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ORIGINAL ARTICLE

Cyclic Stretching Induces Maturation of Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes through Nuclear-Mechanotransduction

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Abstract

BACKGROUND: During cardiogenesis, cardiac cells receive various stimuli, such as biomechanical and chemical cues, from the surrounding microenvironment, and these signals induce the maturation of heart cells. Mechanical force, especially tensile force in the heart, is one of the key stimuli that induce cardiomyocyte (CM) maturation through mechanotransduction, a process through which physical cues are transformed into biological responses. However, the effects and mechanisms of tensile force on cell maturation are poorly studied.

METHODS: In this study, we developed a cyclic stretch system that mimics the mechanical environment of the heart by loading tensile force to human-induced pluripotent stem cell (hiPSC)-derived CMs. hiPSC-CMs cultured with the cyclic stretch system analyzed morphological change, immunofluorescent staining, expression of maturation markers in mRNA, and beating properties compared to static cultures.

RESULTS: hiPSC-CMs cultured with the cyclic stretch system showed increased cell alignment, sarcomere length and expression of maturation markers in mRNA, such as TNNI3, MYL2 and TTN, compared to static cultures. Especially, the expression of genes related to nuclear mechanotransduction, such as Yap1, Lamin A/C, plectin, and desmin, was increased in the cyclically stretched hiPSC-CMs. Furthermore, the volume of the nucleus was increased by as much as 120% in the cyclic stretch group.

CONCLUSION: These results revealed that nuclear mechanotransduction induced by tensile force is involved in CM maturation. Together, these findings provide novel evidence suggesting that nuclear mechanotransduction induced by tensile force is involved in the regulation of cardiac maturation.

Keywords Tensile force · Cyclic stretch · Cardiomyocyte · Maturation · Nuclear-mechanotransduction

1 Introduction

Cardiogenesis, a process of forming a new heart from embryonic cells that is strictly regulated by the interplay between structure and function, is a pivotal process and model for understanding cardiac differentiation, specification and maturation [1–3]. To recapitulate heart development *in vitro*, studies have been conducted to generate the biochemical microenvironment of the heart by including growth factors and cytokines to induce the maturation of cardiomyocytes, CMs [4, 5]. In addition to the biochemical environment of the heart, the importance of biomechanical signals is being recognized due to the nature of the continuously beating heart, such as geometrical guidance, extracellular matrix (ECM) properties, electrical stimulation, and tensile force. For example, biomimicking the geometrical guidance of the myocardium, such as the

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aligned bundle structure, leads to structural and functional maturation, such as myofibril alignment, cell elongation, increased contractile performance, and improved calcium metabolism [6]. The stiffness of the ECM of a tissue varies greatly depending on the tissue characteristics [7]. The stiffness of the left ventricle is approximately 10 kPa, and a left ventricle microenvironment of similar stiffness shows an increased CM differentiation and induces the maturation of cardiac cells through the development of calcium channels and the formation of a myofibrillar structure [8, 9]. Electrical signals transmitted periodically for continual heart beating not only increase the efficiency of CM differentiation but also induces their maturation, as indicated by an adult-like gene expression profile, oxidative metabolism and an organized ultrastructure [10, 11].

Tensile force, one of the major mechanical cues that produces cyclic stretching at the cellular level, is known to affect the structural and functional maturation of CMs [12]. In recent years, to induce CM maturation by recapitulating the myocardium microenvironment, a number of research groups have developed cell culture devices and applied tensile force to CMs. CMs stimulated by cyclic stretching exhibit increased expression of connexin 43 at the gene and protein levels as well as morphological maturation, such as increased cytoskeletal alignment and sarcomere length [13]. Depending on the culture conditions, cyclic stretching produces pressure overload, and therefore, it has been applied to generate disease models, such as pathological hypertrophy models [14, 15]. Applying 15% stretch to CMs confirmed CM molecular-level hypertrophic properties, such as atrial natriuretic peptide (ANP) and B-type natriuretic peptide (BNP) expression, as well as increased CM size and stiffness [15]. Therefore, a cyclic stretch system can serve as a platform to understand the mechanisms of mechanotransduction. However, despite directed research efforts, the relationship between maturation and mechanical forces remains unclear.

Mechanotransduction, in which biomechanical signals are translated into biological responses that trigger intracellular signaling in cells, is one of the key processes that need to be discerned to understand the effect of mechanical cues on cells [16]. Tensile forces induce conformational changes to stretch-activated channels (SACs), including those in the transient receptor potential (TRP) family, altering the concentration of intracellular cations (Ca^{2+} and Na^{+}) [17, 18]. This change not only affects beating properties through depolarization of the cell membrane but also affects gene expression through a signal transduction cascade in the cytoplasm and nucleus mediated by specific transcription factors [18]. In addition to ion channels, tensile forces are also sensed and transmitted through membrane adhesion molecules such as integrins and focal adhesions. The tension transmitted through an adhesion molecule induces structural rearrangement of the cytoskeleton, such as sarcomere alignment, which affects cell function by modulating the beating properties of these cells. Rearrangement of the cytoskeleton by tension also affects gene expression by inducing nuclear deformation through a linker of the nucleoskeleton and cytoskeleton (LINC) complex [19, 20]. However, the effects of tensile force on CM maturation and nuclear signal transmission during this process remain unclear.

Here, we sought to determine how the tensile force acting on CMs affects their maturation and how it is related to changes in the nucleus from a mechanotransduction point of view. We developed a cyclic stretch system and demonstrated that tensile force induces structural and functional maturation of iPSC-derived CMs. In particular, YAP was translocazlied from the cytosol to the nucleus, which led to increased expression of nuclear rigidity-related genes, and the increases in the size and volume of the nucleus were greater in the stretch group than in the static group. Our data suggest that nuclear mechanotransduction induced by tensile force is involved in CM maturation.

2 Materials and methods

2.1 Cyclic stretch system and cell culture

2.1.1 Cyclic stretch system and PDMS chip

The cyclic stretch system, a custom-made device (Mpia, Gyeonggi-do, South Korea), was designed to provide mechanical stimulation to CMs. Briefly, two clamps consisting of a fixed clamp and a shifting clamp to fix the PDMS chip were assembled in parallel 50 mm apart. The shifting clamp connected to the electric motor can modulate the strain and rotation speed through a controller. The strain and rotation speed were fixed at 10% and 1 Hz in this experiment.

The poly(dimethyl siloxane) (PDMS; Sylgard 184, Dow Chemical Co., Midland, MI, USA) chip consists of a top inlet and outlet layer, middle two-channel layer, and bottom cell adhesion layer. After mixing the PDMS precursor and a curing agent at a ratio of 10:1, the mixture was poured into a flat 150 × 150 mm mold and polymerized at 60 °C for 2 h to ensure the bottom layer was 2 mm thick, the middle layer was 2.5 mm thick, and the top layer was 3 mm thick. After cutting all layers to 50 × 20 mm, a plasma treatment was used to generate two 20 × 5 mm channels in the middle layer. These channels were attached to the top layer. The top layer was attached to the bottom layer by plasma treatment after 1 mm inlet and outlet holes were drilled into both ends in contact with the middle layer

channel. The finished PDMS chip was treated with 50 μg/ml fibronectin for 2 h to generate a coating before use.

2.1.2 Cell culture

Human-induced pluripotent stem cell (hiPSC)-CMs (Cardiosight-S®, NEXEL Co., Ltd., Seoul, South Korea) stored in a liquid nitrogen tank were thawed in a 37 °C water bath for 1 min and used immediately in the experiment. hiPSC-CMs were loaded onto the PDMS chip at a concentration of 3×10^5 cells/ml with 400 μl of 10 μM Y27632 (Tocris, Bristol, UK) in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with B27^{TM} (1 ×, without vitamin A) added to each channel and incubated for 24 h. On day 1, the medium used for the PDMS chip was changed to RPMI 1640 medium with B27^{TM} supplement (1 ×, without vitamin A), followed by cyclic stretching at 1 Hz to generate a 10% strain for 48 h.

2.2 Immunofluorescence staining

The chips were washed with PBS (Gibco) and fixed with 4% paraformaldehyde (PFA) solution (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature. The samples were permeabilized with 0.4% Triton X-100 for 20 min and blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich) for 45 min at room temperature. After overnight incubation at 4 °C with primary antibodies, which included mouse sarcomere anti-alpha-actinin (Abcam, Cambridge, UK), anti-cardiac troponin T (anti-cTNT; Thermo Scientific, Waltham, MA, USA), anti-Ki67 (Abcam), rabbit anti-MLC2v (anti-MYL2, Proteintech, Rosemont, IL, USA) or anti-Yap1 (Santa Cruz Biotechnology, Dallas, TX, USA) at dilutions of 1:500, 1:500, 1:250, 1:500 and 1:200 in 5% BSA solution, respectively, each sample was washed five times with 0.01% PBST for 15 min each time. Alexa Fluor 488 goat anti-mouse IgG (H+L) against sarcomere anti-alpha actinin, anti-cardiac troponin T or anti-Yap1; Alexa Fluor 594 goat anti-rabbit IgG (H+L) (Life Technologies, Carlsbad, CA, USA) against anti-Ki67 or anti-MLC2v; or Alexa 488 or 594 phalloidin (Thermo Scientific) against anti-F-actin were added as secondary antibodies at dilutions of 1:500, 1:500, 1:200, 1:250, 1:500 and 1:300 in 5% BSA solution, respectively, and then incubated for 3 h at room temperature. Then, 40,6-diamidino-2-phenylindole, dihydrochloride (DAPI; 1:2000; Molecular Probes, Carlsbad, CA, USA) was also added to each sample for nuclear staining. After staining, the samples were washed again five times with 0.01% PBST for 15 min each time. Samples were then visualized using an LSM 800 (Carl Zeiss, Oberkochen, Germany) confocal fluorescence microscope.

2.3 Image-based analysis

2.3.1 Sarcomere length measurement

Images were taken by confocal microscopy, and sarcomere length was measured through a profile plot in ImageJ software. The intensity profile was obtained by drawing a line in the vertical direction of the sarcomere, and the sarcomere length was measured as the peak-to-peak distance in the intensity profile.

2.3.2 YAP intensity analysis

For quantification of the nuclear/cytoplasmic ratio of YAP, cells stained with anti-YAP antibody (Alexa Fluor 488) and DAPI were obtained by confocal microscopy and analyzed with ImageJ software. We measured the mean fluorescence intensity of YAP staining in the nucleus and cytoplasm. Then, the intensity ratio was calculated after subtracting the background values from the nuclear and cytoplasmic intensity values.

2.3.3 Nucleus size and height measurements

Cells stained with DAPI were imaged through Z-stacking of approximately 30 image slices performed at intervals of 5–7 μm using a confocal microscope, and then, the images were subjected z-projection processing with ZEN3.1 (blue edition) software. The size and height were measured using ImageJ and ZEN3.1 software in the z-projection image of DAPI, respectively.

2.4 Quantitative PCR

Total RNA was extracted by treating CMs in the chip channels with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations with slight modification. Briefly, a PrimeScript (a cDNA synthesis kit; TaKaRa, Shiga, Japan) was used to reverse transcribe extracted RNA (1 μg) to cDNA. cDNA was detected with iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed with a MyiQ 2 real-time PCR detection system (Bio-Rad). Each gene was normalized to the expression of the control group by the DDCt method using β-actin as the reference gene. The primers are listed in Table 1.

2.5 Beating analysis

The contractile properties of the hiPSC-CMs were estimated by analyzing movies taken with a microscope (Nikon, Ti2-E, Tokyo, Japan) at × 20 magnification. Movies captured images at 50 frames per second, and the

Table 1 List of the primers used for the quantitative PCR analysis

Gene	Forward primer	Reverse primer	Company
β-Actin	GGACCTGACTGACTACCTCAT	CGTAGCACAGCTTCTCCTTAAT	Integrated DNA Technologies (USA)
TNNT2	GACCTGCAGGAGAAGTTCAA	GAGGAGCAGATCTTTGGTGA	Integrated DNA Technologies (USA)
TNNI3	GAGGCAAAAGTCACCAAGAA	CTCCTTCTTCACCTGCTTGA	Integrated DNA Technologies (USA)
MYL7	TCCAACGTCTTTTCCATGTT	TCTGTCCCATTGAGCTTCTC	Integrated DNA Technologies (USA)
MYL2	ACCCTGAGGAAACCATTCTC	ATGATGTGCACCAGGTTCTT	Integrated DNA Technologies (USA)
CACNA1C	TGAAGATGAACCATGACACG	CCTTCTGTCGCTTCAGACAT	Integrated DNA Technologies (USA)
RYR2	TGGACAGAGTTCGCACAGTA	TGTACTCGGTTCCACCTGAT	Integrated DNA Technologies (USA)
ANP	GATCTGCCCTCCTAAAAAGC	CCTCTTGCAGTCTGTCCCTA	Integrated DNA Technologies (USA)
BNP	GCAGACACCTGCTTCTGATT	TTTCACCGTGGAAATTTTGT	Integrated DNA Technologies (USA)
MYH7	CCAGTGGAGGACCAAGTATG	CTACGTCCACCATCAAGTCC	Integrated DNA Technologies (USA)
MYH6	CCAGTGGAGGACCAAGTATG	CTACGTCCACCATCAAGTCC	Integrated DNA Technologies (USA)
TTN	GATGACAGTGGAACCTACCG	GTCAGCTCAGGGAAAACAGA	Integrated DNA Technologies (USA)
GJA1	TGGGTCCTGCAGATCATATT	TCGCATTTTCACCTTACCAT	Integrated DNA Technologies (USA)
Ki67	ACGGATTATACCTGGCCTTC	AATACCCCTTCCAAACAAGC	Integrated DNA Technologies (USA)
YAP1	GCA GTT GGG AGC TGT TTC TC	GCC ATG TTG TTG TCT GAT CG	Integrated DNA Technologies (USA)
TAZ	TTG CTG CCT TCT GGA TTC TT	CCC TGC CTA AGC TTC TTC CT	Integrated DNA Technologies (USA)
LMNA	GTGCGTGAGGAGTTTAAGGA	GCAGCATCTCATCCTGAAGT	Integrated DNA Technologies (USA)
PLEC	TCATCGACATGAACAAGGTG	ATACAGCGACGAGACGTAGG	Integrated DNA Technologies (USA)
DES	CAGACCTACTCTGCCCTCAA	TAGAGCACTTCATGCTGCTG	Integrated DNA Technologies (USA)
DCTN1	CTGAGTGACACCGTCGAGAA	GAAGGTCACTTTGCCCATGT	Integrated DNA Technologies (USA)
TEAD1	GGA AGC CTC AAA CTG AGA CG	GGG CTG GAA CAT TCT TTG AA	Integrated DNA Technologies (USA)
TEAD2	TTTTGGTCTGGAGGATCTGG	TTCCACGAAGGCTGAGAACT	Integrated DNA Technologies (USA)
TEAD3	GGACCCTCTCAGGACATCAA	CACCTCCATGAAGGCTGAAT	Integrated DNA Technologies (USA)
TEAD4	GCTCCTTCTATGGGGTCTCC	GTGCTTGAGCTTGTGGATGA	Integrated DNA Technologies (USA)

movies were analyzed in MATLAB (MathWorks, Inc., Natick, MA, USA) using a customized source code, enabling quantification of variations in light intensity within 10 s. The beating kinetics of each sample were estimated to identify beating regularity and homogeneity.

2.6 Statistical analysis

Statistical analysis was performed with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) using Student's *t*-tests to evaluate the differences between experimental groups. The data are presented as the mean value ± standard deviation. Experiments were performed in triplicate. A significant difference was defined as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

3 Results

3.1 Applying mechanical force to stretch hiPSC-CMs alters cell morphology

Cardiac cells are exposed to a microenvironment where cyclic stretching continues throughout the lifespan [21]. To induce maturation of hiPSC-CMs by recapitulating the biomechanical environment of the heart, we developed a uniaxial stretching machine (10% elongation in length; 1 Hz) and applied tensile force to hiPSC-CMs for 48 h (Fig. 1A). In the stretch group exposed to tensile force, the morphology of cells was aligned in the stretching direction; however, in the static (control) group, the cells showed randomly organized morphological structures (Fig. 1B). Immunofluorescence staining of F-actin was performed to confirm that, in addition to cell morphology, actin showed directionality, and the results showed that F-actin in the stretch group was aligned in the stretching direction compared to the static group (Fig. 1C). This actin alignment was quantitatively analyzed through the directionality of ImageJ. In the stretch group, the frequency of actin alignment in the 0° direction was high, whereas in the static group, actin was aligned in various directions (Fig. 1D, 1E). These data suggested that tensile force induces cell alignment along the direction of the applied force.

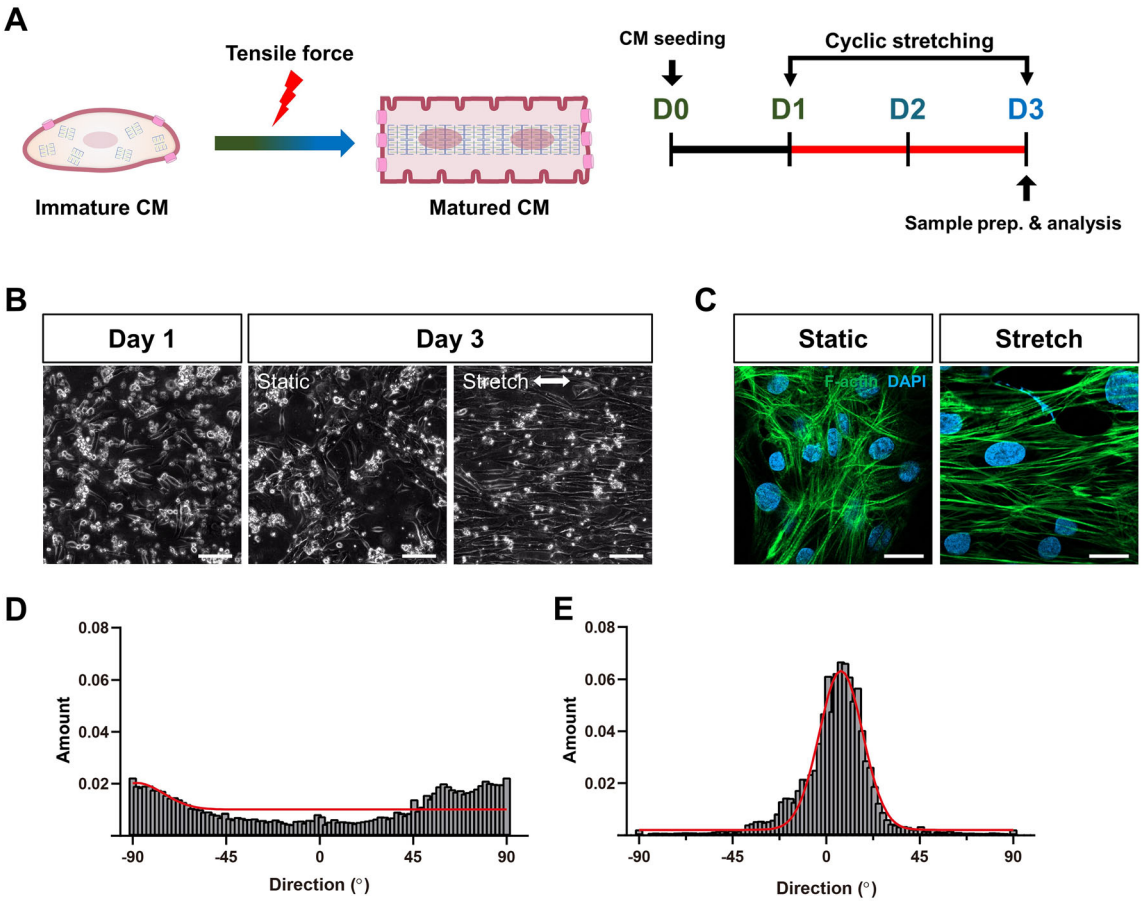


Fig. 1 Effects of cyclic stretching on hiPSC-CMs. **A** Experimental scheme for applying tensile force to hiPSC-CMs. **B** Bright field image of seeded cells on day 1 and after stretching on day 3. **C** immunofluorescence image of the static and stretch groups and **D**, **E** quantitative analysis of cell alignment by F-actin static, stretch). The scale bar represents 100 μm in **B** and 150 μm in **C**

3.2 Mechanical stretching induced structural maturation of hiPSC-CMs

To observe changes in cell morphology induced by mechanical stretching, immunofluorescence staining was performed, and structural changes in the cells were examined. It has been reported that, compared to immature CMs, mature CMs exhibit greater structural alignment and longer sarcomeres [22]. After immunofluorescence staining of sarcomeric α-actinin and Ki67 (Fig. 2), observation revealed that, in addition to sarcomere alignment in the stretch direction, sarcomere length was increased in the stretch group (1.794 ± 0.18 μm) compared to the static group (1.646 ± 0.21 μm, Fig. 2C). Furthermore, it is known that the rate of CM proliferation decreases as CMs matures [23]. By comparing the expression of Ki67, it was confirmed that the proliferation decreased in the stretch group (Fig. 2D). The expression of MLC2v, a ventricle-type marker in CMs, was also confirmed through immunostaining (Fig. 2B). Finally, a colocalization analysis confirmed that more MLC2v proteins were along myosin in the stretch group than in the static group (Fig. 2E). These results suggest that tensile force induces structural maturation of CMs.

3.3 Effects of tensile force on gene expression

To evaluate gene expression changes by tensile force, we performed quantitative PCR analysis (Fig. 3). Expression of cardiac troponin and myosin light chain, as markers of cardiac maturation, was observed. The expression of cardiac troponins (cardiac troponin T, TNNT2; and cardiac troponin I, TNNI3) and myosin light chains (atrial light chain-2, MYL7; and ventricular regulatory myosin light chain, MYL2) was significantly increased in the stretch group. In particular, the expression of MYL2 was increased approximately 2-fold, indicating that the CMs were differentiated into ventricle type cells. In addition, the expression of voltage-dependent L-type calcium channel subunit alpha-C (CACNA1C) and ryanodine receptor 2 (RYR2), which are ion channels regulating the intracellular calcium ions in CMs, was significantly increased in the

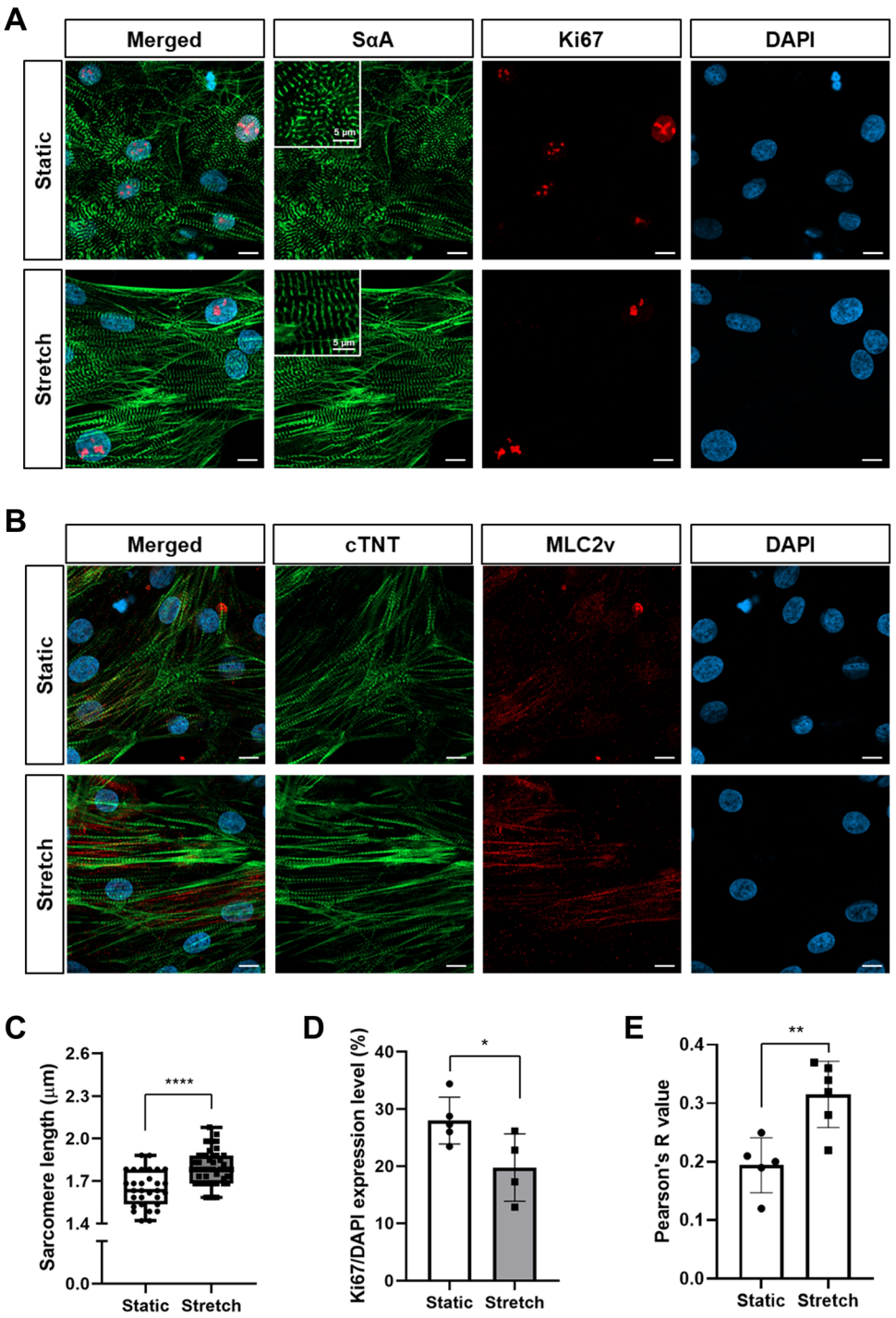


Fig. 2 Expression of maturation markers in hiPSC-CMs as determined by immunofluorescence staining. **A** Immunofluorescence images of the expression levels of sarcomeric α-actinin and Ki67 and **B** of cTNT and MLC2v in the static and stretch groups. **C** Quantitative analysis of sarcomere length, **D** expression level of Ki67/DAPI and **E** colocalization of MLC2v and cTNT in hiPSC-CMs. The scale bar represents 20 μm. A significant difference is indicated by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$

stretch group. As CMs mature, the expression of myosin heavy chain 6 (MYH6) decreases and the expression of MYH7 increases, expressed as a MYH7/MYH6 ratio. The analysis showed that the expression of cardiac structure markers MYH7/MYH6 and titin (TTN) increased by 1.5- and 4-fold in the stretch group, respectively. Furthermore, hypertrophy markers (ANP and BNP) are known to be expressed when force is applied to cells. The expression of Ki67, a proliferation-related marker, as shown by the immunostaining results, which is consistent with the decrease in proliferation observed as immature CMs mature. The expression of gap junction connexin 43 (GJA1) did not differ between the two groups. These data suggest that tensile force facilitates hiPSC-CM maturation at the gene level.

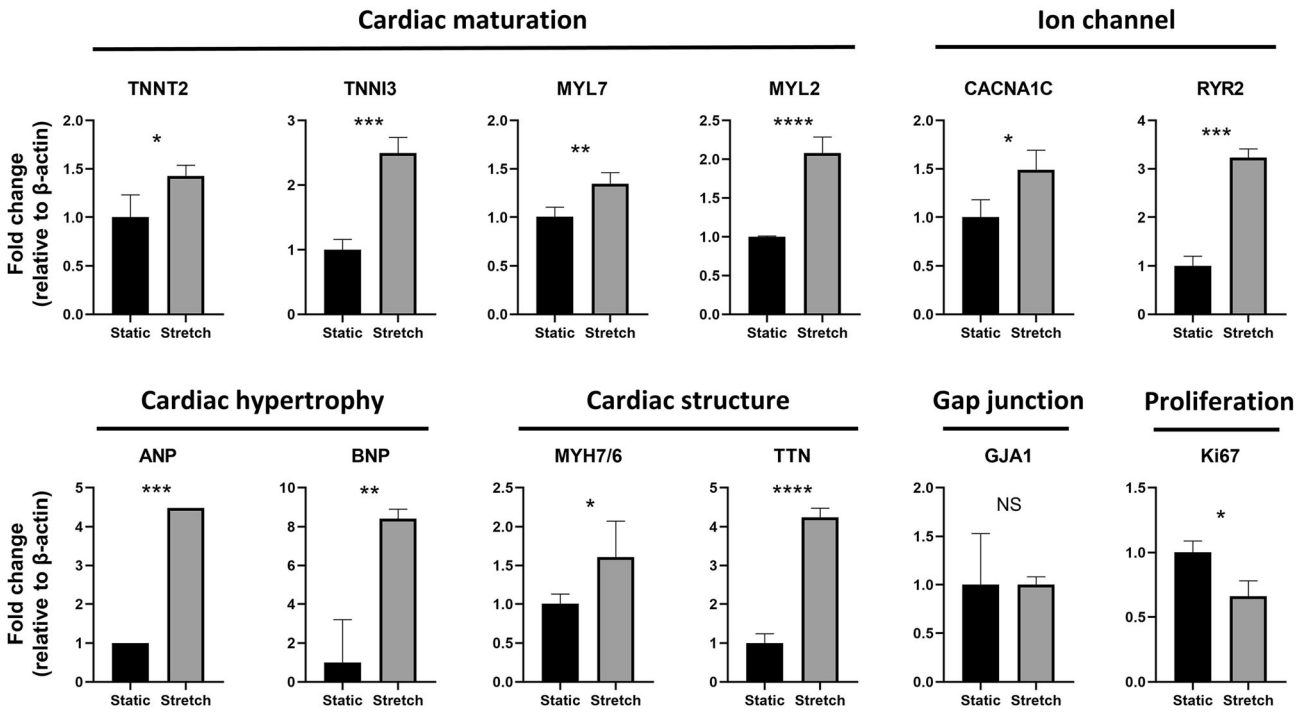


Fig. 3 Comparison of the mRNA expression levels in hiPSC-CMs of the static and stretch groups. Quantitative analysis of mRNA expression levels as performed with real-time PCR. The expression levels of cardiac maturation markers (TNNT2, TNNI3, MYL7, and MYL2), ion channel markers (CACNA1C and RYR2), cardiac hypertrophy markers (ANP and BNP), cardiac structure markers (MYH7/MYH6, and TTN), a gap junction marker (GJA1) and a proliferation marker (Ki67) normalized to that of β-actin. A significant difference is indicated by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$

3.4 Beating characterization

Organized CM alignment and beating are important for maintaining the mechanical properties of the heart. In particular, in addition to the force of contraction, the rate of both contraction and relaxation in CMs is an important determinant of cardiac function. To measure the beating characteristics of the group exposed to tensile force, pixel intensities in the static and stretch groups were recorded using an optical microscope and analyzed for 10 s using MATLAB software (Fig. 4A). Beating rhythms representative of each group are shown in Fig. 4B (static group) and Fig. 4C (stretch group). No significant difference in terms of beats per minute between the groups was detected (Fig. 4D) in a peak-to-peak duration analysis (Fig. 4). However, it was confirmed that the contraction–relaxation duration time was significantly increased in the stretch group (Fig. 4E). This outcome is typical of a mature heart: The heart does not relax quickly, allowing the ventricles time to refill adequately before the next excitatory stimulus is received.

3.5 Nuclear-mechanotransduction

Tensile force applied to hiPSC-CMs facilitates maturation by inducing gene overexpression. We monitored and analyzed nuclear deformation to determine whether it is related to nuclear mechanotransduction. The nuclear deformations of DAPI-stained hiPSC-CMs were captured in a Z-stack by a confocal laser scanner microscope, and the size and height of the nuclei were measured in the z-projection processed image (Fig. 5A). The analysis revealed that the nucleus size was increased 1.2-fold in the stretch group compared with the static group (Fig. 5B), and nucleus height was also increased, by 1.4-fold, in the stretch group (Fig. 5C). This means that the volume of the nucleus increased by as much as 120%. In addition, the expression of LINC-related genes, such as Lamin A/C, plectin (PLEC), desmin (DES), and DCTN1, which are affected by nuclear inner membrane rigidity and mechanical stress, was also evaluated in each group. The stretch group showed significantly higher expression of these genes than the static group, shown as follows with the fold change: Lamin A/C (1.3-fold), PLEC (1.3-fold), DES (2.4-fold), and DCTN1 (1.4-fold) (Fig. 5D).

We examined the localization of YAP and measured the gene expression associated with the YAP-TAZ pathway to

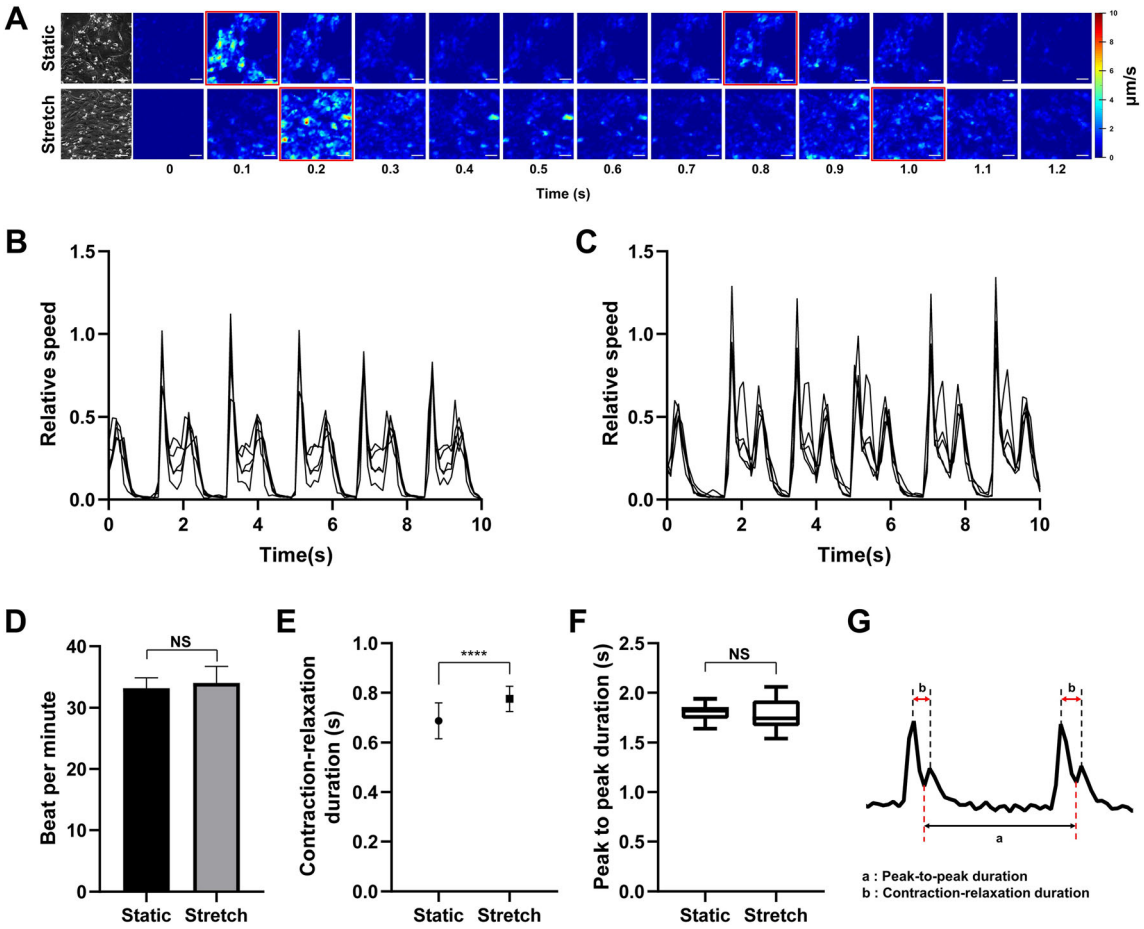


Fig. 4 Beating characterization of hiPSC-CMs in the static and stretch groups. **A** Representative images of contraction–relaxation over time as indicated by image-based analysis. **B**, **C** Beating characteristic analysis was performed by monitoring pixel intensity over a period of 10 s under static and stretch conditions. **D–F** A comparison of BPM (beat per minute, contraction–relaxation duration, and peak-to-peak duration was performed. **G** Schematic figure of peak to peak duration and contraction-relaxati on duration in a beating graph. The scale bar in **A** represents 100 μm. A significant difference is indicated by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$

determine whether nuclear deformation is mediated by tensile force since force induces YAP translocation from the cytoplasm to the nucleus. The expression of YAP in the nucleus and cytoplasm was analyzed through immunostaining (Fig. 5E). The results were quantified by the nuclear/cytoplasmic ratio, and the translocation of YAP from the cytoplasm to the nucleus in the stretch group was higher than that in the static group (Fig. 5F). In addition, quantitative PCR analysis was performed to measure the expression level of YAP-TAZ pathway-related genes such as YAP1, TAZ, and TEAD1, 2, 3 and 4 (Fig. 5D). The results showed that the expression of TEAD1, 2, 3, and 4 was increased in the stretch group, and in particular, YAP and TAZ levels in the stretch group were 1.9- and 2.4-fold higher, respectively, than those in the static group. Together, these results suggested that tensile force induces nuclear deformation of CMs through LINC-related proteins and induces translocation of YAP from the cytoplasm to the nucleus, thereby affecting gene expression, such as those that induce CM maturation, through transcription factors. These outcomes can be explained by the close relationship between nuclear mechanotransduction and mechanical stress in CMs.

4 Discussion

Maturation of CMs is a crucial factor for the development of fully functional hearts and cardiac tissues [23, 24]. In particular, maturation of CMs derived from iPSCs are required to fabricate cardiac tissues that reflect the adult heart phenotype [25, 26]. However, hiPSC-CMs exclusively present the fetal phenotype, which makes these CMs inappropriate for use in toxicity tests of drugs used in adults. Recent studies show that cyclic mechanical forces, such as tensile force, uniaxially contribute to CM

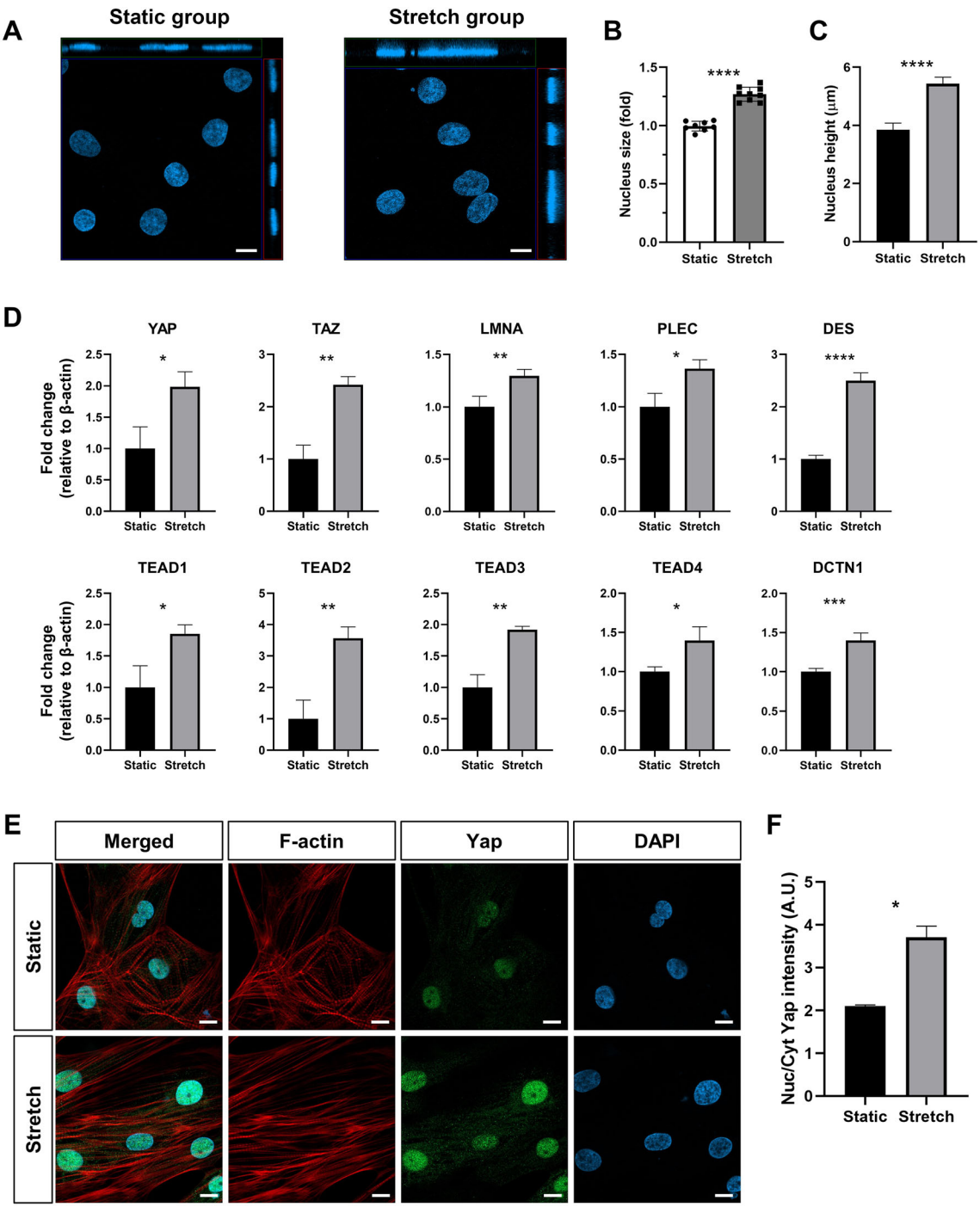


Fig. 5 Analysis of nuclear deformation induced by tensile force. **A** Z-projection images of nuclei as determined by immunofluorescence in the static group and stretch group. **B**, **C** Quantitative analysis of the size and height of nuclei. **D** mRNA expression levels involved in YAP/TAZ signaling and nuclear mechanotransduction. **E**, **F** Immunofluorescence images of Yap and quantitative analysis of Yap translocation based on nuclear/cytosolic fluorescence intensity. The scale bar in **A** and **E** represents 10 μm. A significant difference is indicated by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$

maturation by inducing myofibril and sarcomere alignment, thereby enhancing CM contractile characteristics [11, 27–29]. In this study, we applied tensile force to hiPSC-CMs and analyzed its effects on cardiac maturation. Cyclic stretching increases structural maturation, as indicated by enhanced alignment increased sarcomere length in hiPSC-CMs and expression of genes related to maturation, such as those involved with hypertrophy and those that encode ion channels and structural markers; the results showed that maturation indicators and gene

expression differed between the stretch and static groups. We also found that cells exposed to tensile stress showed an increased nuclear volume, indicating a correlation with nuclear transduction. We further investigated YAP translocation to the nucleus and the expression of nuclear link proteins, known as force transmitters that act through the cytoskeleton. The results suggested that tensile force was transmitted via a cytoskeleton–nuclear linkage and contributed to CM maturation.

The mechanism of tensile force on CM maturation is not known, although tensile force is known to be important in cardiac development. Mechanical stimulation activates stretch-activated ion channels in CMs, which leads to increased membrane calcium influx and greater action potential duration [23, 30, 31]. Our results showed that the expression of calcium channels, namely, CACNA1C and RYR2, was increased by tensile force (Fig. 3). This finding suggests that calcium channels might be activated by force and initiate the activation of downstream signaling pathways. In other studies, calcium provided an important link for increased biomechanical stimulation [32]. Troponin, a key marker of mature CM fibers, is composed of three subunits: troponin T, troponin I, and troponin C [33, 34]. Among these subunits, the mRNA expression of troponin T and troponin I is known to be increased in mature CMs because of mechanical force [35]. Our results show that the expression of the CM maturation-related genes TNNT2 and TNNI3 was increased in the stretch group (Fig. 3). This finding is consistent with that of previous studies shown that troponin, found in skeletal muscles and myocardial fibers, binds to tropomyosin in mature CMs, is localized to actin, and is involved in cardiac muscle contraction [36, 37]. We also measured the expression of myosin light chain genes. Our results showed increased expression of the atrial isoform MYL7 and the ventricle/cardiac muscle isoform MYL2, suggesting that immature CMs without a specific phenotype differentiate into atrial-/ventricle-type CMs upon exposure to mechanical force. The myosin light chain is involved in a central process regulating smooth muscle cell contraction and is an indicator of mechanical force-induced differentiation of mature CMs with specific phenotypes [38]. Cx43, a gap junction channel important for CM maturation, is known to form the basis of direct intercellular communication among CMs, and many studies have reported that the expression of gap junctions between CMs is increased by tensile force [13]. In our experimental results, we found no difference in the expression of Cx43 in the stretch and static groups (Fig. 3). The culture conditions and cell density in our 2D experimental setup were not ideal for monitoring junction formation.

Nuclear mechanotransduction, transmitting forces to the nucleus and resulting in the deformation of the nucleus, is an interesting mechanism that can explain force transmission inside a cell [39]. Our results clearly show that the size of nuclei exposed to tensile forces increased, by as much as 120%, indicating that nuclear deformation is involved in force-mediated CM maturation (Fig. 5A). Size and shape changes upon exposure to external forces affect chromatin structure dynamics, which influences the activation of specific genes (Fig. 5B, C). To elucidate the mechanism of force transmission, we monitored the genes known to be involved in nuclear transduction factors such as Lamin A/C and LINC-mediated proteins (PLEC, DES, and DCTN1). Our results showed that the expression of Lamin A/C was increased in the stretch group compared to that in the static group (Fig. 5D). Lamin A/C is known as an important intermediate filament protein that physically supports the nucleus and regulates nuclear stiffness [40]. This nuclear envelope protein is involved in sensing forces through nuclear structure deformation during mechanotransduction [19]. Recent findings suggest that the expression of Lamin A/C is highly correlated with the stiffness of the nucleus, which affects cellular fate during differentiation [40]. Our results indicate that higher expression of Lamin A/C affects the nuclear stiffness, indicating a mechanosensitive status. In the mechanical environment, Lamin A/C expression levels in cells regulate the expression of LINC-mediated proteins, which affects nuclear stiffness [41]. In particular, tensile forces modulate the stiffness of the cellular inner membrane by altering nuclear envelope proteins that mediate mechanical interactions between the nucleus and the cytoplasm [42, 43]. In our experiment, the expression of the LINC-mediated proteins PLEC, DES, and dynactin was increased in the stretch group. This outcome correlates with previous findings showing that link-mediated proteins anchor cytoskeletal proteins to the nuclear envelope for effective force transmission; however, direct evidence for these anchoring effects needs to be acquired through further investigation.

The expression of maturation and force transmission specific genes via tensile force can be affected during the CM maturation process [44]. We first monitored the localization of YAP, a representative transcriptional activator, upon mechanical stimulation. We shows that YAP was localized in the nucleus in the stretch group (Fig. 5E), which is consistent with previous findings showing that YAP is translocalized from the cytosol to the nucleus through an external mechanical force [45]. We also found that YAP expression was increased upon tensile force. It was reported that translocation of YAP to the nucleus is dependent on nuclear stiffness [46]. Strong anchoring of the cytoskeleton to the nucleus and increased strength of the nucleus via Lamin may facilitate the nuclear localization of YAP via tensile force. The YAP/TAZ protein binds to the promotor of transcriptional enhanced associate

domain (TEAD) family genes, resulting in cell proliferation, differentiation, and migration [47]. Previous studies have shown that the YAP/TAZ complex activates force related genes and cardiac marker proteins. In particular, the MYH7, NPPA, and NPPB genes, which were overexpressed in our study, were previously shown to be activated by YAP/TAZ via the Hippo pathway [48]. It is reasonable to suggest that tensile force affects nuclear transduction pathways by activating force related genes. Further investigation to find direct evidence of nuclear transduction induced by tensile force is needed. Our findings on the transmission of tensile forces via nuclear transduction during CM maturation provide interesting research topics related to cardiac development *in vivo* as well as for fabricating mature cardiac tissues *in vitro*. The exact mechanism and effectors of force transmission will continue to be challenging issues in developmental biology, regenerative medicine and tissue engineering in the near future.

In conclusion, we report that applying a tensile force to CMs using a cyclic stretch system induces structural and functional maturation of CMs and that nuclear deformation and increased expression of LINC-related genes identified in this process suggest that nuclear mechanotransduction is involved in the maturation of CMs subjected to mechanical stimulation. Especially, we suggest a mechanism for understanding the effect of the biophysical environment on CM maturation and for identifying the mechanisms by which externally transmitted forces affect cell functions. We propose that the relationship between the maturation of CMs induced by the mechanical environment and nuclear-mechanical deformation may contribute to understanding the molecular biological mechanisms of mechanotransduction pathways, as well as the underpinning of developmental problems in cardiomyopathy, such as cardiac hypertrophy and fibrosis.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement There are no animal experiments carried out for this article.

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