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Recent Advances in Maturation of Pluripotent Stem Cell-Derived Cardiomyocytes Promoted by Mechanical Stretch

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Stem cells have significant potential use in tissue regeneration, especially for treating cardiac diseases because of their multi-directional differentiation capability. By mimicking the in vivo physiological environment of native cardiomyocytes during their development and maturation, researchers have been able to induce pluripotent stem cell-derived cardiomyocytes (PSC-CMs) at high purity. However, the phenotype of these PSC-CMs is immature compared with that of adult cardiomyocytes. Various strategies have been explored to improve the maturity of PSC-CMs, such as long-term culturing, mechanical stimuli, chemical stimuli, and combinations of these strategies. Among these strategies, mechanical stretch as a key mechanical stimulus plays an important role in PSC-CM maturation. In this review, the optimal parameters of mechanical stretch, the effects of mechanical stretch on maturation of PSC-CMs, underlying molecular mechanisms as well as existing problems are discussed. Mechanical stretch is a powerful approach to promote the maturation of SC-CMs in terms of morphology, structure, and functionality. Nonetheless, further research efforts are needed to reach a satisfactory standard for clinical applications of PSC-CMs in treating cardiac diseases.

Keywords: Engineering • Mechanical Processes • Pluripotent Stem Cells

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Background

For patients with cardiac diseases like myocardial infarction and heart failure, many strategies have been tried to maintain and even reverse the impaired cardiac function in the long term. Among them, cell therapy holds significant promise as it provides the heart with new cells to overcome the fundamental cause of these diseases, the loss of cardiomyocytes [1]. Techniques such as engineered heart tissue (EHT) [2] are also used to avoid the shortcomings existing with early attempts to provide cells through intramyocardial and intravenous injection, like low survival rate and low retention rate of cells at the impaired area [3,4]. In terms of available cell sources, stem cells are generally a better choice than differentiated cells because the latter usually cannot re-differentiate to qualified cardiomyocytes and cannot establish good electrical integration with the host cardiac tissue, thus having limited curative effects [5,6]. Both pluripotent stem cells (PSCs) and adult stem cells, like bone marrow mesenchymal stem cells, are capable of differentiating to cardiomyocytes [7]. PSCs are primarily embryonic stem cells (ESCs) that are derived from an inner-cell mass of the preimplanted blastocyst or from primordial germ cells of the early embryos, and induced pluripotent stem cells (iPSCs) that are derived from somatic cells by using reprogramming factors and resemble ESCs in many ways. Although use of these cells faces ethical and safety concerns [8], they are widely studied for their superior differentiation potential over that of adult stem cells, and can hopefully be used for various therapeutic purposes in regenerative medicine in the future [9]. However, as early work tried to obtain cardiomyocytes from PSCs via different methods, such as the use of exogenous cytokines, growth factors, and extracellular matrix [10], these pluripotent stem cell-derived cardiomyocytes (PSC-CMs) turned out to be significantly more immature compared to adult cardiomyocytes and exhibit a fetal-like phenotype [11]. For example, PSC-CMs are generally round and single-nucleated, while adult cardiomyocytes are elongated, rod-shaped, and partially binucleated. Other differences between them are briefly summarized in **Table 1** [12]. These major shortcomings limited their applications for treating cardiac diseases. Therefore, finding approaches to improve the PSC-CM maturation process remains a key issue to resolve.

Recent strategies have been explored to improve PSC-CM maturation, such as long-term culturing, substrate stiffness, electric stimulation, biochemical cues, and mechanical stimuli [13]. In vivo, the pulsating heart is constantly subjected to the preload caused by filling of the heart cavity by the returning blood flow. As mechanical stimuli may be critical to cardiac development [14], applying mechanical stretch that simulates the normal myocardium preload may effectively promote the maturation of PSC-CMs. As early as 2000, Zimmermann et al found

Table 1. Main differences between PSC-CMs and adult cardiomyocytes.

	PSC-CMs	Adult cardiomyocytes
Morphology	• Round and single-nucleated; • Distributed and random alignment; • 1000-1300 μm^2 surface area	• Elongated, rod-shaped and partially binucleated; • More aligned and anisotropic; • 10 000-14 000 μm^2 surface area
Sarcomere structure	• Short length; • Mostly indistinguishable sarcomere striations, with Z discs and I band; • Random alignment	• Long and wide in size; • Clear Z disks, I band, H bands, A band and M band; • Tight and neat alignment
Cardiac gene expression levels	• Low cTnT, actin, Cx-43; • Low β-MHC/α-MHC, nondeterministic MLC2v/MLC2a, low cTnI/fetal ssTnI	• High cTnT, actin, Cx-43; • High β-MHC/α-MHC, high MLC2v/MLC2a, high cTnI/fetal ssTnI
Function	• 0.1 to 0.5 mN/mm^2 active contraction force and low passive stiffness; • Nonlinear force-length relationship; • Negative force-frequency relationship	• 10-50 mN/mm^2 active contraction force and high passive stiffness; • Linear force-length relationship; • Positive force-frequency relationship
Electrophysiology	• Contract asynchronously and spontaneously; • Minute amount of ion channels like KCNJ2 and SCN5A	• Only excited when provided an electrical stimulus and contract in a synchronous manner; • High expression of KCNJ2 and SCN5A
Calcium handling	• Lack of transverse tubules; • Underdeveloped sarcoplasmic reticulum; • Depolarization velocity are about 6-to-50-fold decreased amount compared to adult cardiomyocytes	• Deep and distributed transverse tubules; • Well-developed sarcoplasmic reticulum around myofibers

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that neonatal rat cardiomyocytes-based EHT retained many of the physiological characteristics of rat cardiac tissue [15]. After phasic mechanical stretching (also termed mechanical strain), cardiac cells in EHT were extensively interconnected and longitudinally oriented. Cardiac muscle bundles also showed morphological features that resembled adult rather than immature native tissue [16], which revealed the potential value of mechanical stretch in the maturation of immature cardiomyocytes. In a subsequent study by Shimko et al, a custom-built device was used to exert mechanical stretch to murine ESC-CMs (mESC-CMs) embedded in collagen and fibronectin scaffolds. These mESC-CMs showed longitudinal alignment within the constructs with long strands of cells containing elongated nuclei. Mechanically loaded constructs also presented thin interwoven strands between the cells, similar to that observed in the neonatal heart. Changes in the expression of particular cardiac genes such as α-skeletal actin, α-cardiac actin, α-myosin heavy chain (MHC), and connexin-43 (Cx-43) were also observed [17]. These results further proved the role of such stimulation in the maturity of PSC-CMs. However, despite these impressive advances in the use of mechanical stimuli to promote PSC-CM maturation, there is no recent review focusing on this topic. In this review, we summarize the parameters of applying mechanical stretch as well as its impact on the maturation of PSC-CMs. Then, the underlying signal transduction pathways and existing problems are discussed.

Parameters of Mechanical Stretch

The main approach to apply mechanical stretch involves fixing 2 ends of the engineered tissue onto flexible mechanical arms to control stretch parameters such as amplitude and frequency. There are also magnet- and vacuum-based devices that are capable of exerting similar preset stretch [18,19]. Finding the best parameter set for mechanical stretch is vital in promoting efficient maturation. As a good example, mechanical overload of human ESC-CMs (hESC-CMs) leads to a hypertrophic state of the cell at the structural, functional, and gene expression levels, and this state is observed in many cardiovascular diseases [20]. Parameters associated with mechanical stretch mainly include stretch direction, intensity (usually defined as the ratio of cells being elongated), frequency, and time [21]. Details of recent associated studies are briefly described in **Table 2**.

(1) *Stretch direction.* Uniaxial stretch is the primary parameter adjusted for mechanical stretching of engineered tissue [17,22,23] and is dependent on the design of the bioreactors. Various axial directions can be used when trying to differentiate stem cells into SC-CMs by mechanical stretch, including biaxial, equiaxial, and multiaxial stretching [24-26]; however, they are rarely used when trying to improve the maturity of PSC-CMs [20]. Currently, no studies have explored differences caused by these axial directions.

(2) *Stretch intensity.* Although data examining stretch intensity in defining the optimal degree of elongation are sparse, setting the stretch intensity to around 10% elongation ensures an efficient improvement in human PSC-CM (hPSC-CMs) maturation [19,22,27]. When Kroll et al applied a 5% cyclic stretch to human iPSC-CMs (hiPSC-CMs) cultured on PDMS membranes, these cells did not mature to cardiac phenotypes in morphology and cardiac marker expression levels [28]. In another study, 15% stretch applied to hESC-CMs caused a significant increase in cell volume and sarcomere size but was also associated with a 1.6-fold slower beating rate. This abnormal beating rate was caused by the greater downregulation of Ca2+, Na+, and K+ channel proteins [20]. Considering that 10%–20% elongation may be physiological [29,30], these studies may provide an approximate range of optimal stretch amplitude for hPSC-CMs. Further studies are needed to confirm these observations.

(3) *Stretch frequency.* As for stretch frequency, cyclic stretching may have a better effect than static stretching (0 Hz) [27,31], and the majority of studies based on hPSC-CMs have selected 1 Hz to simulate the human heart rate [20,32]. For mouse models, a study based on mouse ESC-CMs (mESC-CMs) compared the different effects of 1, 2, and 3 Hz stretching at 10% elongation [17]. Only the 3 Hz stretch significantly upregulated cardiac genes, such as α-skeletal actin, α-cardiac actin, and α-MHC, which indicates that a higher frequency may better mimic the murine heart rate, a point also raised in other reports [33]. In contrast, a recent experiment showed cyclic stretch conditions (2.5%, 5%, and 10% strain) at 0.33 Hz on mESC-CMs were also able to significantly increase the expression of cardiac genes [34]. This controversy may be partly due to the different study designs and different experimental conditions used, including the longer duration of cell culture in the latter study. In addition, because a normal heart does not always keep the same beating rate, it is noteworthy that a dynamic change in stretch frequency may also have some effects on cardiomyocyte maturation [27].

(4) *Stretch time.* As long-term culturing advances maturity of PSC-CMs [35], application of longer stretch time should yield better results if carried out under an appropriate stretch regimen. Typically, the stretch time is selected to be no more than 2 weeks, as is shown in **Table 2**. Considering that excessive stretch time may increase the time and economic burden of its future clinical application, more research is required to explore the balance in between. Importantly, another study revealed that mechanical conditioning should be initiated early during the high cell plasticity period because the responsiveness of hiPSC-CMs to physical stimuli declines as maturation progresses [36].

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Table 2. Recent studies focused on the maturation of PSC-CMs promoted by mechanical stretch since 2015.

Publication year & main author	Cell types	Stimulations	Parameters of mechanical stretch	Impact of stretch on structure	Impact of stretch on function
2020 Kreutzer	hiPSC-CMs	Mechanical stretch only	• Uniaxial; • 8% elongation; • 0.8 Hz; • 7 days	• Successful attachment and survival of cells; • More uniform sarcomere orientation and alignment	–
2019 Labarge	hiPSC-CMs	• Mechanical stretch; • Electrical stimulation	• Uniaxial; • 10% elongation; • 1 Hz; • 7 days	• Formed many Z-band precursors; • Increased ratio of βMHC to αMHC transcript expression; • Improved MLC2v expression	–
2018 Ronaldson-Bouchard	hiPSC-CMs	Electrical stimulation induced mechanical stretch	• Uniaxial; • 2 Hz and variable frequency (2 Hz to 6 Hz to 2 Hz)	• Cell size increased and both the cells and nuclei elongated; • Fraction of cells containing sarcomeres and organization of sarcomere similar to adult type; • Comprehensive changes in genes encoding for adult-like maturation	• Increased contractility; • Positive force-frequency relationship; • Observed frequency-dependent acceleration of relaxation; • Responses to isoproterenol similar to adult type
2018 Ovchinnikova	hESC-CMs	Mechanical stretch only	• Equiaxial; • 15% elongation; • 1 Hz; • 2 days	• Increased size of cells and sarcomeres; • Myofibrils were distributed in parallel and appeared wider	• Slower beating frequency but not significantly affected active force; • Higher passive stiffness
2018 Abilez	hiPSC-CMs; hESC-CMs	Mechanical stretch only	• From its original length 3 mm to 5 mm, 7 mm, and 9 mm, respectively (3 groups); • 28 days	• Demonstrated a heterogeneous phenotype at days 25–28; • More aligned sarcomeres under 7 mm stretch	• Better calcium dynamics, like amplitude, time-to-peak, response to electrical stimulation, especially under 7 mm stretch; • Linear force-length relationship
2017 Zhang	hESC-CMs	Mechanical stretch on hESC-CMs cocultured with Nicole cells	• Uniaxial; • 10% elongation; • Static and 1 Hz; • 3 days	• Integrin β1 and vinculin were upregulated in both stretch groups; • Better alignment and greater sarcomere length in both stretch groups; • Significant changes in the gene expression of maturation markers, like MLC2a, MLC2v, NPPA, and Gja7	• Increased active force under cyclic stretch; • Better passive stiffness in both groups; • Linear force-length relationship; • Positive force-frequency relationship; • Responses to extracellular [Ca2+] similar to adult type
2017 Shen	hESC-CMs; mESC-CMs	• Mechanical stretch; • Fluid sheer stress	• 2.5%, 5%, and 10% elongation; • 0.33 Hz • 12 days (stretch only)	Increased expression of cardiac genes in mESC-CMs, especially under 5% elongation	Proper cardiac electrophysiology, Ca2+ handling and responses to Nifedipine in hESC-CMs under combining stimulation
2017 Kroll	hiPSC-CMs	• Mechanical stretch; • Electrical stimulation	• Uniaxial; • 5% elongation; • 1 Hz; • 7 days	No improvements in neither microstructure nor expression of cardiac genes under single mechanical stretch	Increased percentage of the cells expressing the L-type membrane current under single mechanical stretch

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Table 2 continued. Recent studies focused on the maturation of PSC-CMs promoted by mechanical stretch since 2015.

Publication year & main author	Cell types	Stimulations	Parameters of mechanical stretch	Impact of stretch on structure	Impact of stretch on function
2016 Ruan	hiPSC-CMs	• Mechanical stretch; • Electrical stimulation	• Uniaxial; • Static; • 14 days	• Improved cardiomyocyte density, volume and myofibrillar alignment under single static stretch; • Promoted passive tissue stiffness under single static stretch; • Improved expression of SERCA2 and RYR2 under single static stretch	• Increased active force under single static stretch; • Improvement on force-frequency relationship under single static stretch
2015 Ruan	hESC and hiPSC-derived cardiovascular progenitors (CVP)	Mechanical stretch in 3D microenvironment	• Uniaxial; • 5% elongation; • Static and 1 Hz; • 14 days	• No significant effect on cardiomyocyte fate choice; • Increased cTnT intensity under cyclic stretch; • Increased β-MHC and decreased α-MHC expression in hESC-derived CVP but not in hiPSC-derived CVP under cyclic stretch; • Increased connexin-43 and cadherin under cyclic stretch	• Increased active force and passive stiffness, especially under cyclic stretch; • Positive force-length relationship, especially under cyclic stretch; • Better calcium dynamics, like increased peak calcium flux and departing velocity; • Responses to BDM and isoproterenol similar to adult type
2015 Qi	hESC-CMs	Mechanical stretch only	• Uniaxial; • 10%, 20% or 30% elongation; • 1 Hz	Realignment of hESC-CMs in the direction transverse to the direction of stretch, with the percentage of realigned cells positively correlated with the degree of elongation	–

Effects of Mechanical Stretch on Maturation of PSC-CMs

Structural Level

During the development of cardiomyocytes, the changes in cell morphology, alignment, and microstructures (such as sarcomere, T-tubes, and mitochondria) lay the foundation for future mature contractility and electrophysiological performance. In addition, evaluating their cardiac gene expression profiles is also a powerful tool to determine the maturation stage of PSC-CMs. In this review, the description of structural changes to PSC-CMs under mechanical stretch is divided into 2 parts: structures observable by imaging techniques, and cardiac gene expression levels.

Tulloch et al [37] examined, through imaging, the influence of static and cyclic stretching (5% elongation, 4 days of 1 Hz) on intercellular alignment of hiPSC-CMs and hESC-CMs, which was quantified from the reciprocal of the cell axis angle dispersion, in which a low standard deviation of angles indicated a high degree of alignment. Both static stretch and cyclic stretch of cells increased the alignment value (4.09 for both stretch groups vs 1.96 for the non-stretch group). Orientation analysis with circular variance of hiPSC-CMs receiving a uniaxial (8%, 0.8 Hz) stretch on PDMS membranes for 7 days clearly demonstrated a significant unidirectionality of sarcomere structures that were perpendicular to the stretch axis after 2 days, while sarcomeres were only slightly more oriented at days 4 and 7 [23]. The authors of that study concluded that a time span of 4 days was sufficient to gain the highest degree of orientation. hiPSC-CMs in another study [22] formed many Z-bodies, the precursors to Z-bands, adjacent to contractile fibers under uniaxial (10%, 1 Hz) stretch for 7 days. Interestingly, mechanical stretch has been shown to cause rearrangement of hESC-CMs and human atrial fibroblasts perpendicular to the direction of the stretch [32,38], whereas another study revealed parallel rearrangement of newborn mouse cardiomyocytes [39]. Furthermore, in a long-term study, hPSC-CMs cultured under static stretch showed heterogeneous cellular phenotypes, with ventricular-like cells being the predominant phenotype (57%) along with atrial-like (24%) and

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nodal-like (19%) cells, when the culture time was extended to 25-28 days [40]. Other experiments focusing on hESC-CMs showed similar results in terms of cell morphology and sarcomere structure [20,27,32], which indicates that improved cardiac maturity benefited from mechanical stretch. Apart from stretch-only conditions, Kensah et al investigated the combined role of fibroblasts, ascorbic acid, and mechanical stimuli on both human and murine PSC-CMs, showing that these cells presented even better organization compared with that of a single stimulus [41]. However, despite all these impressive outcomes, the alignment level of such stimulated hPSC-CMs still cannot reach that observed in neonatal or adult rat cardiomyocytes [37]. Although it is inappropriate to compare cells from 2 species, this observation in rats highlights the differences between stretched PSC-CMs and mature cardiomyocytes.

Genes regulated by mechanical stretch within PSC-CMs are related to contractility (eg, cTnI, α-MHC and MLC2v), gap junctions (eg, Cx-43 and N-cadherin), ion channels (eg, SCN9A), and others (eg, ANP) [20,27. Although many genes have been detected by various studies, only those genes that are studied frequently are discussed in this review.

(1) *Myosin heavy chain (MHC)*. In humans, the MHC isoforms MHC-α and MHC-β change with the ratio of MHC-β over MHC-α, increasing as cardiac development matures [42]. Mechanical stretch (1 Hz, 5% elongation for 2 weeks) on hESC-CMs has been shown to increase the expression of MHC-β by 550% to 800% compared with hESC-CMs that experienced non-stretch or static stretch conditions [31]. Concurrently, MHC-α gene expression in hESC-CMs decreased by 62% or 50% under cyclic stretch conditions compared with MHC-α gene expression in cells under non-stretch or static stretch conditions, respectively. Similar increases in the MHC-β/MHC-α ratio by mechanical stretch were observed in other studies examining hiPSC-CMs and hESC-CMs [21,22]. It is noteworthy that in mouse models, although MHC-α is the predominate MHC isoform in the adult heart, this only occurs after the mouse is born. During pregnancy, the amount of MHC-β is still higher and the MHC-β/MHC-α ratio is even progressively increased [43,44]. This special developing pattern may help better evaluate the maturation status of mPSC-CMs under mechanical stretch.

(2) *Connexin 43 (Cx-43)*. Cx-43 is the main gap junction protein expressed in the heart. It has been shown to gradually accumulate during gestational stage in rats [45]. For the mouse and human fetal heart, the increase of Cx-43 during gestation is associated with the development of the right ventricle and ventricular conduction systems, respectively [46-48]. In a study where stretch was applied (10% elongation, 3 Hz) to mESC-CMs cultured in collagen gels, Cx-43 was observed to be more widespread and diffuse throughout the tissue compared with that of the non-stretch group [17]. For mhiPSC-CMs, stretch (10% elongation, 1 Hz) for 7 days also induced the expression of Cx-43 [41]; however, the levels were up to 10-fold lower compared with that of neonatal hearts. These results showed that mechanical stretch accelerates the accumulation of Cx-43, indicating the advanced maturity of mPSC-CMs, while the effects may be still far lag behind naturally developed heart cells. Similar results were also found in hESC-CMs and hiPSC-CMs [22,31].

Functional Level

The main features used to assess the function of PSC-CMs are active force (twitch force), passive force (stiffness, elastic modulus), and ion dynamics. Ruan et al found hESC-CMs in collagen hydrogels under uniaxial stretch (1 Hz, 5% elongation) presented significantly improved active and passive forces. A non-stretch construct had a passive stiffness of 0.22±0.04 kPa, whereas hESC-CMs in collagen hydrogels subjected to static and cyclic stretching increased passive stiffness to 0.47±0.05 kPa and 0.71±0.12 kPa, respectively [31]. Kensah et al explored the combined impact of ascorbic acid and growing static stretch (stepwise elongation every second day) on human and murine PSC-CMs [41]. For miPSC-CMs, maximum active forces almost doubled, measured at 1.42±0.09 mN/mm2, whereas passive forces were about 3 times higher than in untreated controls at day 21. Similar results were found in hPSC-CMs. In addition, the force-length relationship can be evaluated by installing a force transducer on the mechanical arm where the tissue is fixed while gradually stretching the cells. A linear relationship was demonstrated for hESC-CMs subjected to uniaxial (1 Hz, 10%) stretch for 3 days, which is in agreement with the Frank-Starling Law because native myocardia behave in the same way [27]. However, the hESC-CMs in this study did not show a positive force-frequency relationship, which is observed in native myocardia, because their force output declined as the contraction rate accelerated. In another study, mechanical stretch was observed to weaken the original negative correlation of hPSC-CMs and promote the development of a positive correlation [49]. Importantly, if these tests need separation between cells and their culture environment, the outcomes may incorrectly reflect the in situ states of PSC-CMs. Some specific devices have been developed to realize in situ monitoring of cellular functions to solve this problem [50,51]. Moreover, for some drugs such as isoproterenol, hESC-CMs respond to the drugs in a similar manner to native cardiomyocytes, with corresponding increases or decreases in contractile force observed [31,52]. These enhancements may be attributed to the development of the intracellular sarcomere, mitochondria [18], and realignment of cells [39].

In terms of ion dynamics, hiPSC-CMs express the main components of cardiac excitation-contraction coupling, including L-type calcium channels and sodium-calcium exchangers [53-56].

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hiPSC-CMs also generate ion currents for depolarization (*I*Na, *I*CaL, *If*) and repolarization (*I*to, *I*Kr, *I*Ks, *I*K1) of the membrane, which together produce action potential waveforms resembling that of human cardiomyocytes [57]. However, the poor co-localization of calcium channels and ryanodine receptors as well as the non-uniform distribution of calcium release [58,59] caused the upstroke and decline rates of the whole-cell Ca2+ signals to be substantially slower in hiPSC-CMs compared with that observed in adult cardiomyocytes [60,61]. These findings reveal the gap in ion dynamics, especially Ca2+ handling, between hiPSC-CMs and adult cardiomyocytes. A previously mentioned study found that with uniaxial cyclic strain at 1 Hz, 5% elongation on hESC-CMs was able to increase both peak calcium flux and upstroke rate by over 100% [31]. A study by Kroll et al [28], applying the same set of stretch parameters on hiPSC-CMs, also showed an increase in the percentage of cells expressing an L-type membrane current (from 66% to 81%) compared with that of the non-stretch group. Similar to contractility, proper responses of Ca2+ handling to cardiac drugs such as Nifedipine were also observed [34]. Together, these changes in parallel with the expression levels of ion channels [20,21] indicate the improved maturity of PSC-CMs under mechanical stretch in vitro.

Signal Transduction Mechanisms

Exploring how mechanical stretch promotes cardiac maturation of PSC-CMs is critical because it may provide a strategy to improve the efficiency of maturation. Although the internal signal transduction mechanism is currently unclear, the following pathways are introduced to provide a brief overview.

(1) *TRPV4/PI3K/AKT.* The TRPV4 channel is a non-selective cation channel that is Ca2+ permeable and activation of this channel causes an increase in intracellular Ca2+ levels [62]. Qi et al demonstrated that uniaxial cyclic stretching induces an increase Ca2+ influx, AKT phosphorylation, and realignment of hESC-CMs, and all these effects are abolished by TRPV4 inhibitors. Additionally, inhibitors of AKT and PI3K abolished stretch-induced hESC-CM realignment, suggesting the possible role of PI3K/AKT in cell realignment under cyclic stretch [32]. Because TRPV4 should be upstream of AKT, and TRPV4 channels are Ca2+ permeable channels whose activity leads to a rise in the intracellular Ca2+ level, it is likely that Ca2+ is a link between TRPV4 and AKT activity, which has been shown in other studies [63,64]. These studies show that the TRPV4/PI3K/AKT signal pathway may mediate the realignment of hESC-CMs under cyclic stretch.

(2) *Wnt/β-catenin.* The Wnt/β-catenin signal pathway is well known to influence a diverse set of biological processes, especially those processes involved in embryonic development and tissue renewal [65]. Recently, the Wnt/β-catenin signal pathway has been demonstrated to play a biphasic role in cardiogenesis (positive, then negative as the heart develops) [66,67]. Shen et al concluded that after 20 days of cyclic stretch, hESC-CMs expressed a lower level of β-catenin compared with that of static hESC-CMs, which indicates an inhibition of the Wnt/β-catenin signal pathway because of mechanical forces [34]. In their study, hESC-CMs under cyclic stretch displayed advanced maturity of the sarcomere structure and expression of cardiac markers such as Cx-43 and cTnT compared with those for hESC-CMs under static conditions. However, neither the development stage of stretched hESC-CMs nor changes caused by mechanical stretch through the Wnt/β-catenin signal pathway were explored. Further studies are required to characterize the vital role of the Wnt/β-catenin signal pathway in late cardiogenesis.

(3) *Others.* In embryonic mouse cardiomyocytes, stretch decreases the expression of the TGF-β family of proteins and the phosphorylation level of SMAD3, which is located downstream of TGF-β [68]. In bone marrow mesenchymal stem cells, cyclic stretching promoted these stem cells to differentiate into cardiomyocyte-like cells by suppressing the expression of the miR-27 and SCF genes (a target of miR-27). Although these findings did not originate from studies examining PSC-CMs, they may provide new clues for exploring potential molecular mechanisms regulated by mechanical stretch during the maturation of PSC-CMs.

Discussion and Summary

As is discussed in the "Parameters of mechanical stretch" section, even though there has been a growing number of studies based on PSC-CMs in recent years, few of them are focused on finding the appropriate stretch parameters. A noticeable issue is the lack of versatile and universally used devices that release/apply proper mechanical stretch to meet most requirements of different researchers. Another problem is the diversity of study designs, including different stem cell sources, dimensions of extracellular microenvironment (2D or 3D), additional stimuli like electrical stimulation, as well as diverse indicators for assessing the maturation status of PSC-CMs, leading to difficulty in comparing different trials. These shortcomings are partially due to the fact that mechanical stretch alone is not enough to fully promote the maturity of PSC-CMs, an issue that will be addressed later. Based on current progresses, suggestions on developing more standardized devices and study designs are as follow: (1) Since native myocardium is also stretched from different directions and even different planes (like a small part of the surface of an inflating balloon), devices which are pneumatically or vacuum controlled [69] to make the tissue extend nonlinearly may have a better effect

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than flexible pillars that stretch the tissue in a single straight direction; (2) 3D extracellular matrix should be considered, as it not only closely mimic the native environment where cardiomyocytes are subjected to mechanical stretch, but also can promote the maturity of PSC-CMs alone [70]; (3) In addition to those specially designed research purposes, some indicators that are easy to quantify should be universally proposed for maturation assessment, such as sarcomere length, expression levels of specific cardiac genes, active contracting force, and passive stiffness of the tissue; (4) In particular, comparing stretched PSC-CMs to adult cardiomyocytes in each study may be vital in identifying the real maturation stage of the former.

Indeed, mechanical stretch is able to promote the maturity of PSC-CMs to some extent, including increased size and elongated alignment of cells and sarcomeres, improved expression of cardiac markers (eg, actin, MHC-β, Cx-43, MLC2v), stronger contractility and passive stiffness, enhanced calcium handling, as well as ability to respond to some medicines in a way similar to that of adult cardiomyocytes. However, as discussed, the maturity of PSC-CMs under stretch conditioning still cannot reach a satisfactory level for further clinical usage. This is consistent with other studies where PSC-CMs generally remain within the early to late fetal cardiomyocyte stages after applying engineering approaches like electrical stimulation, non-cardiomyocyte interactions, or extracellular matrix interactions [12]. Considering that native heart muscle cells require a long time to mature in a complex physiological environment by receiving various stimuli, these outcomes under single in vitro simulated condition within a restricted culture time seem to be reasonable. Although researchers have been exploring the underlying signal transduction pathway and the combination of different maturation-promoting strategies [71], the number of relevant studies is limited. Further efforts should be focused on these aspects as well. Hopefully, with advances in research examining the effects of mechanical stretch on the maturation of PSC-CMs, we may soon develop more effective stem cell therapies for treating cardiac diseases.

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Conflict of Interest

None.

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