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REVIEW

Biomechanical regulation of *in vitro* cardiogenesis for tissue-engineered heart repair

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Abstract

The heart is a continuously pumping organ with an average lifespan of eight decades. It develops from the onset of embryonic cardiogenesis under biomechanical load, performs optimally within a defined range of hemodynamic load, and fails if acutely or chronically overloaded. Unloading of the heart leads to defective cardiogenesis *in utero*, but can also lead to a desired therapeutic outcome (for example, in patients with heart failure under left ventricular assist device therapy). In light of the well-documented relevance of mechanical loading for cardiac physiology and pathology, it is plausible that tissue engineers have integrated mechanical stimulation regimens into protocols for heart muscle construction. To achieve optimal results, physiological principles of beat-to-beat myocardial loading and unloading should be simulated. In addition, heart muscle engineering, in particular if based on pluripotent stem cell-derived cardiomyocytes, may benefit from staggered tonic loading protocols to simulate viscoelastic properties of the prenatal and postnatal myocardial stroma. This review will provide an overview of heart muscle mechanics, summarize observations on the role of mechanical loading for heart development and postnatal performance, and discuss how physiological loading regimens can be exploited to advance myocardial tissue engineering towards a therapeutic application.

Introduction

The heart muscle engineering field is evolving mainly around three fundamentally different bioengineering concepts: seeding of *bona fide* heart cells or cardiogenic cells on preformed natural or synthetic scaffolds [1-5]; assisted self-assembly of *bona fide* heart cells or cardiogenic cells in hydrogels [6-9]; and matrix-free self-assembly of *bona fide* heart cells or cardiogenic cells [10,11]. Importantly, each of these technologies can be exploited to produce contractile heart muscle. Proof of concept for a wide range of research applications of tissue-engineered heart muscle has been presented; this includes applications in drug screening [12], target validation [13], and disease modeling [14]. Preliminary evidence for therapeutic applications has also been provided [15,16], but clinical translation of engineered heart muscle-based heart repair has not yet been attempted.

Today's challenges for clinical translation pertain to tissue scale, functionality, and maturity. It is anticipated that

roughly 20% of a scarred ventricle would have to be replaced by engineered myocardium for a clinically meaningful outcome. Based on these premises and estimations that approximately one billion cardiomyocytes are lost in patients with hemodynamically relevant myocardial infarction [17], it will be necessary to integrate at least 200 million cardiomyocytes. Heart muscle engineering relies on the propensity of immature cardiomyocytes to assemble into a functional syncytium. However, evidence for terminal maturation within these syncytia with cardiomyocytes of adult dimensions has so far not been provided. Comparisons of adult rat and human cardiomyocytes with cardiomyocytes from 11-day-old rat or 55-day-old human heart, which have a similar volume to cardiomyocytes in engineered heart muscle, have documented an approximately 10-fold volume mismatch between young and adult cardiomyocytes [18,19]. Matching this discrepancy by applying more cells would necessitate engineered heart muscle implants containing two billion cardiomyocytes. This not only seems overambitious, but would also carry the risk that the grafts could outgrow the defect especially if engrafted cardiomyocytes would respond with hypertrophic growth to the *in vivo* environment. Morphological evidence for the propensity of tissue-engineered heart

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grafts to undergo hypertrophic growth *in vivo* has been provided previously [16,20]. Conversely, inducing a fully adult phenotype already *in vitro* to allow for a full match of graft and host cardiomyocytes does not seem desirable either, because of the anticipated higher metabolic and oxygenation demands in mature versus immature heart muscle. Finally, it seems reasonable to argue for a compromise between cell number and maturity to facilitate cell survival *in vitro* and fast scar replacement *in vivo* by a graft with a potential for hypertrophic growth.

Tissue-engineered heart repair will not only require a large number of cardiomyocytes with hypertrophic growth potential, but also strategies and tools to enable smart integration of the newly engineered biomechanically functioning units into the failing heart for effective contractile support. A key requirement will be to align myocyte grafts with the recipient hearts' cardiomyocytes. To ensure anisotropic cardiomyocyte assembly in tissue-engineered heart muscle and thereby facilitate *in vivo* applicability, it seems important to make use of biomechanical conditioning *in vitro* [16,18,21,22]. The following paragraphs summarize important principles of heart muscle/sarcomere structure and mechanics, inform about changes in hemodynamics and its influence on cardiac morphology *in utero* as well as in the adult heart, and discuss possibilities to implement mechanical loading for the allocation of improved engineered heart muscle grafts for therapeutic applications in heart failure repair.

Heart muscle and sarcomere mechanics

The heart ejects blood into the circulation via a wringing motion [23]. This is possible because of the anatomic organization of the heart muscle with obliquely overlaying muscle sheets. The muscle sheets are under physiological conditions composed of strictly anisotropically arranged cardiomyocytes, again containing anisotropically organized sarcomeres (Figure 1). Reduced anisotropy at the cardiomyocyte and sarcomere level is a typical feature of a chronically failing heart. Synchronized activity of heart muscle is established via the formation of a functional syncytium that expands *in utero* mainly by cardiomyocyte proliferation and postnatally mainly by hypertrophy [24,25]. Both mechanisms are adaptations to the increasing hemodynamic load imposed on the myocardium by changes in circulating blood volume, blood viscosity, peripheral resistance, and hydrostatic pressure [26]. Electromechanical synchronization of the heart is finally achieved by an excitation wave originating from defined pacemaker cells and spreading anisotropically along the cardiac muscle sheets.

From beat to beat there is repetitive loading and unloading of the heart, the cardiomyocyte and the individual sarcomere. Handling of the hemodynamic load depends crucially on beating frequency (Bowditch phenomenon [27]) and myofiber length (Frank–Starling mechanism [28]), with higher output at high beating rates (positive force–frequency relationship) and optimal heart distension (positive force–length relationship). The load of the heart can be distinguished as preload and afterload. Under physiological conditions in humans, the heart is preloaded during diastole (right ventricle, 0 to 5 mmHg; left ventricle, 60 to 90 mmHg) and thereby extended. During systole, ventricular pressure rises to overcome the hemodynamic pressure (afterload) that keeps the pulmonary (for the right ventricle) and aortic (for the left ventricle) heart valves closed (20 to 30 mmHg in the right ventricle; 90 to 140 mmHg in the left ventricle). The whole contraction cycle of the heart is best presented as a pressure–volume loop with distinct phases of isotonic and isovolumetric contraction during systole and relaxation during diastole (Figure 1). Pathologically increased preload and afterload can induce distinct phenotypes [29]. The principle features of myocardial loading and unloading introduced here are particularly important for the understanding of the presently applied mechanical loading regimens in heart muscle engineering. There is certainly more to consider, and a large body of literature is available to which the interested reader is referred for a more comprehensive overview on cardiac biomechanics.

Load as an essential factor for heart development and function

The myocardium is always exposed to hemodynamic load. In particular during embryonic heart development, which is paralleled by erythropoiesis and increasing hemodynamic load, massive changes with respect to tissue architecture and function occur (reviewed in [26]). Adaptation to increasing hemodynamic load is a physiological phenomenon that can, however, go awry *in utero* in the case of overloading [30], but also if the ventricles are unloaded [31]. In embryonic cardiomyocytes, load is a strong stimulus for proliferation [32,33]. In contrast, postnatal load adaptation is mainly via physiological hypertrophy [18], and chronic hemodynamic overloading is typically associated with pathological hypertrophy [29]. Several pathways have been associated with these different hypertrophy processes [34]. However, it remains to be determined whether distinct individual factors or complex interconnected signaling networks govern transition from physiological to pathological hypertrophy.

Prenatal heart development and postnatal adaptation to alterations in hemodynamic load not only go along with changes in cardiomyocyte phenotype, but also with biophysical alterations of the myocardial extracellular matrix [35]. Extracellular matrix is secreted and remodeled mainly by cardiac fibroblasts in healthy myocardium and by myofibroblasts in diseased myocardium. Recent evidence suggests that extracellular matrix elasticity has
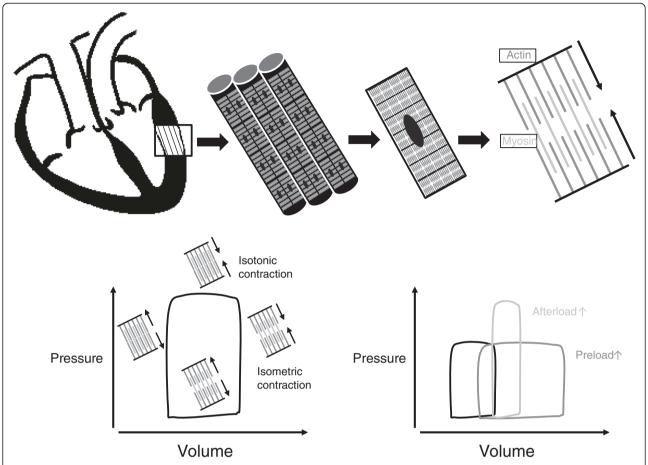
Figure 1 Heart and cardiomyocyte biomechanics. The smallest functional unit of the heart at the micrometer scale is the sarcomere (typically spans 1.5 to 2 μm). Sarcomeres are aligned anisotropically in the cardiomyocytes (typical dimensions: length 110 μm, width 25 μm [18]), which are assembled as continuous muscle sheets. Coordinated contractions of these muscle sheets enable the typical wringing motion or twist of the heart. Each contraction cycle can be displayed as a pressure–volume loop (typically measured by conductance catheterization), comprising phases of iso (volu)metric and isotonic sarcomere contraction (systole) followed by iso(volu)metric and isotonic relaxation (diastole). These phases are shortened or prolonged in case of pathologically increased preload (for example, in case of mitral valve insufficiency) or increased afterload (for example, in case of aortic stenosis); note that in most clinical cases mixed phenotypes are observed. Pressure–volume loops adapted from [29].

a pivotal role also in cell fate decisions [36], and it appears plausible to postulate that stem cell-based myocardial tissue engineering will not only benefit from paracrine inputs [22,37] but also from a permissive biomechanical environment [38].

A key biomechanical event during heart development is the separation of lung and peripheral circulation at birth. This establishes a prominent load imbalance between the left and right ventricles, with left-sided physiological hypertrophy as an adaptation to the load requirements. These differences in heart morphology provide additional evidence for the relevance of biomechanic load for cardiomyocyte hypertrophy and demonstrate how quickly in particular the young heart can adapt to changes in hemodynamic requirements. Pathologic hypertrophy and heart failure are typically the consequences of chronic overloading (for example, after myocardial infarction or in

patients with hypertension). Left ventricular unloading with assist devices has been employed to enable reverse remodeling [39]. Interestingly, unloading and overloading of the heart can elicit similar fetal gene programming [40], a long-described feature thought to be associated with an adverse heart failure outcome.

Exploitation of mechanical loading in myocardial tissue engineering

For tissue engineers, it is on the one hand essential to ensure cardiomyocyte survival in tissue engineered myocardium *in vitro* and *in vivo*. On the other hand, assuring physiological hypertrophy to an extent that does not compromise cardiac metabolic capacity is also advantageous. This may have to be paralleled by innovative mechanisms for oxygenation of the growing engineered myocardium - for example, by inducing vascularization already *in vitro*

[41] or by utilization of perfusion bioreactors for optimal oxygen and nutrient provision [42].

In myocardial tissue engineering, responses to load seem to depend crucially on the developmental stage of the input cardiomyocytes; for example, neonatal rat cardiomyocytes typically undergo dedifferentiation towards a fetal phenotype in monolayer culture and retain/regain a limited proliferation capacity, but in engineered heart muscle abundant proliferation was not observed [18]. In contrast, human embryonic stem cell-derived cardiomyocytes displayed cell cycle activity in a similar tissue engineering format [33]. These findings collectively suggest that embryonic stem cell-derived cardiomyocytes retain their immature phenotype also in three-dimensional engineered heart muscle cultures. Further studies will have to address under what circumstances cardiomyocytes from different developmental stages and also species can be advanced towards terminal differentiation.

Up-to-date different mechanical loading protocols have been tested to morphologically and functionally advance engineered heart muscle [16] (Figure 2). The easiest way to load engineered heart muscle is by suspending it between two fixed holders [6]; the holder distance can be adapted freely to alter the preload (Figure 2A). An advantage of static loading protocols is that they are simple to implement and are also easily conducted for extended periods of time without tissue rupture. Conversely, cyclic stretching (Figure 2B) is experimentally more demanding and only possible for a relatively short period of time (7 to 10 days) without premature rupture. This limitation may be overcome by optimizing the viscoelastic properties of engineered heart muscle – for example, by making use of advanced biomaterials – or by adapting the stretch algorithm to the intrinsic contractile behavior of engineered heart muscle. Motorized cyclic stretching can induce hypertrophy with better contractile performance in some but not all tissue engineering protocols [7,21]. The cycle length has to be adapted to the endogenous beating frequency, which depends on the presence of pacemaker cells within engineered heart muscle and the employed species (rat, ~2 Hz; mouse, ~4 Hz; human, ~1 Hz; author's own unpublished data). Under a proper cycle length, engineered heart muscle adapts the contraction cycle to the stretch cycle so as not to contract actively while being distended; this closely resembles isotonic contractions. Finally, culture of engineered heart muscle was optimized
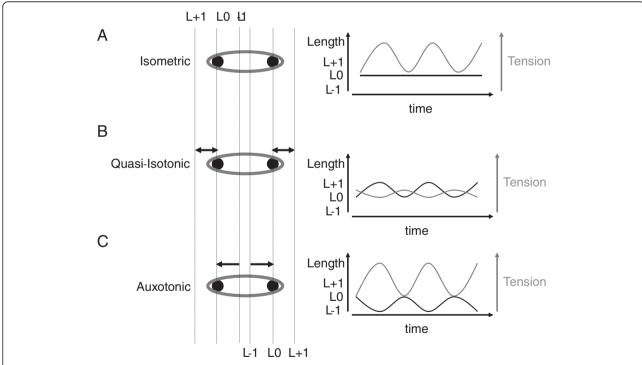

Figure 2 Biomechanical loading in myocardial tissue engineering. Circular engineered heart muscle (gray) can be subjected to different loading protocols [16]. **(A)** Suspension between static holders facilitates isometric contractions under highly controlled conditions; preloading can be adapted by increasing or decreasing the holder distance. **(B)** Suspension between motorized holders enables defined extensions at a defined cycle length; engineered heart muscle ideally adapts to the motorized cycle to contract and relax in phase with the narrowing and widening of the holders (quasi-isotonic contractions). **(C)** Suspension against a bias force supports auxotonic contractions; that is, contraction against increasing load followed by extension under a defined bias force. L0, slack length at diastole; L + 1, extension from slack length; L-1, length at peak systole. Black arrows indicate displacement forced upon engineered heart muscle either by a motorized device with fixed cycle length **(B)** or defined biasing force elicited by a resilient mount **(C)**.

by suspending it between resilient mounts to facilitate auxotonic contractions [16] (Figure 2C). This seems to resemble the physiologic contraction cycle quite closely, but is also most difficult to establish in a way to match with the continuously developing contractile properties. Future developments will probably include automated feedback loops to measure the force developed by engineered heart muscle, and based on this to simulate appropriate preload and afterload to train the heart muscle constructs from beat to beat to develop optimal contractile and structural properties. This will probably also include external electrical stimulation to facilitate syncytial arrangement of cardiomyocytes and further assist morphological and functional maturation.

In addition to the described stretching mechanisms it appears important to also simulate natural viscoelastic tissue properties as closely as possible in tissue-engineered heart muscle. This may be achieved by staggered tonic loading protocols and further supported by defining optimal mixtures of cardiomyocytes and nonmyocytes in the original reconstitution mixture as well as through inclusion of optimized biomaterials [43,44]. Fibroblasts are particularly important in this scenario because they provide the necessary extracellular matrix architecture to establish optimal viscoelastic properties that define, for example, resting (diastolic) tension. Under biomechanically optimized conditions, engineered heart muscle develops specific muscle tension of 30 to 50 mN/mm^2 [16,45]. These forces are similar to what can be expected from native heart muscle [46]. To further increase absolute force, preferably by augmenting the muscle cross-sectional area, it will be essential to enhance cardiomyocyte density and/or volume with anisotropically arranged sarcomeres in engineered heart muscle (please refer to [45] for a more detailed discussion of force generation and assessment in engineered heart muscle).

Perspective

Engineered heart muscle is already today used in simulations of heart muscle development [18], drug screening [12], and target validation [13,47]. Importantly, engineered heart muscle is, in principle, scalable to meet clinical demands [16,43]. The apparent limitations of monolayer cardiomyocyte cultures such as unphysiological contractile performance, low degree of maturation, and inadequate pharmacological responsiveness have facilitated the entry of engineered heart muscle into a wide array of *in vitro* applications [12,13,18,47]. With further improved insight into mechanisms governing tissue maturation, it seems predictable that flat cultures will be exchanged for three-dimensional cultures in particular if they are used as a surrogate for the native heart (for example, in drug development). To find a broad application, simplification of the tissue engineering protocols will be required.

Repair of a failing heart by engineered heart muscle grafts has so far only been attempted in animal models, with encouraging results [16,44]. Here it is important to note that the cell sheet technology is presently under clinical investigation in Japan with skeletal myoblasts as a putatively therapeutic cell source [48]. For optimal therapeutic impact, however, it seems that cardiomyogenic cells or *bona fide* cardiomyocytes will be necessary. The most promising cardiomyocyte sources for tissue-engineered heart repair are pluripotent stem cells, such as embryonic stem cells, induced pluripotent stem cells, and parthenogenetic stem cells [8,33,44,49]. Clinical translation will probably first be attempted with human embryonic stem cell-derived cardiomyocytes. Ongoing clinical studies on embryonic stem cell-derived oligodendrocytes [50,51] and retinal pigment endothelial cells [52] will help to collect pivotal data on the safety of embryonic stem cell-derived cells, and thus help to inform the anticipated first-in-man myocardial tissue engineering trials. Whether the recent developments in direct fibroblast-to-cardiomyocyte programming [53] will provide a new cell source for myocardial tissue engineering remains to be seen. Scaling to clinical size, at least *in vitro*, does not seem to be a major caveat in light of the recent developments in cardiomyocyte-derivation protocols [54] and the possibility for a modular tissue design [43]. In rodent models, fast vascularization of engineered heart muscle grafts with no obvious cell death has been observed [16,33]. Further studies will have to investigate whether this surprising observation can also be made in large animal models and, finally, the human. Appropriate large animal models will also be necessary to establish the risk for teratoma formation. An observation period of 4 to 8 weeks, which is often used in rodent models, will not be sufficient to rule out the risk for teratoma formation from human pluripotent stem cell-derived grafts; instead, 6-month to 12-month observation periods seem appropriate.

To finally establish the therapeutic potential of tissue-engineered myocardium, it will be important to scrutinize carefully whether therapeutic effects are the consequence of functional cardiomyocyte integration, reduction of wall stress leading to improved metabolism according to the LaPlace law, and/or pleiotrophic effects such as stimulation of endogenous repair via paracrine activation of stem cells, angiogenesis, or cardiomyocyte proliferation. Finally, some investigators may argue that a lack of mechanistic insight may be acceptable as long as a therapeutic benefit can be affirmed; and in fact for many of the classical heart failure drugs, there is until today uncertainty on how, when, and for whom they work best. For a (bio) engineer, however, it appears unacceptable to not be able to exactly define and simulate the role of an engineered part in the context of its (bio)mechanic environment.

Conclusion

Engineering of functional myocardium benefits greatly from biomechanical stimulation protocols. Physiological loading to facilitate auxotonic contractions appears ideal, but optimal conditions for resilient loading and mechanisms underlying the observed beneficial effects remain to be clarified. For therapeutic applications, it will be necessary to construct clinical scale heart muscle from approximately 200 million cardiomyocytes with appropriate sarcomere content and alignment to achieve a therapeutic impact in hearts with hemodynamically relevant defects. Scaling tissue-engineered heart muscle toward clinical needs can be achieved, for example, by fusing single elements into larger geometries [43]. Whether these geometries will also survive in a clinical setting cannot be answered today. Large animal experiments will not only be important to address whether engineered heart muscle grafting is feasible; they will mainly have to provide an answer regarding the necessity of prevascularization, proper electromechanical integration, and risk for tumor formation. In addition, immunological barriers must be overcome ideally by employing major histocompatibility complex-matched allografts or autografts. A fully unanswered question is what level of tissue maturity needs to be achieved prior to implantation. Immature myocardium with the propensity to undergo hypertrophic growth if subjected to a proper *in vivo* milieu appears to be ideal from the metabolic point of view. Mechanical integration into diseased hearts has to be achieved according to biomechanical demands so as to not deteriorate myocardial performance. This may require additional (transient) electrical stimulation to synchronize the graft with the native myocardium until electrical contacts have been established. Exploitation of advanced imaging and mathematical modeling to predict outcome and guide the development of personalized grafts may turn out to be an attractive addition to the ongoing efforts to engineer myocardium for heart failure repair.

> **Note:** This article is part of a thematic series on *Physical influences on stem cells* edited by Gordana Vunjak-Novakovic. Other articles in the series can be found online at http://stemcellres.com/series/physical.

Competing interests

W-HZ is founder and shareholder of Tissue Systems Holding GmbH and Myriamed GmbH, which have either acquired or licensed intellectual property rights pertaining to myocardial tissue-engineering technologies from the Zimmermann laboratory. The University Medical Center Göttingen has filed for additional patents related to myocardial tissue engineering technologies developed by the Zimmermann laboratory.

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