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Original article

Cyclic stretch of embryonic cardiomyocytes increases proliferation, growth, and expression while repressing Tgf-β signaling

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ABSTRACT

Perturbed biomechanical stimuli are thought to be critical for the pathogenesis of a number of congenital heart defects, including Hypoplastic Left Heart Syndrome (HLHS). While embryonic cardiomyocytes experience biomechanical stretch every heart beat, their molecular responses to biomechanical stimuli during heart development are poorly understood. We hypothesized that biomechanical stimuli activate specific signaling pathways that impact proliferation, gene expression and myocyte contraction. The objective of this study was to expose embryonic mouse cardiomyocytes (EMCM) to cyclic stretch and examine key molecular and phenotypic responses. Analysis of RNA-Sequencing data demonstrated that gene ontology groups associated with myofibril and cardiac development were significantly modulated. Stretch increased EMCM proliferation, size, cardiac gene expression, and myofibril protein levels. Stretch also repressed several components belonging to the Transforming Growth Factor-β (Tgf-β) signaling pathway. EMCMs undergoing cyclic stretch had decreased Tgf-β expression, protein levels, and signaling. Furthermore, treatment of EMCMs with a Tgf-β inhibitor resulted in increased EMCM size. Functionally, Tgf-β signaling repressed EMCM proliferation and contractile function, as assayed via dynamic monolayer force microscopy (DMFM). Taken together, these data support the hypothesis that biomechanical stimuli play a vital role in normal cardiac development and for cardiac pathology, including HLHS.

1. Introduction

Understanding the biomechanical and biochemical mechanisms of congenital heart disease is critical to reducing patient morbidity and mortality. Hypoplastic Left Heart Syndrome (HLHS), which occurs ~4 out of 10,000 live births, is one of the most severe and costly congenital defects [1]. HLHS presents with an insufficiently sized left ventricle that is unable to provide proper blood flow to the systemic circulation. While some genetic mutations have been correlated with HLHS [2–5], the majority of HLHS cases are idiopathic. This lack of understanding

Abbreviations: (HLHS), Hypoplastic Left Heart Syndrome; (GO), Gene ontology; (DMFM), Dynamic monolayer force microscopy; (RNA-Seq), mRNA-sequencing; (FDR), False discovery rate; (EdU), 5-ethynyl-2′-deoxyuridine; (qPCR), Quantitative Real Time PCR; (SSC), Flow cytometry side scatter.

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regarding the pathogenic drivers involved in this syndrome has impaired both the development of treatments and prognostic tools for HLHS. Interestingly, the proposed involvement of abrogated mechanical stimuli in the development of HLHS has led to the intriguing possibility that novel biomechanically modulated molecular pathways are causative for this disease.

Observations of fetal echocardiograms in HLHS patients have led to the hypothesis that disruption of biomechanical stimuli results in perturbed growth of the left ventricle [6–8]. Narrowing of the foramen ovale or mitral valve *in utero* decreases the diastolic filling of the left ventricle, reducing mechanical stretch stimuli on developing cardiomyocytes, and impairing left ventricular growth. This hypothesis is supported by data from model organisms (*e.g.* embryonic sheep and chicken), in which a reduction of left atrial size resulted in decreased diastolic filling of the left ventricle and development of a HLHS phenotype [9–13]. In addition to physiological changes, examination of postnatal cardiomyocytes from HLHS patients revealed a decrease in proliferation-related genes [14]. At the cellular level, animal models for HLHS were also shown to have decreased embryonic cardiomyocyte proliferation and increased apoptosis, recapitulating key features of the disease [10,12]. Despite the progress in modeling HLHS, there is little information about the specific molecular signals that are impacted by biomechanical stimuli at the cellular level. Given this lack of knowledge about the molecular pathways involved in the pathogenesis of HLHS, understanding the response of embryonic cardiomyocytes under biomechanical stimuli is critical.

In this effort, we hypothesized that biomechanical stimuli promote embryonic cardiomyocyte growth via stretch-activated signaling pathways. To test this hypothesis, we utilized an *in vitro* model in which embryonic mouse cardiomyocytes (EMCMs) were exposed to biomechanical stretch. Our results demonstrated that *in vitro* stretch increased both proliferation and size, indicating a direct link of stretch loading to EMCM growth and proliferation. Additionally, stretch modulated the levels of key myofibrillar factors such as myosin heavy chain and Titin. Bioinformatic analyses of mRNA-sequencing (RNA-Seq) data from stretched and static cells demonstrated significant enrichment of gene ontology groups (GO) involved in myofibrillogenesis and heart development. In addition, previously identified stretch-responsive pathways (*e.g.* focal adhesion, GTPase, integrin, cytoskeletal, calcium ion binding, oxidoreductase activity) were modulated under biomechanical stretch. Together, these data demonstrated that cyclic stretch is sufficient to promote phenotypic and gene expression changes in EMCMs.

One molecular pathway that is suggested to be involved in HLHS pathology is the Tgf-β/SMAD signaling pathway [15,16]. Tgf-β signaling has long been known to play crucial roles in development and disease. Indeed, activation of Tgf-β receptors controls the expression of Tgf-β-dependent genes by way of the SMAD proteins, which shuttle from the membrane-bound receptor to the nucleus to modulate gene-expression in a phosphorylation-dependent mechanism. During embryonic development, signaling through Tgf-β receptors is thought to play important roles in the selection of cell-lineage and cell-fate, as well as in the migration and homing of cells. Characterization of the Tgf-β/SMAD signaling pathway has provided insights into the plasticity of cell differentiation. Indeed, cells may undergo Tgf-β-dependent lineage transitions, for example epithelial–mesenchymal transdifferentiation (EMT), which is integral for normal embryo development and organogenesis [17]. In the heart, EMT is known to contribute to valve development [18]. Tgf-β2-knockout mice display perinatal lethality and congenital heart defects, with a hypercellular myocardium and an enlarged right ventricle [19]. Abnormal EMT caused by pathological Tgf-β signaling was shown to cause fibrosis and to play a role in tumor metastasis [17] During cardiomyopathy, Tgf-β signaling is thought to activate resident cardiac fibroblasts, leading to excessive fibroblast proliferation, cardiac fibrosis, and stiffening of the heart through excessive deposition of extracellular matrix. There is ongoing discussion that physiologic growth and pathologic hypertrophy of cardiomyocytes represent different pathways [20,21]. Moreover, there may be a developmental stage specific (embryonic vs. neonatal/adult) difference in the cardiomyocyte response to Tgf-β signaling.

Intriguingly, pathological alterations to Tgf-β signaling have been implicated in the development of HLHS in humans. Specifically, the expression levels of Tgf-β pathway genes were lower in the right ventricles of HLHS patients compared to tissue samples from left and right ventricles of patients without heart disease [15]. Right ventricular tissue of HLHS patients experiences more stretch since these right ventricles have to pump the blood volume that is normally handled by both ventricles. Conversely, a recent report shows that left ventricular tissue from fetal HLHS patients, which experiences decreased stretch, has increased expression and activation of Tgf-β1 [16]. Indeed, data from stem cell experiment provide evidence that activated Tgf-β signaling inhibits cardiomyogenesis [22–24]. Furthermore, data from cultured neonatal rat cardiomyocytes suggest that Tgf-β signaling inhibits cardiomyocyte proliferation *in vitro* [25,26]. Together, these data support the concept that downregulation of Tgf-β signaling could play a role in proper cardiac development, and that pathological deregulation of this signaling pathway during development may drive the pathogenesis of HLHS.

Given these data, we chose to examine the Tgf-β pathway in detail to better understand the mechanism of biomechanical stretch. Herein, we observed that stretch stimulation was sufficient to decrease Tgf-β family member expression, protein levels, and signaling *in vitro*. EMCM size was increased when Tgf-β signaling was inhibited. In contrast, activation of Tgf-β signaling led to decreased proliferation, contractile force, and beating frequency of EMCMs. Bioinformatic analyses predicted SMAD3 binding sites within 5 kb the transcriptional start sites of the majority of stretch responsive genes that are responsible for myofibrillogenesis and normal heart development. Taken together, our findings presented in this study demonstrate that the *in vitro* stretch of EMCMs can serve as a model to study the contribution that biomechanical stimuli play in normal cardiac development and in the development HLHS.

2. Methods

2.1. Embryonic mouse cardiomyocyte (EMCM) culture

EMCMs were harvested from E16.5 wild-type Swiss Webster mouse embryos (Charles River) using standard methods [27] under an IACUC-approved protocol.

2.2. Biomechanical EMCM stretch analyses

EMCMs were grown on Collagen-I-coated Bioflex plates (BF-3001C Flexcell International). EMCMs were concurrently exposed to cyclic stretch of 16% at 1 Hz for 24 h using a Flexcell FX-5000 Tension system (Flexcell International), and to static condition (control) on Bioflex plates.

2.3. RNA extraction

RNA was extracted from the EMCMs using the RNeasy Mini kit (Qiagen). The concentration of RNA was determined at 260 nm using ND-1000 (Nanodrop) and all RNA was assessed for integrity using an Agilent 2100 Bioanalyzer. Only samples with a RNA Integrity Number of >8 were used for mRNA sequencing experiments.

2.4. Library construction

Libraries were generated from three biologic replicates for each condition. Purified RNA was sheared and used to prepare an Illumina sequencing library using the Illumina TruSeq™ RNA Sample lit (Illumina,