidental [54]. After calculating the enrichment p-values, they were adjusted by applying the Benjamini-Hochberg approach for multiple-testing and false discovery control.

Additionally, to measure the regulatory impact of the interplay between the DEGs, we used the bias-corrected activation z-score, representing the predicted level of activation or inhibition of the pathway of interest.

### 3.3   Study III: Frequency-dependent signaling in cardiac myocytes

*FRET technique to monitor the spatio-temporal activity of Src kinase in NRCMs*

The same method for isolating and purifying NRCMs was used as described in the first study. Hearts were quickly removed from Sprague-Dawley rats that were two days old. The NRCMs were then placed onto 35 mm MatTek glass bottom dishes that had been coated with 2 μg/cm2 fibronectin, at a density of $7 \times 10^3$ cells/mm2. After 24 hours, the plating medium was exchanged with serum-free maintenance medium. Next, the NRCMs that were being cultured were exposed to an adenoviral vector of KRas-Src FRET reporter [55] with a multiplicity of infection (MOI) of 10, and then were incubated at 37°C for 48 hours. This FRET-based biosensor, which detects Src activation, was developed by Wang et al. [56] and is comprised of ECFP (Enhanced Cyan Fluorescent Protein) and YPet (an improved version of Yellow Fluorescent Protein) that are linked together by the c-Src SH2 domain (a flexible linker), and the Src-specific substrate peptide, derived from c-Src substrate p130Cas. The ratio of donor (ECFP) to acceptor (YPet) in the biosensor is fixed at 1:1. When Src is activated and becomes phosphorylated, a change in conformation occurs that alters the distance between the donor and acceptor molecules, resulting in a change in FRET. In the resting state of Src, the conformation of the biosensor causes ECFP and YPet to be in close proximity, resulting higher FRET signal. However, when Src is activated and the substrate domain at tyrosine sites is phosphorylated, the biosensor unfolds, causing ECFP and YPet to separate and decreasing the FRET emission.

Following 72 hours of culturing, we performed FRET microscopy of Src activity in response to 30 ng/ml Platelet-derived growth factor (PDGF) stimulation of NRCMs that had been serum-starved. The imaging was carried out in a chamber that was temperature-controlled, and the NRCMs of interest were imaged using a 63X/1.4 oil-immersion objective. The fluorophores were excited using a 457 nm laser, and both the ECFP and FRET emissions were detected simultaneously at 5-second intervals, starting at 5, 10, and 20 minutes after the PDGF stimulation. Subsequently, the ratio of the ECFP to FRET fluorescence intensities were generated against time to investigate the spatial and temporal patterns of Src activity following PDGF treatment.

*Mathematical model of a contracting sarcomeric disc*

We developed a COMSOL Multiphysics [57] program to study how changes in the contraction rate of a cardiac muscle (CM) can impact membrane signal transduction. The program simulated the CM dynamic geometry throughout both the contraction and relaxation phases.

The model assumed that the adult human CM had a cylindrical shape composed of multiple symmetric "Sarcomeric Discs" (SDs). As sarcomeres are repeating units that are similar in structure, we modeled one contracting SD to simplify the computational complication. Additionally, we presumed that one SD had axial symmetry to reduce the simulations to a two-dimensional longitudinal section. During the contraction of the sarcomere and accordingly the whole cell, the shape of the SD changes over time as the Z-discs move towards each other. As the SD shortens, the Z-discs expand to a larger diameter to maintain a constant intracellular volume, resulting in an increase in the surface area of the SD membrane.

In our model, we allowed for continuous computation of the reshaping of the SD membrane, while maintaining isovolumetric cytosol. The dynamic deformation of the SD membrane caused the

cytosolic substances to flow towards and away from the membrane. To accomplish this, we generated a finite-element mesh that divided the model into smaller elements, over which we solved the Navier-Stokes equations. To test the hypothesis, we conducted a mathematical study using numerical simulations of the Src-mediated PDGFR signaling cascade. The movement of different molecules within the cytosol is ruled by advection and diffusion. Therefore, in our model, we calculated the concentration of molecules interplaying in the PDGFR signaling at each mesh node, using numerical solutions of the reaction-advection-diffusion partial differential equations. Specifically, we evaluated the concentration of phospho-Src over a 20-minute period following PDGF treatment and ran the simulation separately for beating frequencies of 0, 1, 2, and 4 Hz.

# 4 Results and Discussion

## 4.1 Study I: An approach to study shape-dependent transcriptomics at a single cell level

### *Results*

Cardiac hypertrophy is commonly linked with an enlargement in the size of the heart cells, which results in varying gene expression patterns when compared to normal-sized heart cells [18]. However, the impact of cell shape on whole gene expression in CMs is not analyzed.

To investigate how cell shape impacts gene expression, we created a dependable technique for growing CMs with different ratios of length to width. We achieved this by using a coverglass chip tailored with fibronectin micropatterns that dictate the shape of trapped cells. Our custom-designed chips had three different zones with specific length-to-width ratios (11:1, 7:1, 1:1), and each zone allowed the cells to grow into a particular AR. Studies have demonstrated a strong correlation between variations in the length-to-width ratio of cardiac myocytes and various forms of cardiomyopathy. We selected AR7 as the reference shape because it had a similar shape to a normal cardiac myocyte. As we were interested whether gene expression is associated with CM shape, we had to take out the impact of the cellular volume. Therefore, we conducted 3D reconstructions of 100 z-stack images to measure the volume of single cells with different ARs. Based on our analysis, we found no significant difference in the volumes of cardiac myocytes with different ARs.

After using the designed microchip, we could successfully grow engineered NRCMs with ARs of 11:1, 7:1, and 1:1 by culturing ventricular NRCMs from two-day-old rats for 72 hours, seeded on the fibronectin patches. The patterned CMs were easily identifiable through direct observation using a microscope. Additionally, the immune-fluorescent staining of the α-actinin sarcomeric structure confirmed their morphology. Notably, we observed changes in the orientation of sarcomeres in the cells with an AR of 1:1.

Second, we devised a method to find and pick mononucleated single CMs that have fully grown to the shape of the micropattern that they have been accidentally adhered to. Then, we exploited single cell RNA sequencing to detect differences in gene expression between cells with various ARs. We could successfully extract good cDNA libraries as evident by the distribution of cDNA length. To ensure the quality of the purified cDNA, an automated electrophoresis analyzer was used. An electropherogram of the pre-amplified cDNA of a single cell that was picked is included in the article. The electropherogram displays a clear band in the gel-like densitometry plot, corresponding to the peak near 1850 bp. The average size of the fragments was 1588 bp. The presence of only a small number of fragments shorter than 300 bp is indicative of a high-quality cDNA library.

### *Discussion*

Two critical factors in the experimental design led us to opt for scRNA-seq instead of bulk RNA sequencing. Firstly, only a small proportion of the micropatterns can be filled with a single cell. Secondly, there are instances where a single cell may not entirely cover the surface of its corresponding micropattern. CMs which thoroughly acquired their pattern shape need to be collected for gene expression analysis. Since only a subset of plated cells on the chip satisfied both selection criteria, it was not practical to trypsinize the entire chip, collect all cells and perform bulk RNA

sequencing. Therefore, individual qualified cells had to be sorted using a semi-automated cell picker for further single cell RNA sequencing.

To aid the process of fluidic vacuum cell picking, we utilized TryplE to weaken the cell attachment from fibronectin. Immediately thereafter, we promptly scanned the entire chip using a fluorescent microscope to see the nucleus of attached cells. Our selection process only focused on micropatterns that trapped a single mononucleated cell, and only if the cell thoroughly covered its corresponding fibronectin pattern. The scanning and selection process needs to be performed as quick as possible, before CMs deform due to TryplE treatment.

There were some limitations to the study. For instance, due to the difficulty in culturing enough adult CMs in defined shapes, NRCMs had to be used to generate different morphotypes. Additionally, culturing CMs *ex vivo* for 72 hours may have influenced the gene expression pattern. Nevertheless, this culturing duration was necessary to allow the cells to form specific morphotypes.

The qualified CMs of one chip need to be sorted in just one round. Eventually, approximately one-third of the selected cells, which is about 50 cells, were successfully picked from each CYTOOchip. The number of correctly picked CMs was limited due to two reasons. Firstly, some CMs were firmly attached to their fibronectin patch, and the pickup flow, which relies on vacuum pressure, could not pick them without causing damage to the cell membrane. Second, because of the TryplE addition, the attachment between some CMs and fibronectin become too weak. As a result, when the microcapillary moved towards these CMs, they were propelled from their fibronectin patches and could not be picked up.

This method can widely be applied to study different cell types, such as human induced pluripotent stem cell-derived CMs (hiPS-CMs) or other cell types, although some factors may need further optimization. For instance, appropriate ECM adhesive molecules should be used to coat the micropatterns to facilitate attachment of the specific cell type. Additionally, the geometry of the micropatterns would need to be adapted based on the research question and cell type being studied. The duration of the culturing period should be adjusted depending on the research question being addressed. The choice of detachment reagent and the duration of its incubation might be optimized specifically for the cell type being studied. For example, Accutase may be used rather than TryplE to detach neuronal and embryonic stem cells. Additionally, the parameters for valve opening time should be carefully examined to ensure successful and gentle picking of cells.

To summarize, we developed an innovative platform for investigating the impact of cell shape on gene expression, which can be a valuable tool for researchers in this field. Our experimental approach replicated the *in vivo* characteristic shapes of cardiac myocytes that are imposed by hemodynamic constraints to explore the relationship between the CM geometry and gene expression *in vitro*. Furthermore, we established a new platform for studying HF *in vitro*, and discovered that cell shape is a significant factor in determining gene expression. This is a groundbreaking finding with significant implications for the fields of medicine and biology.

The methods proposed in this paper could be used to study the interplay of cell shape and gene expression *in vitro* with great implications in developing our understanding of HF mechanisms.

## 4.2   Study II: Cell shape determines gene expression: cardiomyocyte morphotypic transcriptomes

### *Results*

In this study, we sequenced a total of 213 single cells exhibiting distinct morphotypes and identified an average of 4852 genes per cell, considering a gene detected if it had a read per kilobase million (RPKM) value > 1. The mean number of detected genes in AR7 was 5332, which was significantly higher than that observed in both AR1 and AR11, indicating lower gene expression in these morphotypes compared to AR7.

To determine if CM AR were the main factor influencing the differences in gene expression, we used unsupervised clustering to group cells based on their expressed genes, and found that CMs with similar AR tended to cluster together in three-dimensional t-distributed Stochastic Neighbor Embedding (t-SNE) and Principal Component Analysis (PCA) plots.

To further examine the effects of morphotypic heterogeneity, we compared different CM ARs to identify significant DEGs. The analysis indicates that pathologic CM ARs had fewer upregulated genes. This, along with the observation of reduced gene expression in AR1 and AR11, supports the idea that elongation or squaring of CMs had a significant impact on the number of genes being expressed.

We used IPA software to conduct pairwise comparisons of different cell shapes (AR1 vs AR7, AR11 vs AR7, and AR1 vs AR11). This allowed us to identify several enriched canonical pathways. When compared to AR7, both AR1 and AR11 showed inhibition of oxidative phosphorylation, cardiac beta-adrenergic signaling pathways and protein kinase A, indicating that these pathological conditions share common affected pathways. In addition, the results predicted that CM apoptosis, muscle necrosis and heart degeneration were activated in AR1 compared to AR7. On the other hand, we observed a lower flux of ions in AR11 compared to AR7.

We were interested to study if the effects resulted by altering the AR of CMs can be boosted or diminished by modifying mechanotransduction cascades. $\beta_1$-integrin/Src cascade is one of the well-known pathways in cardiac mechanotransduction. To address our study question, we conducted three sets of functional experiments. In the first set of experiments, we inhibited $\beta_1$-integrin by blocking anti β1-integrin antibody. As the second set, Src kinase was inhibited by saracatinib treatment, a known Src inhibitor [58]. For the third set, we over-expressed Src by transduction of patterned CMs with the KRas–Src adenovirus vector. Then, we conducted intra-condition studies, which means that we compared three morphotypes of one condition with each other and not with the morphotypes of other condition. Interestingly, we found that there was no significant difference in the average number of detected genes between morphotypes of β1-integrin-inhibited condition and also between morphotypes of Src-inhibited condition. Whereas, in the Src-overexpressed condition, the average was significantly less in AR1 and AR11, compared to AR7. In addition, we performed intra-conditional PCA. Importantly, morphotypes in β1-integrin-inhibited condition and in Src-inhibited condition were mixed, whereas morphotypes of the Src-overexpressed condition were clustered in accordance with their AR.

### Discussion

The fundamental biological processes involve sensing biophysical stress, strain and forces along with conversion of these stimuli into chemical signals. These processes are linked to the atrophic and hypertrophic responses. Deficiencies in these biological processes are associated with different diseases, particularly in the circulation system. Although CMs are exposed to significant hemodynamic stimuli that alter their shapes, it was not known until recently whether changes in CM shape impact gene expression. However, recent progress in scRNA-seq have enabled the profiling of transcriptomes of individual CMs with engineered geometries, which are specific to normal or pathological conditions such as preload or afterload.

The primary objective of this study was to examine the impact of distinct cell shapes on gene expression. Although, the *in vitro* experiments differ from *in vivo* ones, comparing *in vivo* cells with various morphology is extremely challenging [59]. The research was motivated and inspired by a previous study conducted by Kuo et al. [36], which employed a comparable method and reported changes in physiological parameters attributed to modifications in cell shape. In addition, the overall idea to study the effects of cell shape on cellular behavior was inspired by Rangamani et al. [33].

The key finding of our study was that the three distinct morphotypic CMs had noticeably varied gene expression patterns, implying that the shape of a CM plays a role in gene expression. This was apparent from the separate groupings we detected in unsupervised clustering using PCA and t-SNE analysis, which considered all the expressed genes. Additionally, we observed qualitative differences in gene expression levels among pathological cell shapes, particularly in pathways related to cell death or survival, energy and calcium metabolisms and beta-adrenergic signaling.

The functional studies illustrated that β1-integrin/Src cascade can modify the shape-dependent impacts on gene expression. This suggest that mechanotransduction and morphotype-dependent gene expression might be associated. It is noteworthy that the response of cells to inhibition of β1-integrin and Src kinase is almost identical. Blocking this pathway eliminates the cell shape-dependent gene expression, whereas activating Src kinase maintains this effect. These observations strongly suggest that this pathway plays a crucial role in regulating the mRNA transcriptome in a morphotype-specific manner.

In summary, our major finding was that cell shape determines gene expression, a previously unrecognized finding with far reaching implications for biology and medicine. While hemodynamic constraints impose changes in cardiac cell shape, HF-causing mutations change cell shape and hence feedback to hemodynamic consequences. Although far reaching, changing cell shapes can be used to treat HF.

### Limitations

To generate different morphotypes, we exclusively utilized NRCMs because adult CMs are extremely challenging to culture and keep in a specified shape [59]. Additionally, we cultured the CMs *ex vivo* for 72 hours, that may have influenced the overall gene expression profile. Nevertheless, the culturing was essential to arrange specified shapes. We employed the Smart-Seq2 protocol for RNA sequencing, which is only able to target poly-A tail containing mRNAs. This caused that almost half of the reads being uniquely mapped. Lastly, we just selected single CMs that were thoroughly captured their patches and were mononucleated. These stringent criteria, along with the difficulty in successfully loosening adherent cells for picking, resulted in a limited number of qualified and then analyzed CMs per morphotype.

## 4.3  Study III: Frequency-dependent signaling in cardiac myocytes

*Results*

Initially, we examined how Src kinase is activated in NRCMs in response to PDGF stimulation. Src kinase is normally present in an inactive state. However, when a cell is treated with PDGF, it attaches to the membrane-bound receptor, PDGFR, and activates intracellular Src. The activated transmembrane PDGFR then employs PTP (Protein Tyrosine Phosphatase) to activate (i.e., phosphorylate) the inactive Src. The activated Src (phospho-Src) can then be deactivated by Csk (C-terminal Src Kinase), a non-receptor tyrosine kinase [60, 61].

As outlined in the Methods part, we analyzed the features of Src activation in NRCMs through ECFP/FRET ratiometric analysis. Due to ECFP's high susceptibility to bleaching, it was necessary to limit laser exposure as much as possible to avoid any potential impact on FRET analysis. To this end, we measured the intensities of both the FRET and ECFP channels at four specific time intervals: 0+, 5, 10, and 20 minutes following PDGF stimulation.

The recorded microscopy images demonstrate that the costamere sites exhibit the strongest fluorescence intensities, indicating that Src kinase is predominantly localized at the costameres in CMs. While lower fluorescence signals were detected from other areas of the cell, we specifically focused on analyzing Src kinase activity at the costameres due to their significance in CM signaling. Additionally, when analyzing lower fluorescence signals from non-costamere regions, the autofluorescence bias was found to be more prominent.

Next, we computed the ratio of ECFP/FRET intensities for each time point. Although the ECFP/FRET values at time points t = 5, 10, and 20 minutes did not exhibit significant differences when compared to each other (according to an unpaired two-tailed t-test), they were all significantly higher than the value at t = 0+. This suggests Src kinase activation following PDGF stimulation, which is consistent with prior findings in various cell types [33, 62-64]. We conducted the ECFP/FRET measurement at the costamere pixels, using the methodology outlined in the methods section. Therefore, we monitored costameres to assess our hypothesis mathematically.

By employing FRET microscopy alongside our hypothesis, we were able to concentrate on Src activity specifically at the costameres. As mentioned earlier, Src kinase is primarily localized at the costameres in CMs. Hence, we utilized the modeled SD to evaluate the progression of phospho-Src concentration beneath the membrane at the costamere site. Our simulations revealed that although temporal activation of Src is approximately similar for all contraction frequencies, phospho-Src concentration is higher when sarcomere contracts by higher rate. Specifically, the maximum concentration of phospho-Src was 21.5%, 9.4%, and 4.7% higher at contraction frequencies of 4, 2, and 1 Hz, respectively, compared to 0 Hz.

To differentiate between the effects of membrane stretch and lattice expansion, we compared the previously mentioned SD model with an impractical model where we presumed that the membrane was not elastic and therefore could not be stretched. This led to greater expansion of the Z-line lattice in order to maintain a constant cytosolic volume. Under this assumption, at a contraction frequency of 4 Hz, the concentration of phospho-Src at the climax point was only 2.2% higher than that of the 0 Hz case. Therefore, membrane stretch has a more significant effect than lattice expansion in raising the concentration of phospho-Src.

## Discussion

The importance of membrane curvature as a key characteristic of cellular membranes has been widely recognized. Cells utilize various types of local membrane curvature to perform specific functions, such as filopodia for forming adhesions, caveolae for regulating membrane tension, and endosomes for intracellular signaling. The protrusion or invagination of membranes has been illustrated to respond differently to stimuli, by an increase in membrane eccentricity. This is because of temporary accumulation of active receptors at parts of the membrane with higher curvature. This temporary inhomogeneity in the concentration of active receptors is because the equilibrium between the diffusion and reaction of receptors and ligands in the membrane is locally disrupted, as described in Rangamani et al. [33]. A recent study has shown that fluctuations in local membrane curvature and the regulation of myosin II have a feedback relationship in endothelial cells [65].

The adult human CMs are almost in the two-dimensional shapes and not in their *in vivo* three-dimensional geometry. It is virtually unfeasible to assess our hypothesis by the *in vitro* experiments, since dynamic deformation of membrane is different in two-dimensions. In addition, it is very challenging to keep the transduced adult human CM in culture for long enough time to conduct the accurate experiment [59]. At this point, the computational modeling offers an alternative approach.

The main finding of this study was that an increase in the rate of CM contraction leads to an increase in the concentration of activated Src kinase, especially underneath the costameres. Since hypertrophy of CM modifies the ratio of surface to volume at the plane of membrane, the finding of this study suggests that hypertrophy might be considered as part of a feedback, equilibrating membrane-mediated signaling cascades.

## Limitations of the proposed model

Although we postulated that local cell geometry mediates signaling cascade, it is still based on theoretical factors which largely depends on the parameters of the cascade of interest. Therefore, the demonstrated impact may vary between distinct signaling pathways. In this study, we basically concentrated on Src-mediated PDGF signaling to examine our hypothesis, since Src plays critical role for cardiac mechanotransduction. Moreover, Src is crucial in mechanically-induced CM hypertrophy [66] and it has been implicated as a main effector downstream of PDGF-BB signal transduction.

Although, our computational model continuously measures the SD membrane deformation, constrained to the displacement of the isovolumetric cytosol, we assumed that the geometry transformation of the SD membrane sticks to a sinusoidal function. This means that two-dimensional projection of the SD membrane is similar to a positive half-sine shape, but with varying magnitude and width. Albeit the in-vivo geometry transformation is unlikely to accurately follow a sinusoidal function, this assumption seems uncontentious.

Another simplifying assumption used in our model is that no organelles or other obstacles were individually implemented in the model. At first glance, this assumption seems unfeasible, as the density of organelles significantly affects the diffusibility of molecules. However, as we used the realistic diffusion coefficient of all interacting molecules, we could simplify the model by neglecting the organelles density.

The geometry of a SD is not exactly disc-shaped. The main drawback of modeling of a SD in the form of disc-shaped is CM membrane invaginations known as T-tubules. The tortuous structure and the strait lumen of the T-tubules impede diffusion at the plane of membrane.

Another limitation of the proposed approach was that it was technically challenging to simulate the secondary effects of the proposed hypothesis on the cascade of interest. For example, it is known that STAT5 competes with Src for the PDGFR-β binding site.

# 5 Conclusions and Points of Perspective

Here we combined fundamental biophysical concepts, including reaction-advection-diffusion partial differential equations, with experimental cardiology and derived novel insights into cardiovascular biology. One of our major discoveries was that cell shape determines gene expression, a previously unrecognized finding with far reaching implications for biology and medicine. For example, it is well known that hemodynamic constraints have powerful effects on cardiac cell shape and hence physiology. Vice versa, HF causing mutations also induce changes in cell shapes which feedback to hemodynamic consequences. Therefore, in future it might be possible to change primarily the genome or epigenome with the aim of changing cell shapes to cure HF or other human diseases.

Apart from that, we engineered and designed novel experimental approaches to study single cell biology. Our approach, whereby we forced single cells into different shapes, can be used to study "HF on a chip" with implications for large scale and high throughput applications in big pharma.

Moreover, when we studied the effects of membrane curvature and hence frequency on signaling, we developed a novel algorithm to study intracellular signaling, program potentially useful to predict effects of different experimental interventions, including the study of novel drugs in the pharmaceutical industry.

Mechanobiology and its implications in medicine, cardiovascular biology, and especially for the HF, remain to be of significant importance as can be seen in various recent publications [67]. Mechanobiology and its medical applications hold great promise for the future.

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