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Chapter 4

Modeling Human Cardiac Hypertrophy in Stem Cell-Derived Cardiomyocytes

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ABSTRACT

Cardiac hypertrophy accompanies many forms of cardiovascular diseases. The mechanisms behind the development and regulation of cardiac hypertrophy in the human setting are poorly understood, which can be partially attributed to the lack of human cardiomyocyte-based pre-clinical test system recapitulating features of diseased myocardium. The objective of our study is to determine whether human embryonic stem cells - derived cardiomyocytes (hESC-CM) subjected to mechanical stretch can be used as an adequate \textit{in vitro} model for studying molecular mechanisms of cardiac hypertrophy. We show that hESC-CM subjected to cyclic stretch, which mimics mechanical overload, exhibit essential features of a hypertrophic state on structural, functional, and gene expression levels. The presented hESC-CM stretch approach provides novel insight into molecular mechanisms behind mechanotransduction and cardiac hypertrophy and lays ground for the development of new pharmacological approaches as well as for discovering new potential circulating biomarkers of cardiac dysfunction.
INTRODUCTION

Heart diseases are among the leading causes of death worldwide\(^1\). Cardiac hypertrophy is common manifestation of many forms of cardiac disease including heart failure, myocardial infarction and hypertension\(^2\).

An important cause of cardiac hypertrophy is mechanical stretch\(^2,3\). At the moment when this stretch becomes excessive due to the volume or pressure overload, the heart starts sending stress signals activating a hypertrophic response program to compensate the wall stress. Persistent wall stress leads to a progressive cardiac remodelling and eventually the heart goes into a failing state\(^2,3\). Understanding the molecular mechanisms underlying development of cardiac hypertrophy is essential for advancing treatments of cardiac diseases. However, the absence of adequate research models is a major obstacle to functional studies of molecular mechanisms of cardiac hypertrophy. Human \textit{in vitro} models are limited by the amount of patient-derived primary cells of cardiovascular lineage and poor consistency, while existing animal models are not always accurately representing the mechanisms responsible for cardiac hypertrophy development due to the significant inter-species differences in contractile features, stress response, ion channels expression and distribution\(^4-6\). Additionally, extensive animal studies are limited by ethical reasons and there are strong incentives to limit the use of animal testing in European Union and to replace it with alternatives, including the development of technologies such “human-on-a-chip” via employment of stem cell derived organ specific cells (report on a European Commission scientific conference, Cronin, 2017).

Cardiomyocytes (CM) differentiated from human embryonic stem cells (hESC) are a powerful tool for investigating cardiac development, function and pathophysiology\(^8,9\). Subsequently, the availability of human embryonic stem cell-derived CM (hESC-CM) provided a relatively cheap platform for drug testing\(^10,11\). However, the use of hESC-CM for studies of cardiac hypertrophy and downstream molecular effects is not clearly demonstrated\(^12,13\). The mechanic force overload, or stretch model, is based on induction of mechanical stress by physical stretching of CM. This is a sustained \textit{in vitro} model that mimics volume overload on the heart during cardiac hypertrophy development\(^3,14,15\). Additional advantage of stretch \textit{in vitro} model compare to neurohumoral stimulation of CM (i.e. phenylephrine stimulation) that it allows to dissect direct effects of increased biomechanical load from the secondary neurohumoral activation.

The aim of this study is to determine whether hESC-CM subjected to mechanical stretch can be used as an informative \textit{in vitro} model for investigation of molecular mechanisms of cardiomyocyte hypertrophy and identification of new targets involved in this process.
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METHODS

Cardiac Differentiation of hESC
To differentiate hESC into CM a small molecule-based protocol utilizing modulation of Wnt/β-catenin signaling was employed. hESC were treated with 6 µM GSK3-β inhibitor CHIR99021 (Cayman Chemicals) for two days, followed by inhibition of WNT signaling using 2 µM of Wnt-C59 (Tocris Bioscience). To further increase CM population purity, metabolic differences between CM and non-CM were exploit and cells were subjected to glucose starvation in presence of 5mM of dL-lactate for 6 days, as was published previously67.

Application of Mechanical Stretch
CM were cultured on a BioFlex six-well plate (Flexcell international) coated with Geltrex (Flex I Culture Plates Collagen I, Flexcell International) and were stretched using an FX-4000 (Flexcell International). Cyclic equiaxial stretching at a frequency of 1 Hz, mimicking humans heart rate (1 Hz = 60 beats per min), and 15% elongation was applied for 48 hours. Control CM were cultured on identical plates and kept under identical conditions, without mechanical stretch application.

Atomic Force Microscopy (AFM)
All beating force and stiffness values were determined by atomic force microscopy (AFM) nanoindentation. All measurements were performed with uncoated silicon nitride DNP-10 cantilever with pyramidal tip (Bruker). According to previously published data this type of cantilevers provided most stable force reading results in case of CM biosensing70. Cantilevers were first calibrated by the thermal tuning method71. Spring constants were always within the range given by the manufacturer (0.053 to 0.077 N/m). AFM measurements were carried out at room temperature in CDM3 media supplemented with 10 mM HEPES using a BioScope Catalyst AFM (Bruker) with z-scan rates 1.0 Hz for beating force measurements and 3.0 Hz for stiffness measurements, under a maximal loading force of 2 nN, and indentation depth of 100 nm. Force curves were measured between the cantilever and CM and Young’s modulus values were derived using the Sneddon model72,73. We used a Poisson’s value of 0.5 for the cell. Indentation module of NanoScope Analysis software (Bruker) were used to calculate Young’s modulus (Version 3.0.0.19). CM contractions were measured during 60-120 seconds. Typical noise levels during these measurements were up to 200 pN. The contraction force and rate were calculated. The contraction force of each beat is calculated as a difference between peak value and baseline, the beat rate is obtained from the reciprocal of the time interval to the next beat.
RNA-seq Libraries Preparation and Sequencing

RNA-sequencing was performed on 14 HUES9-CM total RNA samples; CM from 7 independent differentiations were divided into a control or stretched group. Total RNA was extracted using Trizol reagent. Quality of extracted RNA were accessed on Bioanalyzer 2100 (Agilent) using Pico RNA chip (Agilent). All samples passed QualityControl; all exceeded a minimum RIN of 8. Then, 1 µg of total RNA from each sample was processed with the NEXTflex Poly(A) Beads kit (Bioo Life Science) in order to pull down the mRNA from the total RNA samples. Eluted mRNA was further used for library preparation. Libraries were generated using NEXTflex™ Illumina qRNA-Seq Library Prep v2 kit (Bioo Life Science). At the end of the procedure, libraries were purified with Ampure XP beads (Beckman Coulter) and the DNA eluted with 17 µL resuspension buffer. The size and quality of the libraries were controlled by running them on a Bioanalyzer 2100 using the High Sensitivity DNA assay. For sequencing, all 14 samples were pooled and sequenced on Illumina HiSeq2500 machine. RNA-seq data were deposited to NCBI Short Read Archive under acc. no. SRR5875410-SRR5875423.

Gene Expression Analysis

PCR duplicate reads were removed using molecular indexes present in RNA-seq libraries made with NEXTflex qRNA-Seq kits. Nonredundant reads were mapped to human genome (GRCh38) with STAR v.2.5.2b and gene counts were obtained with STAR’s option ‘quantMode GeneCounts’ and gene annotations from GENCODE v. 26. Obtained gene counts were normalized with RUVSeq using parameter k=5, and differential gene expression was calculated with edgeR package. Gene Ontology (GO)-term enrichment analysis was performed with GOrilla and visualized with REVIGO. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed genes was performed with STRING database.

Statistical Analysis

Values are displayed as medians of at least three independently performed experiments. For all graphs: each dot represents results of independent experiments performed on the independently derived CM, each dot is a mean of at least two technical replicates. Unless stated otherwise statistical comparisons were performed using two-tailed, unpaired Student’s t-test (Prism, GraphPad Software). For qRT-PCR, analysis was performed using GenEx software (MultiD Analyses AB). The following indications of significance were used throughout the manuscript: * p < 0.05, ** p < 0.01, *** p < 0.001.
RESULTS

Generation and Characterization of hESC-CM.

CM were generated from two independent previously characterized hESC lines, HUES9 and H9, using small molecule-modulated differentiation and subsequent lactate purification\textsuperscript{16,17}. Purified CMs were subjected to mechanical stretch (Figure S1A) and examined for cardiac hypertrophy-related alterations with several assays (Figure 1A). CM derived from both lines expressed standard CM-specific markers (Figure 1B) and exhibited spontaneous beating (Movies S1 and S2).

Mechanical Stretch Induces Hypertrophy, ROS Production, Cell Death and Fetal Genes Expression in hESC-CM.

Heart cells are exposed to rhythmic contraction or regular cyclic stretch throughout adult life. However, chronic biomechanical stress is also an attribute of arterial hypertension or valvular heart disease, eventually leading to cardiac remodeling and cardiac hypertrophy development\textsuperscript{18}.

There are several stretch parameters that could influence the hypertrophy response, including the duration of stretch and the amount of stretch. Before extensive phenotyping was started, we studied several conditions, including different durations of stretch (7, 24, and 48 hr) and different amounts of stretch (5% versus 15%). We applied 15% stretch to monitor the release of cardiac-specific biomarkers after 7, 24, and 48 hoursof HUES9-CM stretch. Interestingly, the most robust release of the N-terminal prohormone of brain natriuretic peptide (NT-proBNP), a marker of cardiac stretch, as well as of troponin T (TnT), a marker of CM damage, appeared after 48 hours of 15% stretch (Figure S1B). In addition, mRNA expression level of TnT was not changed during the stretch at different time points, but the expression of NTproBNP significantly increased after 7 hours of stretch and remained significantly upregulated (Figure S1C). We also looked at two different stretch regimes: 5% and 15%. In our setup, application of 5% to HUES9-CMs for 48 hours did not induce a clear phenotype. We did not record significant changes in TnT release, increase of CM size, or clear induction of stress genes expression (see Figure S2). Therefore, for further experiments, 15% cyclic stretch was applied for 48 hr.

Exposure of hESC-CMs to mechanical stretch for 48 hours caused a significant increase in cell size in HUES9-CMs (26% ± 9.7%, p < 0.001) (Figures 2A and S3). In order to define whether inherited variabilities between hESC lines have an impact on the results, we validated the model using a different hESC line, H9. Cyclic stretch applied to H9-CM resulted in increase of CM size (37% ± 19.6%, p < 0.001), which indicated the robustness of the data (Figure 2A).

For both HUES9- and H9-derived CMs we observed a more than 2-fold increase of NT-pro-BNP and 1.5-fold increase of TnT release after 48 hours of stretch (Figure 2B). After 48 hours of stretch, a significant increase in reactive oxygen species (ROS) production in
HUES9- and H9-derived CMs was observed (Figure 2C). In order to further substantiate our claim that cellular hypertrophy is present, we measured CM volume with the use of confocal laser scanning microscopy (Zeiss LSM 7MP) on live wheat germ agglutinin (WGA)/Hoechst stained cells. Confocal imaging was further combined with three-dimensional (3D) image processing software (Imaris 6.3.5) to generate a 3D structure of the CMs and determine cell volume. We chose not to fix CM and performed experiments on live cells, since fixation causes shrinkage of the cells in the z-direction. Our results show that application of 15% stretch leads to a significant increase in CM volume compared with control.

**Figure 1 - Study Workflow.** (A) CMs were subjected to mechanical stress for 48 hours and assessed for changes in cell size, gene expression, cell stiffness, reactive oxygen species (ROS) production, and stress marker release. AFM, atomic force microscopy; RNA-seq, RNA sequencing; hESC-dCMs, human embryonic stem cell-derived cardiomyocytes; KD, knockdown. **p < 0.01, *** p < 0.001. (B) Fluorescent microscopy images of differentiated hESC-CMs expressing cardiac-specific markers cardiac troponin T (TnT) and α-actinin (ACTN2). DAPI staining was used to label nuclei. Scale bar = 50 μm. See also Movies S1 and S2.
Figure 2 - Effects of Mechanical Stretch on hESC-CMs. (A) Changes in CM size. Areas of at least 200 cells per condition per experiment were measured; each experiment represents independent differentiation of hESCs into CMs. Two different hESC lines were used: HUES9 and H9. Scale bar = 50 μm. **p < 0.001 by
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We performed an additional analysis of orthogonal projections of the confocal images in order to determine the height or thickness of the stretched and control cells. This analysis revealed that the height of stretched CMs was not changed compared with controls (10.78 ± 0.5 mm [stretched] versus 9.97 ± 0.56 mm [controls], p > 0.1, n = 4; Figures 2E and S4D).

To assess CM apoptosis, an annexin V assay was used. Stretched CMs displayed a 4-fold (p < 0.01) increased staining of annexin V compared with control (Figures 3A and 3B). After 48 hours of stretching we observed a significant 2-fold increase of lactate dehydrogenase (LDH), a marker of necrosis, in the supernatant from stretched cells (Figure 3C).

Cardiac hypertrophy is often accompanied by reactivation of fetal genes, i.e. genes that are active during fetal cardiac development and quiescent in adult heart. We tested several such genes and showed that expression of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) but not TnT was altered by mechanical stretch in HUES9- and H9-derived CM (Figure 3D). In addition, 48 hours of stretch shifted the balance between α- and β-cardiac myosin heavy chains (MYH6 and MYH7, respectively) expression (Figure 3D), which is a common response to the cardiac injury and hallmark of cardiac hypertrophy.

Mechanical Stretch Leads to Contractility Dysfunction and Increases Stiffness of hESC-CM.

In order to determine whether stretch leads to the development of functional defects (e.g., contractile dysfunction), we assessed CM contraction force with atomic force microscopy (AFM) (Figure 4A). CMs subjected to mechanical stress showed a 1.6-fold slower beating frequency but not significantly affected beating force amplitude compared with control (Figures 4B and 4C). However, AFM observations of CM beating behavior have some limitations, since all the measurements were performed at room temperature. We also noticed changes in myofibril structure and sarcomere length after 48 hours of stretch.
(Figures 4D and 4E). The size of the sarcomeres was significantly increased after stretch (Figures 4D and 4E). We found that myofibrils in stretched hESC-CMs were distributed in parallel and appeared wider, whereas in control CMs myofibrils exhibited a more branched pattern and appeared more spatially separated (Figure 4D). In addition, the elastic modulus, measured with AFM, was significantly higher in stretched CMs compared

![Image](image_url)

**Figure 3 - Effect of Mechanical Stretch on hESC-CM Viability.** (A) Annexin V and Hoechst staining. Scale bar = 50 μm. (B) The total number of annexin V-positive cells as a percentage of total cell number shown as median of three independent experiments. (C) Level of LDH after 48 hours stretch of hESC-CMs, shown as a fold change between control and stretched samples obtained from four independent experiments. (D) qPCR analysis of the mRNA levels of ANP, BNP, TnT, and α- and β-myosin heavy chain (MHC) isoforms in HUES9- and H9-derived CMs after 48 hours stretch. Each dot represents the mean Ct (cycle threshold) values of triplicate measurements normalized against the values obtained for the 36b4 gene for the same sample. For all graphs, each dot represents an independent experiment and independent differentiation of hESCs into CMs. Median values for each condition are shown. *p < 0.05, **p < 0.01.
Figure 4 - Effect of Mechanical Stretch on HUES9-CM Contraction and Sarcomere Length. (A) AFM cantilever brought into contact with cardiac cell. The gray box shows typical beating force trajectory. Peaks represent contraction of the CM: height of the peaks characterizes force of the CM beat (nN) and beat-to-beat distance represents beating rate (dT, seconds). (B) Effect of 48 hours mechanical stretch on CM beating rate, beats per minute (BPM). (C) Effect of 48 hours mechanical stretch on CM beating force. (D) Myofibrillar organization in control and stretched HUES9-CM. CMs were stained for troponin T and α-actinin with specific antibodies (red and green, respectively). Nuclei were stained with DAPI. Magnification, 63× (oil). Scale bar = 10 μm. (E) Effect of mechanical stretch on CM sarcomere length measured as a distance between z-discs. (F) Young’s modulus of the HUES9-CMs subjected to mechanical stretch for 48 hr. The plots show the difference in elasticity for the untreated control cells and stretched CMs. All graphs show results of at least four independent experiments. Median values for each condition are shown. *p < 0.05, **p < 0.01; n.s., not significant.
Figure 5

(A) Control vs Stretch

(B) Pathway
- Metabolic pathways (HSA1100): 0.00605
- Protein processing in endoplasmic reticulum (HSA4414): 0.0103
- p53 signaling pathway (HSA4415): 0.000247
- Ribosome biogenesis in eukaryotes (HSA3006): 0.000512
- Apoptosis (HSA4210): 0.00697
- Steroid biosynthesis (HSA1000): 4.58e-07
- Terpenoid backbone biosynthesis (HSA900): 0.000184

(C) Pathway
- PI3-K-Akt signaling pathway (HSA4151): 0.017
- Focal adhesion (HSA6510): 0.0104
- Adrenergic signaling in cardiomyocytes (HSA4261): 0.00096
- cGMP-PKG signaling pathway (HSA4022): 0.00265
- Protein digestion and absorption (HSA974): 4.06e-06
- ECM-receptor interaction (HSA4512): 1.44e-05
- Cell cycle (HSA44110): 0.00098
- Cytokine signaling pathway (HSA4921): 0.0116
- Dilated cardiomyopathy (HSA5414): 0.00098
- Oocyte meiosis (HSA4114): 0.0123
- Amoebiasis (HSA5146): 0.0123
- Arrhythmogenic right ventricular cardiomyopathy (HSA5412): 0.00858
- Hypertrophic cardiomyopathy (HSA5410): 0.0123
- DNA replication (HSA3030): 0.000387
- Fanconi anemia pathway (HSA3460): 0.0134
- Valine, leucine and isoleucine degradation (HSA280): 0.0154
- Propanoate metabolism (HSA6440): 0.0134
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with untreated control cells, demonstrating that mechanical stretch leads to an increase of CM stiffness (Figure 4F). It is important to note that thinner cells potentially lead to inaccurate measurements of the elastic modulus. However, as described above, stretching did not result in flatter cells (Figures 2D and 2E). Moreover, we were able to image CMs by contact-mode atomic force scan using colloidal probe cantilever and show that there is no significant difference in CM height of control versus stretched CMs (6.1 ± 0.5 versus 6.4 ± 1, p > 0.1, Figure S4). In addition, as was shown before, substrate contributions to the cell’s elastic modulus measurement can be neglected if the AFM tip indents less than 10%-20% of the cell thickness24,25.

Effects of Mechanical Stretch on Gene Expression.

In order to determine genome-wide gene expression changes induced by mechanical stretch we performed RNA sequencing (RNA-seq) of seven independently derived pairs of control and stretched HUES9-CMs. Analysis of RNA-seq data identified 622 upregulated genes and 1022 downregulated genes with a false discovery rate (FDR) below 0.01 (Figure 5A, Tables S1 and S2, a Web interface for data search and visualization is available at http://cardio.genomes.nl). Gene Ontology (GO) term enrichment analysis revealed that downregulated genes were mainly enriched in GOs such as cell cycle, chromosome organization, DNA replication, and animal organ development (Table 1, Figure S4). Interestingly, animal organ development includes such GO terms as regulation of heart contraction, muscle structure development, and muscle system process (Figure S5). Upregulated GO terms were mainly enriched in regulation of apoptotic process, sterol biosynthetic process, and cytoskeleton organization (Table 1, Figure S5). In addition, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis identified seven pathways that were upregulated (FDR < 0.02, Figure 5C, Table S4). Several of the downregulated pathways were enriched for a common set of regulatory (i.e., channels and mediators of Ca²⁺ concentration across the cell and contractility modulators) and structural genes (i.e., dystrophin, sarcoglycan alpha, members of integrin and collagen families) related to various cardiomyopathies (Table S4). KEGG pathway analysis also pointed toward significant upregulation of apoptosis and of p53 signaling pathways, as well as upregulation of steroid biosynthesis (Figure 5B, Table S3). The KEGG pathway maps (obtained via KEGG Mapper v3.1 and available at http://cardio.genomes.nl) allow further detailed exploration of the networks and the effects of stretch on gene expression.
Dysregulation of SP6 and FSTL3 Expressions Play a Role in Stretched-Induced Hypertrophy Development in hESC-CM.

We chose to further explore two genes of particular interest. The first gene, specificity protein 6 (SP6), was the most upregulated gene and transcription factor identified in our RNA-seq dataset (Figure 5A). SP6 is a member of the SP family of transcription factors, which contains zinc finger DNA-binding domains in its structure and localizes in the nucleus. We tested the hypothesis that knockdown of SP6 expression in human...
HUES9-CM would prevent the development of stretch-induced hypertrophy. The second gene, follistatin-like 3 (FSTL3), was chosen to validate already known molecular targets of cardiac hypertrophy development. Follistatin (FST) and follistatin-like genes (FSTL1, FSTL3) play an important role in heart failure development, and were linked to both disease severity and mechanisms underlying recovery.\(^{28-32}\) Interestingly, all of these genes were dysregulated upon stretch (Tables S1 and S2), with FSTL3 being the most dysregulated among them at 2-fold upregulation upon stretch (Figure 5A). Changes in expression of FSTL3 and SP6 upon stretch were also validated on H9-CM (Figure S6A). HUES9-CMs were transduced by lentiviral vector expressing anti-FSTL3 short hairpin RNA (shRNA), anti-SP6 shRNA, and scrambled control (SCR) shRNA. qRT-PCR analysis confirmed that transduction resulted in more than 70% reduction in FSTL3 and SP6 gene expression compared with the SCR (Figure S6B) and SCR subjected to mechanical stretch (Figure S6C). hESC-CMs expressing SCR-, FSTL3-, and SP6-specific shRNAs were stretched for 48 hours and assessed for cell size changes (Figure 6A). In CMs expressing SCR-shRNA, exposure to 15% mechanical stretch led, as expected, to a significant increase of cell size area (Figure 6B), TnT release (Figure 6C), and ROS production (Figure 6D). Knockdown of FSTL3 and SP6 in human CMs resulted in resilience to stretch-induced cardiac hypertrophy and resilience to oxidative stress (Figures 6B-6D).

**DISCUSSION**

In the present study, we employ mechanical stretching of hESC-CMs as a human disease model for cardiac hypertrophy. We show dysregulation at the cellular, functional, and genomic levels upon stretching. All specific hypertrophic hallmarks, such as increase of cell size, elevated levels of stress biomarkers, and shift in fetal genes expression, are observed. In addition, we notice an increase in cellular stiffness and decreased contractility accompanied by changes of the sarcomeric structure. Intervention via knockdown of genes of interest resulted in resilience to the hypertrophic phenotype upon stretch.

Several proteins are involved in sensing and responding to stretch in the heart, the so-called mechanosensors.\(^{33,34}\) Upon mechanical load, alterations in these proteins result in mechanical signaling leading to hypertrophy.\(^{33,35}\) These changes can take place at various sites in the CM, including the sarcomere, sarcolemma, and intercalated disc.\(^{33,36}\) To determine the global gene expression changes involved in the hypertrophic response to mechanical stress, we used an RNA-seq approach. For instance, the ANKRD1 gene, which is 1.5-fold upregulated in our dataset (Table S1), is a transcription factor that interacts with the sarcomeric protein titin and plays a role in the myofibrillar stretchsensor system. Increased levels of this protein have been detected in heart tissue of patients suffering from ischemic cardiomyopathy and dilated cardiomyopathy.\(^{37,38}\) It was also demonstrated...
that ANKRD1 upregulation is associated with altered systolic/diastolic function and that mutations in this gene result in a differential stretch-induced gene expression pattern. \(^{37,39}\) FHL1 is another titin-binding protein that has been shown to be upregulated in mouse models in response to pressure-overload-induced hypertrophy and in the hearts of human patients exhibiting hypertrophic. \(^{40,41}\) This is in line with our RNA-seq data that show a significant 1.5-fold upregulation of FHL1 in stretched hESC-CMs (Table S1). Also, other members of the LIM domain family that have been implicated in mechanical strain signaling, such as PDLIM3 and LDB3, were significantly dysregulated upon stretch (2-fold up and 1.5-fold down, respectively). LDB3 and PDLIM3 are known to interact with the α-actinin rod domain within z-disc and ablation of these proteins in mice resulted in abnormal cardiac function and severe myopathy. \(^{38,42}\)

Our AFM data showed that hESC-CMs subjected to mechanical stretch were significantly stiffer. Increased CM stiffness is one of the hallmarks of myocardial remodeling. \(^{43,44}\) There are several factors that influence cardiac stiffness, such as changes in sarcomere length, myofibril density, shift in N2A to N2B titin’s isoforms expression, and titin phosphorylation. \(^{43-45}\) Even though titin expression was not changed upon stretch, KEGG pathway analysis revealed downregulation of cGMP-PKG signaling pathway. This downregulation further contributes to the deficit of titin phosphorylation and overall increase of CM stiffness. \(^{46}\) Also, stiffness increases under oxidative stress conditions due to disulfide-bridge formation, which can occur in titin filaments. \(^{47}\) We believe that changes in sarcomeric organization observed upon CM stretch could also contribute to the changes in cardiac stiffness. There is a significant dysregulation in the expression of MYOM2, MYPN, FHL1, SQSTM1, CRYAB, OBSCN, PBK, and MDM2. All these genes encode proteins, interact with titin, and affect myofibril formation. \(^{34,47}\)

In addition to changes in myofibril architecture, regulation of cardiac muscle contraction was one of the significantly downregulated processes according to the GO term enrichment analysis. Our RNA-seq data showed significant deregulation of ion homeostasis, one of the essential regulators of cardiac contraction. \(^{48}\) We observed significant downregulation of Ca\(^{2+}\)-channels, such as RYR2, CACNA1D, CACNG6, CACNA1H, and CACNB2 (Table S2). We also found a downregulation of potassium (K\(^+\)) and sodium (Na\(^+\)) channels encoding genes such as SCN9A, SCN8A, ATP1A2, KCNQ1, HCN1, KCNN2, KCNAB2, and KCNH7 (Table S2). Multiple studies demonstrated that alterations in Ca\(^{2+}\) release and disturbance of K\(^+\) and Na\(^+\) ion channels cause abnormalities in CM beating behavior. \(^{49-51}\) These observations are also in line with our AFM results showing that stretched CMs need more time to generate beats of the same force amplitude compared with control.

The GO term enrichment analysis of differentially expressed genes showed that mechanical stretch upregulates biological processes such as apoptosis, sterol biosynthesis, and cytoskeleton organization. This is in line with our phenotyping data, which also demonstrate that mechanical stretch causes increase of ROS production, necrosis, as well
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as apoptosis in CMs, and could be responsible for cell loss. Similar findings have been reported for rat CM and human biopsy studies, in which cardiac hypertrophy development was associated with induction of significant apoptotic and necrotic responses\textsuperscript{43,52-54}.

As the heart begins to fail, disruption of its homeostasis leads to the release of stress-related cytokines, proteins, or peptides, into the circulation. In our in vitro model system NT-pro-BNP and TnT are released in the supernatant upon stretching. Another well-known cardiac biomarker is GDF-15. It has been shown to be upregulated in patients with various forms of heart failure and is associated with an impaired prognosis\textsuperscript{55,56}. Interestingly, GDF-15 was among the top ten upregulated genes identified in our RNA-seq dataset. These findings suggest that our in vitro model combined with the RNA-seq approach can be used for identification and validation of circulating biomarkers involved in cardiac failure.

We used obtained RNA-seq data not only for studying potential candidates, such as SP6, the most induced transcription factor in the dataset, but also for validation of previously identified candidate genes such as FSTL3. This gene has been reported as an important player in cardiac remodeling. Expression of FSTL3 was shown to be elevated in tissue biopsies of patients with heart failure and levels correlated with α-skeletal actin and BNP, both markers of disease development\textsuperscript{28-30}. As well as BNP, plasma levels of FSTL3 were shown to be increased in heart failure patients and associated with the severity of the disease, suggesting its potential role as a circulating biomarker\textsuperscript{57}. In addition to being secreted, FSTL3 can be found in both the nucleus and cytoplasm, which implies its involvement in transcriptional regulation of cardiac remodeling\textsuperscript{28,29}. Knockdown of FSTL3, as well as SP6, caused significant desensitization of CMs toward stretch-induced stress. This is in line with previously obtained in vivo and in vitro studies that show FSTL3 induction under myocardial stress and its negative effect on CM survival. A cardiac-specific FSTL3 knockout mouse model showed significantly smaller infarct sizes and lower number of apoptotic myocytes after ischemia/reperfusion injury. Knockdown of FSTL3 in cultured rat CMs inhibited phenylephrine-induced cardiac hypertrophy\textsuperscript{29,30,32}. Similarly, in our study, FSTL3 ablation in human CMs subjected to mechanical stretch led to the reduced level of cardiac hypertrophy and less cardiac damage assessed by level of TnT release and ROS production. Transcription factor SP6 has never been studied in relation to cardiac hypertrophy development. However, two other members of the SP family, SP1 and SP3, are both significantly overexpressed in hypertrophied and fetal murine hearts and are believed to be involved in cardiac hypertrophy development\textsuperscript{58}.

Current advances in tissue engineering techniques made 3D cardiac tissue cultures feasible and potentially useful for pharmacological and clinical applications\textsuperscript{59-62}. In addition, two-dimensional (2D) and 3D stem cell-derived CM cultures were used in a number of studies in order to address the immaturity of stem cell-derived CMs by employing a variety of biochemical and biophysical signals\textsuperscript{59-62}. Furthermore, there are several studies that applied 3D tissue culturing for studying stretch- or phenylephrine-induced cardiac
hypertrophy. We elected to use 2D CM cultures as a basis for our study for several reasons. While 3D tissues provide a more physiologically relevant structure, the major incentive for using this model is the requirement of non-CMs to be incorporated in 3D tissues for optimal function and tissue functionality. This would impede the attribution of stretch-induced effects specifically to CM. Moreover, constructed 3D tissues are based on various compositions of extracellular matrix (ECM), which undergoes cell-mediated remodeling at all times. Consequently, changes in ECM characteristics might alter the strain on specific parts of a tissue, thereby making the tissue unstable and prone to break, and reproducibility is rendered difficult. 3D tissues also have different diffusion rates of soluble factors depending on the location of individual cells within the tissue, resulting in heterogeneous cell behavior and therefore matrix remodeling. Combined, these traits of 3D tissues make characterization (of tissue as a whole) more complicated than with 2D cultures.

To summarize, in the present study we show that mechanical stretching of hESC-CMs can serve as an in vitro disease model for studying human CM hypertrophy. This model broadens our understanding of the molecular mechanisms behind mechanotransduction and cardiac hypertrophy and can serve as a launch platform for development of pharmacological approaches as well as for discovering p

**AUTHOR CONTRIBUTIONS**

EO, MFH, EB, PvdM designed the experiments. EO, MFH, KU, EB performed experiments, collected and analyzed the data. TVdJ and NB provided technical support. HvdM gave conceptual advice. EO, MFH, EB, PvdM wrote the manuscript.

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SUPPLEMENTARY METHODS

Immunostaining, Cell Size and Sarcomere Length Determination
CM subjected to mechanical stretch and controls were fixed directly on Geltrex-coated silicone membranes of BioFlex six-well plates. After stretch CM were washed twice with ice-cold PBS and fixed in 4% paraformaldehyde (PFA) for 5 minutes on ice, permeabilized with ice-cold PBS + 0.3% Triton X100 for 5 minutes and then washed twice with PBS. Cells were incubated for 1 hour in blocking solution: 3% BSA (Serva), 2% goat serum (Sigma Aldrich), 0.1% Tween 80 (Sigma-Aldrich) in PBS. Primary antibodies were diluted in blocking solution and incubated with the cells for 1 hour at room temperature. All used primary and secondary antibodies are listed in Table S5. Coverslips were mounted using Vectashield mounting medium with DAPI (Vector Laboratories). 4 to 7 random pictures were taken with 20x magnification and 150-200 cells/per condition were analyzed in order to determine cell size. Image processing software, manual pipeline (CellProfiler, Broad Institute of MIT and Harvard in Cambridge) was used to determine the effect of stretch on cell size. The error of the measurements was determined and did not exceed 2% (Figure S5). Sarcomere length was determined as a distance between z-bands on images taken with 63x magnification. Sarcomere length was analyzed using Fiji software. All slides were imaged on a Leica AF-6000 microscope.

Quantitative Real-Time PCR
Total RNA was isolated using Trizol reagent (Invitrogen), according to the manufacturers’ instructions. Superscript III Reverse Transcription Kit (Life Technologies) was used for cDNA synthesis. Quantitative real-time PCR was performed on Roche LC480 (Roche) using SYBRGreen Master Mix (BioRad). Each sample was run in triplicate in a total volume of 15 μL. Ribosomal protein lateral stalk subunit P0 (RPLP0 or 36B4) was used as a reference gene for normalization. Primer sequences are listed in Table S6.

Oxidative Stress Quantification
Total ROS production was quantified using CellROX Orange assay (Thermo Fisher Scientific). The intensity of CellROX Orange fluorescence is proportional to the level of free radical oxidation. Control and stretched CM were loaded with 5 μM CellROX Orange in culture medium and stained in the dark for 60 minutes at 37°C. Stained cells were washed once with PBS, dissociate with TrypLE and suspended in PBS. Cells were centrifuged for 5 minutes at 2000x g and suspended in 600 µl of PBS. 100 µl of cell suspension were transferred to a black 96 well plate (5 wells per condition) and analyzed immediately on a Synergy H1 microplate reader (Biotek) using 540/570 nm excitation/emission wavelength. For each condition the signal intensity was corrected to the number of cells as counted manually with a Fuchs-Rosenthal hemocytometer.
Annexin V Staining
After 48 hours of stretch, cells were washed once with PBS and once with binding buffer (BD Bioscience), piece of the membrane was cut out and incubated in 150 μL of staining solution (used ratio: 5 μL of Annexin V-FITC (BD Bioscience) in 100 μL of binding buffer) in the dark for 15 minutes at room temperature. Cells were washed once with binding buffer, counterstained with Hoechst and fixed with 2% PFA in binding buffer before analysis.

shRNA-encoding Lentiviral Particles Production
HEK 293T cells, grown at 37°C and 5% CO2 to 70% confluency in Dulbecco modified Eagle medium (DMEM, Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS, Sigma Aldrich) were co-transfected with a constant amount of pCMV Δ8.91-transfer plasmid, VSV-G-packaging plasmid and pLKO.1-plasmid expressing short hairpin RNA (shRNA) of interest. Fugene HD (Promega) transfection agent was used to improve transfection efficiency. After 24 hours media were replaced with fresh CDM3 media. After 24 hours CDM3 medium containing viral particles was harvested and filtered with 0.45 nm Nalgene filter to prevent HEK293T cells to be transferred to the solution. Clean viral supernatant was aliquoted and frozen at −80°C. pLKO.1 is a replication-incompetent lentiviral vector used for expression of shRNAs in CM. pLKO.1.shSP6, pLKO.1.shFSTL3, pLKO.1.shSCR (scrambled control) were purchased from Sigma MISSION RNAi. See shRNA probe sequences in Table S7.

Stable Transduction hESC-derived CM with shRNA-encoding Lentivirus
hECS-CM were plated on BioFlex six-well plates to have approximately 80% confluence at the time of transduction. For transduction, 1 ml of viral supernatant and 1 ml of fresh CDM3 media supplemented with 4 μg of polybrene (Sigma Aldrich) were added to the cells and incubated at 37°C for 18 hours. Next day viral particles were washed and 3 ml of fresh CDM3 media was added to the cells. Medium was changed every other day. On day 3 after transduction medium was aspirated and 3 ml of CDM3 medium supplemented with 4 μg/ml puromycin (Thermo Fisher Scientific) was placed into each well. Medium supplemented with puromycin was changed every other day and after 4 days of puromycin selection, cells were used for the downstream assays.

Levels of TNT, NT-proBNP, LDH in Cell Culture Media
Levels of damage-related biomarkers Troponin T (TnT), lactate dehydrogenase (LDH) and the N-terminal fragment of the prohormone of brain natriuretic peptide (NT-proBNP) were analyzed in the culturing media. LDH levels have been determined using an UV assay LDH kit (11822837, Roche Diagnostics). TnT and NT-proBNP levels were analyzed using electrochemiluminescence immunoassay kits (0509277 and 04842464 respectively, Roche Diagnostics). For every independent experiment, measurements were done at least in duplicate.
**Supplementary Figure S1 - Effects of Mechanical Stretch Duration on HUES9-CM.** Related to Figure 2. (A) Equiaxial strain application on hESC-derived CM. (B) Levels of TnT, NT-proBNP measured in CM's media after mechanical stretch at 7hrs, 24hrs and 48-hour time-points. (C) qPCR analysis of the mRNA levels of TnT and BNP in HUES9-derived CM after 7, 24 and 48 hours of stretch. Each dot represents the mean Ct values of triplicate measurements normalized against the values obtained for 36b4 gene for the same sample. For all graphs each dot represents independent experiment and independent differentiation of HUES9-hESCs into CM. Median values for each condition are shown. *p<0.05, **p<0.01, ***p<0.001; by unpaired two-tailed Student’s test.
**Supplementary Figure S2 - Effects of 5% Mechanical Stretch on HUES9-derived CM.** (A) Immunostaining of HUES9-CM with phalloidin. Nuclei stained with DAPI. Images were taken on the control samples and samples subjected to 5% stretch for 48 hrs. Scale bar = 50 μm. (B) Cell size changes of HUES9-CM subjected to 5% stretch for 48 hrs. At least 80 cells were analyzed per condition. n.s., not significant; by Mann-Whitney-U-test. (C) Level of troponin T, measured in CM’s media after 5% mechanical stretch. (D) qPCR analysis of the mRNA levels of ANP, BNP, TnT, α- and β-MHC in CM after 48 hours of 5% stretch. Each dot represents the mean Ct values of triplicate measurements normalized against the values obtained for 36b4 gene for the same sample. For all graphs each dot represents independent experiment and independent differentiation of hESCs into CM.

**Supplementary Figure S3 - Accuracy of CM Size Measurement.** Related to Figure 2. (A) Determination of the error of the CM size measurements. Sizes of at least six cells from the same image were repeatedly measured for at least 9 times, standard deviation (SD) of the measurements were used to calculate error mean. Results are based on 2 independent experiments, 2 independent HUES9-CM cultures. (B) Example of how the measurements were performed.
Supplementary Figure S4 - Effects of 15 % Mechanical Stretch on HUES9-CM height via Confocal Microscopy and Atomic Force Microscopy (AFM). Related to Figures 2 and 4. AFM image of control (A) and stretched (B) hESC-CM in contact mode overlaid with AFM height image. Examples of height measurements along the lines displayed in far-right panels for control and stretched CM. (C) Changes in CM height derived from AFM images of control and stretched CMs. Measurements are based on 3 independent experiments. (D) Examples of orthogonal projections derived from the confocal images of control and stretched hESC-derived CM stained with WGA and Hoechst. Scale bar = 50 μm.
Chapter 4

A

Up-regulated upon stretch

B

Downregulated upon stretch

Supplementary Figure S5 - Gene Ontology Analysis of Genes Up- and Down-regulated upon Stretch. Related to Table 1. The analysis was performed using GOrilla and visualized with REVIGO, which performed removal of redundant GO terms and generated tree maps of the functional annotations associated with the up-regulated (A) and the down-regulated genes (B).
Supplementary Figure S6 - Validation of FSTL3 and SP6 Knockdowns. Related to Figure 6. (A) Validation of FSTL3 and SP6 gene expression in H9-CM. FSTL3 and SP6 are significantly up-regulated in H9-CM upon 48 hours stretch. (B) mRNA expression levels of FSTL3 and SP6 in the shRNA transduced HUES9-CM. Expression levels are relative to untreated scramble (SCR) control. (C) mRNA expression levels of FSTL3 and SP6 in the shRNA transduced HUES9-CM subjected to mechanical stretch. Expression levels are relative to stretched scramble control (Ctrl). For all graphs each dot represents the mean Ct values of triplicate measurements normalized against the values obtained for 36b4 gene for the same sample. Measurements are based on at least 3 independent experiments.
**SUPPLEMENTARY TABLES**

**Table S1 - List of up-regulated genes upon stretch identified by the RNA-seq analysis.** The table represents fold change of gene expression, significance (p-value and FDR) and gene description. Available in online version.

**Table S2 - List of down-regulated genes upon stretch identified by the RNA-seq analysis.** The table represents fold change of gene expression, significance (p-value and FDR) and gene description. Available in online version.

**Table S3 - Pathways up-regulated by 48 hours mechanical stretch.**

| #pathway ID | Pathway description                                      | Observed gene count | FDR            | matching proteins in analyzed network                                                                 |
|------------|----------------------------------------------------------|---------------------|----------------|-------------------------------------------------------------------------------------------------------|
| 100        | Steroid biosynthesis                                     | 9                   | 4.58e-07       | CYP2R1, DHCR24, DHCR7, EBP, FDF1, HSD17B7, MSMO1, NSDHL, SQLE                                        |
| 900        | Terpenoid backbone biosynthesis                          | 7                   | 0.000184       | ACAT2, FDPS, HMGCR, HMGCS1, ICMT, IDI1, MVD                                                          |
| 4115       | p53 signaling pathway                                    | 11                  | 0.000247       | BAX, CDKN1A, FAS, GADD45A, MDM2, PPM1D, RRM2B, SERPINE1, SESN2, THBS1, TNFRSF10B                     |
| 3008       | Ribosome biogenesis in eukaryotes                        | 11                  | 0.000512       | BMS1, EIF6, GTPBP4, HEATR1, LSG1, MPHOSPH10, NOL6, RPP25, RPP38, TCOF1, UTP6                          |
| 1100       | Metabolic pathways                                       | 56                  | 0.00605        | ACAT2, ACLY, ACOX3, ACSS2, AGPAT9, AKR1C3, ALAD, ALG8, ARG2, ATP6V0E1, ATP6V1D, CHPF2, CKB, COASY, CYP2R1, DHCR24, DHCR7, EBP, ENO2, ENO3, ENPP1, FASN, FDF1, FDPS, G6PD, GCLC, HMGCR, HMGCS1, HSD17B7, ID11, INPP1, INPP5K, ITPKB, LIPG, LPIN1, ME1, MMAB, MSMO1, MVD, NSDHL, OCLR, P4HA1, P4HA2, PC, PNPLA3, POLG, POLR1C, RPN2, RRM2B, SHMT2, SMPD2, SQLE, ST3GAL2, ST6GAL1, SYNJ2, TKT |
| 4210       | Apoptosis                                                | 10                  | 0.00697        | AIFM1, BAX, BCL2L1, CAPN2, FAS, RIPK1, TNFRSF10A, TNFRSF10B, TNFRSF10D, TNFRSF11A                   |
| 4141       | Protein processing in endoplasmic reticulum             | 14                  | 0.0103         | ATF4, BAX, CAPN2, CRYAB, DDIT3, DNAJA1, BIXO2, HSP90AA1, HSPH1, NPLOC4, RPN2, UBE2J2, UBQLN4, UFD1L |
Table S4 - Pathways down-regulated by 48 hours mechanical stretch.

| #pathway ID | Pathway description                                      | Observed gene count | FDR       | matching proteins in analyzed network                                                                 |
|-------------|----------------------------------------------------------|---------------------|-----------|--------------------------------------------------------------------------------------------------------|
| 4974        | Protein digestion and absorption                         | 19                  | 4.06e-06 | ATP1A3, ATP1B2, COL1A4A1, COL1B1A, COL1A1, COL2A1, COL27A1, COL2A1, COL3A1, COL4A2, COL4A5, COL4A6, COL6A1, COL6A2, COL6A3, COL7A1, KCNQ1, SLC16A10, SLC8A1 |
| 4512        | ECM-receptor interaction                                 | 18                  | 1.44e-05 | AGRN, COL1A1, COL27A1, COL2A1, COL3A1, COL4A2, COL4A5, COL4A6, COL6A1, COL6A2, COL6A3, HMMR, ITGA10, ITGA11, ITGA8, LAMA4, LAM5, LAMC1 |
| 3030        | DNA replication                                          | 10                  | 0.000387 | LIG1, MCM2, MCM4, MCM5, MCM6, POLE2, RFC3, RNA5EH2A, RNA5EH2B, RPA1                                  |
| 4110        | Cell cycle                                              | 18                  | 0.00098  | BUB1, BUB1B, CCNA2, CCNB1, CCNB2, CDC20, CDC45, CDC6, ESPL1, MAD2L1, MCM2, MCM4, MCM5, MCM6, PLK1, PTTG1, TFGB2, TTK |
| 4261        | Adrenergic signaling in cardiomyocytes                  | 20                  | 0.00098  | ADCY3, ADCY7, ATP1A3, ATP1B2, CACNA1D, CACNB2, CACNG6, CAMK2B, CREB3L4, KCNQ1, MYH6, PLCB2, PLN, PPP1CC, PPP1R1A, PPP2R2B, PPP2R3A, RPS6KAS, RYR2, SL8A1 |
| 5414        | Dilated cardiomyopathy                                  | 15                  | 0.00098  | ADCY3, ADCY7, CACNA1D, CACNB2, CACNG6, DMD, ITGA10, ITGA11, ITGA8, MYH6, PLN, RYR2, SGCA, SLC8A1, TFGB2 |
| 4022        | cGMP-PKG signaling pathway                              | 20                  | 0.00295  | ADCY3, ADCY7, ATP1A3, ATP1B2, CACNA1D, CREB3L4, GUCY1A2, MEF2A, MEF2C, NFAT1, NFAT2, PDE3A, PDE3B, PDE5A, PLCB2, PLN, PPP1CC, PPP3R1, PRKCE, SL8A1 |
| 5412        | Arrhythmogenic cardiomyopathy (ACM)                     | 12                  | 0.00658  | CACNA1D, CACNB2, CACNG6, CTNNAS3, DMD, GJA1, ITGA10, ITGA11, ITGA8, RYR2, SGCA, SLC8A1 |
| 4510        | Focal adhesion                                          | 22                  | 0.0104   | COL1A1, COL2A1, COL2A1, COL3A1, COL4A2, COL4A5, COL4A6, COL6A1, COL6A2, COL6A3, Dock1, ITGA10, ITGA11, ITGA8, KDR, LAMA4, LAM5, LAMC1, PDGFD, PDGFRB, PPP1CC, TTN2 |
| 4921        | Oxytocin signaling pathway                              | 18                  | 0.0116   | ADCY3, ADCY7, CACNA1D, CACNB2, CACNG6, CAMK2B, CAMK2K, EEF2K, GUCY1A2, KCNQ5, MAP2K5, MEF2C, NFAT1, NFAT2, PLCB2, PPP1CC, PPP3R1, RYR2 |
| 4114        | Oocyte meiosis                                          | 14                  | 0.0123   | ADCY3, ADCY7, BUB1, CAMK2B, CCNB1, CCNB2, CDC20, ESPL1, MAD2L1, PLK1, PPP1CC, PPP3R1, PTTG1, SGOL1 |
| 5146        | Amoebiasis                                              | 14                  | 0.0123   | COL1A1, COL2A1, COL2A1, COL3A1, COL4A2, COL4A5, COL4A6, GNAL, LAMA4, LAM5, LAMC1, PLCB2, SERPINB9, TFGB2 |
| 5410        | Hypertrophic cardiomyopathy (HCM)                       | 12                  | 0.0123   | CACNA1D, CACNB2, CACNG6, DMD, ITGA10, ITGA11, ITGA8, MYH6, RYR2, SGCA, SLC8A1, TFGB2 |
| 640         | Propanoate metabolism                                   | 7                   | 0.0134   | ABAT, ACS5S3, ALDH6A1, ALDH7A1, ALDH9A1, EHHADH, SUCLG2 |
Table S5 - List of primary and secondary antibodies used in the study. IF: immunofluorescence.

| Primary AB      | Host  | Source     | Application |
|-----------------|-------|------------|-------------|
| Anti-Troponin T | Rabbit| Abcam      | IF (1:100)  |
| Anti-α-actinin  | Mouse | Sigma Aldrich | IF (1:100) |

| Secondary AB    | Host  | Source     | Application |
|-----------------|-------|------------|-------------|
| Anti-rabbit IgG-FITC | Goat | Southern Biotech | IF (1:200) |
| Anti-rabbit IgG-TRITC | Goat | Southern Biotech | IF (1:100) |
| Anti-mouse IgG-FITC | Goat | Southern Biotech | IF (1:200) |
| Anti-mouse IgG-TRITC | Goat | Southern Biotech | IF (1:100) |

Table S6 - List of RT-qPCR primers used in this study.

| Primer name     | Sequence                                      |
|-----------------|-----------------------------------------------|
| TnT Forward     | 5'-TGGAGGCAGAGAAGTTCCG-3                     |
| TnT Reverse     | 5'-CCTGTTTCGGAAGAATTCAT-3                    |
| ANP Forward     | 5'-CAGGATGGACAGGATTGGA-3                     |
| ANP Reverse     | 5'-TGTCCTCCCTGGCTGATATC-3                    |
| BNP Forward     | 5'-TGGAACGCTCCGTTAC-3                        |
| BNP Reverse     | 5'-GGACTTCCAGACCCCTGG-3                      |
| MYH6 Forward    | 5'-GATAGAGAGACTCCCTGCGGC-3                   |
| MYH6 Reverse    | 5'-TCGTCATCTCCTGGCTCC-3                      |
| MYH7 Forward    | 5'-CAAGGGGTCTTGAATGAGGATG-3                  |
| MYH7 Reverse    | 5'-TCCTCCAGAGACCGCT-3                        |
| SP6 Forward     | 5'-GAGGACCTGGAACGACAG-3                      |
| SP6 Reverse     | 5'-GATGAACTGCTCCACGAG-3                      |
| FSTL3 Forward   | 5'-CACCCGGGGAACAAGATCAA-3                    |
| FSTL3 Reverse   | 5'-GTCGCCAAGATCTTGTGAGGG-3                   |
| 36b4 Forward    | 5'-AACGGGTTACAACGAGTGC-3                     |
| 36b4 Reverse    | 5'-AGATGGATGACGGCAAGAG-3                     |

Table S7 - List of shRNA probes used in the study. All additional information about probes and pLKO1 plasmid can be found on manufacturer website.

| Gene name   | Clone ID       | Sequence                                      | Region |
|-------------|----------------|-----------------------------------------------|--------|
| Sp6         | NM_199262.2-1229s21c1 | 5'-CCGGAGCTCAGCCGCTCTCTCATTTCAGAGATGACGCGG-3 | CDS    |
| Fstl3       | NM_005860.2-309s21c1 | 5'-CCGGAGCTCAGCCGCTCTCTCATTTCAGAGATGACGCGG-3 | CDS    |
