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Graphical abstract

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miR-486 is modulated by stretch and increases ventricular growth

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Abstract

Perturbations in biomechanical stimuli during cardiac development contribute to congenital cardiac defects such as Hypoplastic Left Heart Syndrome (HLHS). This study sought to identify stretch-responsive pathways involved in cardiac development. microRNA (miRNA)-Sequencing identified miR-486 as being increased in cardiomyocytes exposed to cyclic stretch in vitro (63%, p<0.002). The right ventricles of HLHS patients experience increased stretch and have a trend towards higher miR-486 levels 4.9-fold (p=0.08). Sheep RVs dilated from excessive pulmonary blood flow have 60% more miR-486 vs. control RVs (p<0.05). The left ventricles of newborn mice treated with miR-486 mimic are 16.9%-24.6% larger (p<0.01) and display 2.48 fold increase in cardiomyocyte proliferation (p<0.01). miR-486 treatment decreases FoxO1 and Smad signaling, while increasing the protein levels of Stat1. Stat1 associates with Gata4 and Serum Response Factor (Srf), two key cardiac transcription factors whose protein levels increase in response to miR-486. This is the first report of a stretch-responsive miRNA that increases the growth of the ventricle in vivo.
Introduction

Biomechanical stretch stimuli are critically important in the heart, where ventricular cardiomyocytes are stretched every heartbeat during diastolic filling. Disruption of biomechanical stretch \textit{in utero} can result in severe cardiac defects such as Hypoplastic Left Heart Syndrome (HLHS). Patients with foramen ovale or mitral/aortic valve stenosis have impaired filling and/or emptying of the left ventricle (LV) \textit{in utero}, and frequently develop hypoplastic LVs that are unable to support the postnatal systemic circulation (Figure 1)(1-3).

Given poor outcomes and challenges associated with the current management of HLHS(4), there is an unmet need to understand the etiology of perturbed growth \textit{in utero} and to identify small molecules that improve LV growth in HLHS patients. However, while existing animal models(5-11) and computational studies(12) support the theory that HLHS results from perturbed biomechanical stimuli \textit{in utero}, there is a paucity of data regarding molecular responses to biomechanical stretch.

microRNAs (miRNAs) are small non-coding RNAs that bind mRNAs to attenuate mRNA’s translation and/or stability, thereby modulating protein levels of the target genes. To our knowledge, only two publications(13, 14) have reported unbiased miRNA profiling of cardiac cells exposed to stretch. In this report, we demonstrate that miR-486 is a stretch-responsive miRNA that is sufficient to increase ventricular growth, cardiomyocyte proliferation, and Stat1 levels \textit{in vivo}. miR-486 is enriched in striated muscle(15-17) but little is known about its role in the heart. We have identified miR-486 as being upregulated by stretch \textit{in vitro, in vivo}, and it trends to be increased in the RVs of HLHS patients. Specifically, an unbiased miRNA profiling of embryonic mouse cardiomyocytes exposed to cyclic stretch \textit{in vitro} demonstrated that miR-486 levels were increased by stretch as compared to static controls. This upregulation by stretch was confirmed by qPCR on samples from dilated sheep RVs as compared control RVs. There
was also a trend of increased miR-486 levels in HLHS RVs, which experience increased stretch, as compared to controls. *In vitro*, cardiomyocytes treated with miR-486 were more contractile than scramble treated controls, and displayed reduced levels of FoxO1 and Smad2/3 known targets for this miRNA (18-20). Increasing miR-486 in newborn mice for three days was sufficient to significantly increase ventricular growth and cardiomyocyte proliferation. Moreover, proteomic screening of miR-486 treated murine hearts as compared to control hearts identified Stat1 as one of the most upregulated proteins by miR-486 treatment. Given these data, we propose a novel molecular mechanism by which miR-486 promotes stretch-responsive cardiac growth.
Results

miR-486 levels are increased in response to stretch in vitro, in patient samples, and in an animal model of ventricular dilation.

To identify stretch-responsive miRNAs in an unbiased manner, we performed miRNA-Seq on RNA isolated from embryonic mouse cardiomyocytes exposed to cyclic stretch in vitro (16% at 1 Hz for 24 h) vs. static controls. miRNA-Seq identified 34 miRNAs as being stretch responsive based upon a FDR < 0.05 (Table 1). 11 of these miRNAs were upregulated by stretch while the remaining 23 were downregulated.

These data were compared to miRNA profiling, performed by miRNA qPCR array, of RV samples from neonatal HLHS patients undergoing cardiac surgery. In fetal and newborn HLHS patients, the RV experiences increased stretch since it is providing both the pulmonary and systemic blood flows. RVs of HLHS patients generate the systemic blood pressure, which is significantly higher than pulmonary arterial pressures. Hence, HLHS RVs are increasingly stretched due to the elevated pressure load. Increased blood volume and pressure load causes the RV to become stretched and dilated (Figure 2A). As a result of this physiology, RV samples from neonatal HLHS patients can provide insight into the stretch-responsive molecular changes that occur in human hearts. miRNA qPCR array analysis was performed on RV samples from HLHS patients removed during the initial surgical palliation, which can involve placement of a shunt connecting the right ventricle (RV) to the pulmonary artery (PA), as compared to control RV tissue. For several years, albeit not current practice, the surgeon would remove a small piece of the RV in order to connect the RV/PA shunt to the RV; therefore, there were a limited number of HLHS patient samples available for the miRNA analysis. Given the small sample size (N=3 for both groups), we focused on miRNAs that were changed with a p-value < 0.1. miR-486
was the only miRNA that was modulated by stretch in the same direction in the patient HLHS RV compared to control RV qPCR array dataset as the *in vitro* miRNA-Seq data. Specifically, there was a trend for miR-486 levels to be 4.9-fold higher in the HLHS RVs than in control RVs (p=0.08, average control cQ=9.46 vs average HLHS cQ=7.17; Figure 2A, Supplemental Data Table 2). Furthermore, we decided to investigate miR-486 because it has been demonstrated to have a role in striated muscle(15-17) and because of its role in attenuating TGF-β signaling(16, 21), which has been shown to repress stretch-mediated cardiomyocyte proliferation and growth(22).

To confirm if stretch was sufficient to increase miR-486 levels *in vivo*, we compared miR-486 levels in control and dilated sheep RVs. Sheep with an aorto-pulmonary shunt develop RV dilation secondary to elevated pulmonary artery pressures(23). This dilation results in increased stretch of the RV cardiomyocytes. Shunted RVs have 60% more miR-486 as compared to sham operated RVs (p<0.05; average control cQ=-7.52 vs average shunt RV cQ=-8.2; Figure 2B). Based upon our *in vitro*, patient, and sheep data—we determined that miR-486 levels are increased by stretch.

**miR-486 is sufficient to increase cardiomyocyte contractility *in vitro***

In order to examine the effects of increased miR-486 levels on cardiomyocyte contractility *in vitro*, we transfected confluent embryonic mouse cardiomyocyte (EMCM) cultures with either miR-486 mimic or scramble control (BlockIt) and quantified their beating motion using in-house image analysis methods (Figure 3A)(24). On average, the temporal profiles of contractility derived from these measurements (*D(t)*, Figure 3B) experienced similar temporal dynamics but the miR-486 profiles (N=19) were above control ones (N=14). Statistical analysis of these data showed that miR-486 treated EMCMs generated 35% higher peak contractility (p=0.003; Figure 3C) and 52% higher average contractility (p=0.002; Figure 3D) than controls. Beating periods
and contraction times for miR-486 treated EMCMs however remained unchanged (Figures 3E and 3F; p=0.51 and p=0.31 respectively). Overall, these data demonstrate that miR-486 can improve the contractile function of cardiomyocytes.

**miR-486 is sufficient to increase left ventricular growth and cardiomyocyte proliferation in vivo in newborn mice**

To examine effects of increased miR-486 levels on the heart in vivo, we treated newborn mice with a systemically delivered miR-486 mimic or scramble control (CTL, BlockIT). Echocardiographic data (Figure 4) demonstrated that miR-486 treated mice (N=9) had a 16.9% larger end-diastolic LV internal diameter (LVIDd, 1.52 mm vs 1.30 mm, p<0.01) and a 24.6% larger end-systolic LV internal diameter (LVIDs, 0.71 mm vs 0.57 mm, p<0.01) than controls (N=7) 3 days after treatment. In addition, end-diastolic diameter/posterior wall dimensions (EDD/PWD) were 20.7% larger in miR-486 mice (3.61 vs 2.99 p<0.01), and end-diastolic LV dimension/body weight ratio was increased by 12.5% (0.36 vs 0.32 p<0.05). Calculated LV mass was increased by 17.7% (10.36 mg vs 8.80 mg p<0.05). Calculated LV mass to body mass ratio was also increased in miR-486 treated mice by 15.4% (2.47 mg/gm vs 2.14 mg/gm, p=0.05). There were no significant differences in ventricular wall thicknesses (LVPWd), heart rate, or fractional shortening (%FS) between the two groups (Figure 4; Supplemental Table 3). The observed increases in LV size without changes in wall thicknesses support the finding that increasing miR-486 is sufficient to increase ventricular growth in vivo.

**miR-486 promotes cardiomyocyte proliferation in vivo**

In order to study the cardiomyocyte proliferation rate in miR-486 hearts, we stained cardiac sections for phalloidin, DAPI, and phospho-histone h3 (p-H3 S10). Phalloidin staining allowed us to identify cardiomyocytes based the actin-staining pattern. DAPI was used to identify the nuclei. Phospho-histone h3 staining demarcated proliferating cells. Histological examination of
the hearts demonstrated that the miR-486 treated mice had a 2.48 fold increase in cardiomyocyte proliferation (2.75% vs. 1.11% p=0.002, N=3; Figures 4H and 4I).

**miR-486 increases Stat1 protein levels *in vivo* and *in vitro***

We performed iTRAQ-based mass spectrometry proteomics(25) to compare protein samples from miR-486 mimic and scramble control (BlockIT) treated mouse hearts. We identified 116 proteins as being modulated (73 upregulated, 43 downregulated) by miR-486 mimic treatment with p<0.2 (Figure 5A, Supplemental Data Table 4). Gene ontology (GO-term) enrichment analysis identified muscle fiber development (-log10(p) = -7.39) and striated muscle contraction (-log10(p) = -7.22) as among the most significant GO-terms modulated by miR-486 treatment (Figure 5B). We focused on Stat1, given it was one of the most upregulated proteins in the iTRAQ data (Figure 5A and Table 2) and literature indicating stretch stimulation of cardiomyocytes activates Stat1 signaling(26, 27).

We confirmed that miR-486 is sufficient to increase Stat1 protein levels by immunoblotting on total protein extracted from treated mouse hearts (Figure 5C). Since phosphorylation of Stat1 is key for the activity of the protein and transcription of target genes(28, 29), we performed immunoblot analysis for p-Stat1(Ser727). Indeed, miR-486 treated hearts displayed increased p-Stat1(Ser727) compared to scramble treated controls (BlockIT). Of note, we examined the protein levels for Stat3 and Jak1 since canonically Stat1 is part of a signaling cascade involving Jak1/Stat3. Interestingly, there was no change in total Stat3 or Jak1 levels (Figure 5C). Finally, to test if increased Stat1 levels originate from cardiac myocytes or other tissue types present in hearts, we isolated and transfected neonatal mouse cardiomyocytes with miR-486 mimic or scrambled control (BlockIT). Similar to our *in vivo* data, transfected cardiomyocytes also significantly increased total Stat1 (1.74 ± 0.1249, N=3, p=0.006) and p-Stat1 (2.425 ± 0.3063, N=3, p=0.03) levels after 3 days (Figure 5D and 5E). To further elucidate Stat1’s potential role in
modulating the transcriptional response to biomechanical stretch, we examined for enrichment of predicted Stat1 binding sites within 5kb of the genes activated by stretch in vitro and in HLHS patient samples. oPOSSUM analysis(30) of the genes upregulated by cyclic stretch in vitro (FDR < 0.05, >1.2 fold increase) (22) showed 37.6% (384/1020 genes) were predicted to have at least one Stat1 binding site (z-score 3.4, Fisher score of 49.7) (Figure 5F; left panel). Similarly, 36.1% of the genes upregulated in HLHS RVs(31) were predicted to have at least one STAT1 binding sites (z-score for Stat1 was 8.9 with a Fisher score of 14.8; Figure 5F; right panel).

miR-486 represses Tgf-β/Smad signaling.

Prior reports from our group demonstrated that TGF-β signaling is repressed in EMCM stimulated via cyclic stretch(22), and in RVs of HLHS patient ventricles that experience increased stretch compared to controls(32). TGF-β and Stat1 pathways can act in a repressive manner on each other(33-35) and part of this negative feedback loop involves TGF-β repression of total Stat1 protein levels(36). Moreover, published data have implied a negative feedback circuit between miR-486 and Tgf-β/Smad(16, 21). We sought to further elucidate this mechanism, and the interplay between Tgf-β/Smad, Stat1 and miR-486.

First, we examined the feedback between miR-486 and Tgf-β/Smad in cardiomyocytes. Cardiomyocytes treated with miR-486 mimic have significantly lower Smad2 (0.71 ± 0.08, N=3, p=0.03) and Smad3 (0.69 ± 0.06121, N=3, p=0.04) levels as compared to scramble treated controls (Figure 6A and 6B). These data are consistent with recent reports describing direct regulation of Smad2/3 by miR-486 (19, 20). In order to examine if Tgf-β/Smad signaling is involved in the stretch-mediated increase in miR-486 levels, we treated EMCMs with TGF-β2 during cyclic stretch. TGF-β2 treated EMCMs had 53.3% less miR-486 as compared to untreated cells exposed to cyclic stretch (p<0.05, average control cQ=0.22 vs average TGF-β2
treated EMCM cQ=1.32; Figure 6C). These data suggest that the stretch-repression of Tgf-β/Smad signaling results in increased miR-486 expression. Cardiomyocytes treated with TGFβ2 have decreased p-Stat1 S727 levels (0.44 ± 0.06, N=3, p=0.0099) levels as compared to untreated controls (Figure 6D and E).

miR-486 increases Gata-4 and Srf levels.

Besides repressing Tgf-β/Smad signaling, biomechanical stretch also activates cardiac transcription factors Srf (22, 37) and Gata-4 (38) that have key roles in promoting cardiomyocyte proliferation and growth (39-42). Given that stretch increases miR-486 levels, we tested if miR-486 alone may positively modulate Srf and Gata-4. Indeed, treatment of cardiomyocyte cultures with miR-486 increased protein levels of Srf (1.658 ± 0.046, N=3, p=0.0019) and Gata-4 (1.573 ± 0.1603, N=3, p=0.047) compared to BlockIT treated controls (Figure 7A).

Stat1 interacts with Gata4 and Srf in vivo.

Stat1 has been shown to directly interact with Gata-4 and synergistically activate cardiac gene expression with Srf (41, 43, 44). We aimed to validate if Stat1 associates with Gata-4 in vivo, and test for additional association with Srf. Co-immunoprecipitation experiments using endogenous Srf, Gata-4 and Stat1 demonstrate association of the transcription factors in cardiac lysates (Figures 7B and 7C), suggesting that Stat1 may form complexes with Srf and/or Gata-4 to modulate cardiac gene expression.
Discussion

Biomechanical stretch stimuli are important in the development and pathophysiology of the heart because cardiomyocytes experience stretch during diastolic filling of the cardiac chambers. The role of biomechanical stretch in cardiac development is underscored by congenital cardiac defects such as HLHS, which result from perturbed biomechanical loading of the ventricle in utero. The driver of this pathology is impaired filling and/or emptying of the LV during cardiac development. Based upon fetal echocardiographic data, patients with narrowed or obstruction of the foramen ovale, mitral valve, and/or aortic valve frequently develop HLHS (1-3). Embryonic sheep, chicken and zebrafish models with decreased ventricular filling also develop ventricular hypoplasia (5-11). These data, along with finite element modeling of human fetal left ventricular development (12) support the theory that HLHS is the result of perturbed biomechanical stimuli in utero. However, molecular responses to biomechanical stretch are poorly understood. Therefore, it has not been possible to develop non-invasive treatment modalities for this cardiac syndrome, which is associated with substantial morbidity and mortality, 1-year transplant-free survival is 68.7% (4). This study shows for the first time that the stretch-responsive miRNA miR-486 is sufficient to promote ventricular growth, demonstrates that this miRNA is sufficient to increase ventricular growth in vivo, and shows that it is a novel activator of Stat1 expression. These findings provide evidence that miRNAs could be used as a novel treatment to improve the ventricular growth in patients born with ventricular hypoplasia and thereby altering the treatment paradigm for these challenging patients.

Diastolic filling stretches the ventricular cardiomyocytes, thereby increasing expression and signaling of mechanical activated transcription factors Srf (22, 37) and Gata-4 (38). Srf has been shown to control miR-486 transcription (15), and our data suggest that miR-486 in turn may
indirectly enhance expression of Srf and Gata-4 through increased contractility and biomechanical stretch in a positive feedback loop (Figure 7D). miR-486 forms a negative feedback loop with Tgf-β/Smad signaling(16, 21), partially by directly acting on Smad2/3 and suppressing their expression(19, 20). This feedback loop may help explain our previous findings that stretch represses Tgf-β/Smad signaling \textit{in vitro} and in HLHS RVs(22, 32). miR-486 mediated decrease in Tgf-β/Smad signaling releases the TGF-β repression on Stat1 expression(36), which may explain how miR-486 increases Stat1 protein levels. The baseline Tgf-β/Smad activity in the cardiomyocytes may account for why we did not demonstrate signification repression of Stat1 levels in the TGFβ2 treated cells (Figure 6D and 6E). Stat1 may promote proliferation via its association with Gata4 and Srf transcription factors (Figure 7B and 7C). Indeed, these results support previous findings that show direct association of Stat1 with Gata4, which synergistically activates cardiac gene expression together with Srf(43), promoting cardiomyocyte proliferation and growth(39-42). Taken together, we propose a mechanism that outlines how stretch may modulate miR-486 expression to promote cardiomyocyte proliferation and ventricular growth (Figure 7D).

Besides Smad2/3(19, 20), there are some reports in the literature describing other direct targets for miR-486. Specifically, FoxO1 (18, 45) and PTEN (17, 46) were shown to be direct miR-486 targets in various cancer cell lines and tissues other than the heart. However, very little is known whether these targets are also regulated by miR-486 in heart and cardiomyocytes. When investigated in our miR-486 transfected cardiomyocyte cultures, FoxO1 protein levels were significantly downregulated (Supplemental Figures 1A and 1B), while PTEN was slightly decreased without reaching statistical significance (p=0.18). These data support the efficacy of our miR-486 transfection in cardiomyocytes and that its effects are comparable to previous reports on direct targets for this miRNA.
Mechanistically, we focused on Stat1 because it was among the most upregulated proteins in the miR-486 treated hearts as compared to scramble control treated hearts. Although not being predicted as a direct target of this miRNA, Stat1 has been previously reported as being stretch responsive\(^{(26, 27)}\), similar to miR-486. miR-486’s attenuation of TGF-β may release the TGF-β-mediated repression of Stat1 expression. Furthermore, bioinformatic analyses of the 5kb flanking the genes upregulated in cardiomyocyte exposed to cyclic stretch \textit{in vitro} and the RVs of HLHS patients showed significant enrichment of STAT1 binding sites. These data indicate that STAT1, in conjunction with important cardiac transcription factors such as Gata-4 or Srf, plays a key role in the stretch-mediated increase of important genes involved in cardiac growth.

While there have been some efforts to identify stretch-responsive miRNAs in cardiomyocytes\(^{(14, 47, 48)}\), this report identified stretch-responsive miRNAs based upon the correlation of \textit{in vitro} miRNA-Seq dataset with data from patients and an animal model of increased ventricular stretch. We focused on miR-486 because of its role in striated muscle\(^{(15-17)}\) and modulation of TGF-β signaling\(^{(16, 21)}\), which represses proliferation, growth and contraction of cardiomyocytes\(^{(22)}\). Additionally, several miRNAs—miR-10a, miR-19a/b, miR-99b, miR-208, miR-335, miR-412, and miR-429—were significantly modulated by stretch \textit{in vitro} and were changed in the same direction with a \(p\)-value between 0.1 and 0.2 in the HLHS RV qPCR array (Supplemental table 2). Of note miR-99b has potential of being a stretch responsive miRNA worth studying more detail since it also is up in the RVs of shunted sheep\(^{(49)}\). It is possible that a larger number of patient samples would have allowed us to identify some of these miRNAs as being stretch modulated \textit{in vivo}. However, the number of patient samples in our study was limited due to improvements in the surgical palliation of HLHS, by which RV tissue is no longer removed from the patient. As a result, it is not feasible at this time to expand the number of similar neonatal HLHS RV samples.
Echocardiographic data demonstrates that miR-486 is sufficient to increase left ventricular growth *in vivo*. These data are consistent with growth instead of ventricular dilation given that the LV internal dimensions at the end of systole and diastole as well as EDD/PW were increased while the ventricular wall thicknesses were unchanged. Indeed, based upon the increased cardiomyocyte proliferation seen in the miR-486 treated mice, it appears that at least a component of the ventricular growth is the result of increased number of cardiomyocytes. While a number of miRNAs have been implicated as modulators of ventricular hypertrophy (50-54), the finding that miR-486 is sufficient to increase the growth of the ventricles *in vivo* is novel. Furthermore, miR-486 treated mice did not have altered heart rate or systolic function as compared to controls, supporting that miR-486 promotes LV growth and not LV dilation. While our *in vitro* data supported the *in vivo* finding that miR-486 treatment does not cause chronotropic alterations, it demonstrated that miR-486 treatment increased cardiomyocyte contractility even if we could not observe improved LV systolic function *in vivo*. This partial discrepancy is due to both the miR-486 mimic and scramble control mice having robust systolic function so that the LV walls touch during systole (Figure 4A). Therefore, it would not be possible to discern any potential improvement in shortening fraction in response to miR-486 treatment.

Our mass spectrometry proteomics of hearts treated with miR-486 also identified a possible reduction in sarcomeric proteins titin, myosin (Myh6) and myosin light chain (Myl3) (Figure 5A). However, analysis of sarcomeric protein levels in whole cardiac lysates from miR-486 treated mice did not reveal any significant reduction in the expression of titin, myosin, α-actinin-2, troponin-I or cardiac actin (Supplemental Figures 1C and 1D). The contradictory results between our proteome and immunoblot analyses are likely due to a methodological bias stemming from the necessary depletion of acto-myosin components prior to mass-spectrometry
that allowed for detection of less abundant non-structural proteins in our cardiac lysates. This depletion may have more significantly affected titin and its sarcomeric binding partners including myosin heavy chain 6 (Myh6) (55-57) and its associated myosin light chain (Myl3) (58) in the iTRAQ preparation, as there appears to be a trend towards increased overall titin levels in miR-486 hearts (Supplemental Figure 1C). Moreover, expression of the larger and more compliant N-2BA isoform of titin that would allow for better diastolic filling of the heart seems to be increased. The N-2BA isoform includes additional spring-like elements, like an enlarged PEVK-region through alternative splicing (59). This ratio shift towards more compliant titin isoforms is similar to what we have previously reported occurring in cardiomyocytes exposed to cyclic stretch in vitro (22). Based upon the immunoblot data, we do not believe that miR-486 decreases titin levels and levels of other associated sarcomeric proteins.

Our data suggest that modulation of miRNA levels, such as miR-486, in vivo may be a potential innovative approach to increase ventricular growth in patients with ventricular hypoplasia as an alternative to the current surgical palliation for single ventricle patients. Since HLHS patients have decreased expression of proliferation related genes (60) and animal models for HLHS were also shown to have decreased cardiomyocyte proliferation (7, 8), efforts to improve cardiomyocyte proliferation may improve the size of the LVs in HLHS patients thereby improving their clinical course. Given the pressing need to develop new treatment modalities, part of our future efforts will be to determine if increased miR-486 is sufficient to increase ventricular growth in animal models of ventricular hypoplasia.
Methods

Mouse cardiomyocyte cultures
Cardiomyocytes from hearts of wildtype embryonic (E16.5) or newborn mice (CD1 background, Charles River) were isolated and cultured as previously described (61, 62) under IACUC-approved protocols.

Biomechanical stretching of cardiomyocytes
Embryonic mouse cardiomyocytes (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 24h using a Flexcell FX-5000 Tension system (Flexcell International), and to static condition (control) on Bioflex plates(22).

RNA Extraction
RNA was extracted from the EMCMs using the RNeasy Mini kit (Qiagen). RNA concentration was determined at 260 nm using ND-1000 (Nanodrop) and RNA integrity assessed using an Agilent 2100 Bioanalyzer.

miRNA-Sequencing
1 µg of total RNA from six stretched and six static EMCM samples were used for small RNA library preparation using the TruSeq® Small RNA protocol (Illumina, San Diego, CA, USA) similar to methods that we have previously used(13). All RNA was validated using an Agilent Bioanalyzer and only samples with an RNA integrity number of >8 were used. Illumina adapters were ligated to each end of the RNA molecule and a reverse transcriptase reaction was used to create single stranded cDNA. The cDNA was subsequently PCR amplified using a universal primer and a primer containing one of 48 Illumina index sequences.
The small RNA libraries were loaded onto the Illumina cBot Cluster Station where they bind to complementary adapter oligos grafted onto a proprietary flow cell substrate. Isothermal amplification of the cDNA construct was carried out creating clonal template clusters of approximately 1,000 copies each. The Illumina HiSeq2500 directly sequences the resulting high-density array of template clusters on the flow cell using sequencing by synthesis (SBS). Four proprietary fluorescently labeled, reversible terminator nucleotides were utilized to sequence the millions of clusters base by base in parallel.

For deep-sequencing reads produced by the Illumina HiSeq2500, low quality reads were filtered out to exclude those most likely to represent sequencing errors and adaptor sequences were subsequently trimmed into clean full-length reads formatted into a non-redundant Fasta format. The occurrences of each unique sequence reads were counted as sequence tags (the number of reads for each tag reflects relative expression level) and only small RNA sequences of 18 to 30 nt were retained for further analysis. The miRNA-Seq data has been archived at GEO (GSE120676).

All unique sequence tags that passed above filters were mapped onto the reference mouse genome using the Bowtie2 program(63). The hits were counted using in-house scripts written in Perl, and then translating the RNA ID’s to gene ID’s using the NCBI database. All counts that came from the same gene under that gene ID were added. The edgeR algorithm implemented under the Bioconductor suite was used to obtain expression levels and significance (p-values, q-values)(64).

Human miRNA profiling

The HLHS patient and control ventricular samples were obtained under an IRB approved protocol that has been previously described in detail(32). HLHS RV samples were obtained from
neonatal patients at the time of the RV to pulmonary artery shunt placement. The control RV tissue was obtained from infants (oldest was 135 days old) who died from non-cardiac causes. We used the Taqman Array Human MicroRNA from Applied Biosystems (A v2, V v3). cDNA was generated from 500ng Trizol extracted tRNA using the Megaplex RT Primers and Taqman MicroRNA Reverse Transcription Kit from Applied Biosystems. Human (cDNA kit) from Applied Biosystems. 50ng of miRNA RT for each miRNA qPCR reaction. Samples were normalized to a U6 endogenous control and then used to calculate the $2^{- \Delta \Delta CT}$ values to determine the relative fold changes.

**Sheep RV**

Late gestation fetal sheep had an aortopulmonary vascular graft placed as previously described (65). Four-six weeks after spontaneous delivery, these lambs have biventricular hypertrophy compared to twin controls (66). All procedures were done with IACUC approval.

**miRNA qPCR.**

RNA was isolated using RNAeasy kit (Qiagen). qPCR was performed using Taqman primers. For the sheep samples, miR-103 was used for normalization. For the EMCMs, we used U6 as the endogenous control.

**TGF-β2 treatment**

EMCMs or neonatal mouse cardiomyocytes were treated with 1ng/ml of TGF-β2 (R&D Systems) and then exposed to stretch conditions for 24 h, or to study long term effects cultured in static conditions for 3 days, with daily changes of medium supplemented with 1ng/ml of TGF-β2 before analysis. Untreated cardiomyocytes were used as controls.
miRNA transfection.

Neonatal mouse cardiomyocytes were transfected with either miR-486-5p mimic (Ambion #MC10546, Invitrogen) or scramble control (BlockIT #14750-100; Life Technologies) using Escort III (Sigma-Aldrich) (67) according to the manufacturer's instructions. Cells were harvested for protein analysis or processed for immunofluorescence staining 72 h after transfection.

Contractility assays using dynamic monolayer force microscopy (DMFM)

EMCMs were transfected with either miR-486 mimic (Invitrogen) or scramble control using Lipofectamine 2000(67). Contractility was assessed 48 h after transfection as previously described(24). Phase-contrast image acquisition was performed at 20Hz using a spinning-disk confocal microscope with 20X magnification. Time-dependent cell deformation vector maps with respect to diastolic relaxation, \( u(x, y, t) \), were derived by in-house particle image velocimetry scripts written for MATLAB (Natick, MA). Suitable reference frames representing EMCM diastolic relaxation were selected by performing PIV on consecutive frames to identify the frames with minimal cell velocity. The divergence of \( u(x, y, t) \) is a non-dimensional variable that quantifies relative changes in area, i.e. contractility, of the EMCMs in the field of view every time the beat. Likewise, its square root, i.e. \( \sqrt{|u(x, y, t)|} \) could be interpreted as a measure of cardiomyocyte shortening during contraction. The spatial average of the divergence, \( D(t) = \frac{1}{lxly} \int_0^{lx} \int_0^{ly} |\nabla \cdot u(x, y, t)| dx dy \), was calculated to obtain temporal tracings of cellular contractility for each experiment. These contractility vs. time signals were analyzed to calculate chronotropic and inotropic parameters for quantitative and statistical comparisons of samples, such as peak cycle period, \( T_{cycle} \), the duration of cell contraction within each beating cycle, \( T_{contract} = \)
\[ \int_0^{T_{\text{cycle}}} H(t) \, dt \] where \( H(t) = 1 \) if \( D(t) > 0.1 \, D_{\text{peak}} \) and zero otherwise, peak contractility \( D_{\text{peak}} = \max(D(t)) \), and mean contractility \( D_{\text{mean}} = \frac{1}{T_{\text{cycle}}} \int_0^{T_{\text{cycle}}} D(t) \).

In vivo injections

Newborn mice were injected intraperitoneal with either 10\( \mu \)g of miR-486-5p miRNA mimic (Ambion #MC10546, Life Technologies) or scramble control (BlockIT #14750-100; Life Technologies) in combination with in vivo Jet PEI (Polyplus transfection #201-10G), according to the manufacturers’ instructions. Three days following the injection, cardiac functions and morphological parameters of control, BlockIT and miR-486 injected neonatal mice were recorded, before isolation and processing of tissues for further analysis.

Echocardiography

For analysis of cardiac functions, postnatal mice were measured by trans-thoracic echocardiography using a FUJIFILM VisualSonics SonoSite Vevo 2100 ultrasound system with a 32-55-MHz linear transducer. Fractional shortening was used as an indicator of systolic cardiac function. In addition, heart rate (HR), interventricular septal thickness during diastole (IVSd), the left ventricular internal dimension in diastole and systole (LVIDd and LVIDs), the left ventricular posterior wall thickness (LVPWd) during diastole were measured and analyzed as described previously(68, 69).

Immunofluorescence staining and analyses

Acetone fixed cardiac cryosections were processed for immunofluorescence staining as described earlier(70). Briefly, sections were permeabilized with 0.2% Triton-X100 in 1x PBS for 5 min at room temperature, followed by blocking of unspecific binding sites by 5% donkey serum and 1% BSA dissolved in gold buffer (155 mM NaCl, 2 mM EGTA, 2 mM MgCl2, 20 mM...
Tris-HCl, pH 7.5) for a minimum of 1 h. After blocking, tissue sections were incubated with primary antibodies diluted in gold buffer over night at 4° C. Following incubation, specimens were washed three times with 1x PBS at room temperature, before incubation of with appropriate secondary antibodies diluted in gold buffer for 1 h at room temperature. Subsequently, sections were washed three times with 1x PBS and mounted using fluorescent mounting medium (DAKO). Immunofluorescently labeled specimens were imaged using a Fluoview 1000 confocal microscope (Olympus), in sequential scanning mode using 10x air or 40x oil objectives and zoom rates between 1x and 3x. Image analysis was performed using ImageJ (NIH).

**Antibodies**

The following primary antibodies were used for immunofluorescence and immunoblot analyses as well as co-immunoprecipitation experiments: Stat1 (Cell Signaling; #9172), p-Stat1 S727 (Cell Signaling; #9177), p-Stat1 Y701 (Cell Signaling; #9167), Smad2/3 (Cell Signaling; #5678); Smad3 and p-Smad3 (Cell Signaling(22)), SRF (Cell Signaling; #5147), Gata-4 (Cell Signaling; #36966 or Developmental Studies Hybridoma Bank [DSHB]; CRP-GATA4-1A7, deposited to the DSHB by Protein Capture Reagents Program, produced by JHU/CDI), p-Histone H3 S10 (Cell Signaling; #3377), JAK1 (Cell Signaling; #3332), STAT3 (Cell Signaling; #9404), GAPDH (Santa Cruz Biotechnology; sc-32233), titin T11 (Sigma Aldrich; T9030), sarcomeric myosin (DSHB; A4.1025, deposited to the DSHB by Blau, H.M.), troponin-I (Cell Signaling; #4002), sarcomeric α-actinin-2 (clone EA-53; Mob 227-05; Diagnostic Biosystems), cardiac actin (Progen Biotechnik; Ac1–20.4.2), PTEN (Cell Signaling; #9188), FoxO1 (Cell Signaling; #2880), normal rabbit IgG (Santa Cruz Biotechnology; sc-2027), normal mouse IgG (Santa Cruz Biotechnology; sc-2025). Secondary fluorescence dye-linked or horseradish peroxidase-linked antibodies were obtained from Jackson ImmunoResearch, DAKO, Cell Signaling or Santa Cruz Biotechnology.
Fluorescently labeled phallolidin and DAPI was purchased from Molecular Probes (Life Technologies).

**Proteomics**

Proteins were isolated from whole hearts and lysed into ice-cold isolation buffer (300 mM KCl, 30 mM PIPES pH 6.6, 0.5% NP-40, 1x protease inhibitor (Roche), 1x phos-stop (Roche). Insoluble proteins were removed by centrifugation (14,000 rpm, 10 min at 4°C), and the supernatant was diluted 1:4 with ice cold dilution buffer (1x Phos-stop (Roche), 0.5% NP-40, 1mM DTT). Precipitation of acto-myosin components was done by centrifugation (14,000 rpm, 15 min at 4°C), and the remaining supernatant snap-frozen for further analysis by mass-spectrometry and immunoblot analyses.

Analysis and identification of peptides via mass spectrometry was done as described previously(71). Immediately prior to mass-spectrometry, protein solutions were diluted in TNE buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA). RapiGest SF reagent (Waters Corp.) was added to the mix to a final concentration of 0.1% and samples were boiled for 5 min. TCEP (Tris (2-carboxyethyl) phosphine) was added to a final concentration of 1 mM, and the samples were incubated at 37°C for 30 min. Subsequently, the samples were carboxymethylated with 0.5 mg/ml of iodoacetamide for 30 min at 37°C followed by neutralization with 2 mM TCEP (final concentration). Protein samples prepared as above were digested with trypsin (trypsin:protein ratio of 1:50) overnight at 37°C. RapiGest was degraded and removed by treating the samples with 250 mM HCl at 37°C for 1 h followed by centrifugation at 14000 rpm for 30 min at 4°C. The soluble fraction was then added to a new tube and the peptides were extracted and desalted using C18 desalting tips (Thermo Scientific).
The trypsinized samples (8 samples) were labeled with isobaric tags (iTRAQ8, ABSCIEX)(72), where each sample was labeled with a specific tag to its peptides as described in the manufacturer's instructions. Each set of experiments where then pooled and fractionated using high pH reverse phase chromatography (HPRP-Xterra C18 reverse phase, 4.6 mm x 10 mm, 5 µm particle (Waters). The chromatography conditions were as follows; the column was heated to 37° C and a linear gradient from 5-35% B (Buffer A-20 mM ammonium formate pH 10 aqueous, Buffer B-20mM ammonium formate pH 10 in 80% acetonitrile-water) was applied for 80 min at 0.5 ml/min flow rate. A total of 42 fractions of 0.5 ml volume where collected. For LC-MSMS analysis some fractions were pooled to create a final 16 pooled samples. Each of the pooled fractions were analyzed by high pressure liquid chromatography (HPLC) coupled with tandem mass spectroscopy (LC-MS/MS) using nano-spray ionization.

The nano-spray ionization experiments were performed using a TripleTof 5600 hybrid mass spectrometer (ABSCIEX) interfaced with nano-scale reversed-phase UPLC (Waters Corp. nano ACQUITY) using a 20 cm 75 µm ID glass capillary packed with 2.5 µm C18 (130) CSHTM beads (Waters Corp.). Peptides were eluted from the C18 column into the mass spectrometer using a linear gradient (5–80%) of ACN (Acetonitrile) at a flow rate of 250 µl/min for 1h. The buffers used to create the ACN gradient were: Buffer A (98% H2O, 2% ACN, 0.1% formic acid, and 0.005% TFA) and Buffer B (100% ACN, 0.1% formic acid, and 0.005% TFA). MS/MS data were acquired in a data-dependent manner in which the MS1 data was acquired for 250 ms at m/z of 400 to 1250 Da and the MS/MS data was acquired from m/z of 50 to 2,000 Da. The independent data acquisition (IDA) parameters were as follows; MS1-TOF acquisition time of 250 ms, followed by 50 MS2 events of 48 ms acquisition time for each event. The threshold to trigger a MS2 event was set to 150 counts when the ion had the charge state +2, +3 and +4. The ion exclusion time was set to 4 seconds. The collision energy was set to iTRAQ experiment setting. Finally, the collected data were analyzed using Protein Pilot 5.0 (ABSCIEX) for peptide
identifications and Peaks(73). Observed protein changes with a p-value of <0.2 were considered significant(74). Bioinformatic enrichment and pathway analysis was done using Metascape (http://metascape.org/)(75), Morpheus (https://software.broadinstitute.org/morpheus/) and the BioGRID (https://thebiogrid.org/)(76).

Co-immunoprecipitation and Immunoblot analysis

For co-immunoprecipitation experiments, hearts of adult wildtype mice were lysed into ice-cold lysis buffer (150mM NaCl, 10mM Tris-HCl pH8, 0.2% NP-40, 0.05% SDS, 1x protease inhibitor cocktail (Roche), 1x phos-stop (Roche), 1mM DTT). Samples were briefly sonicated at 4°C before centrifugation at 13,000rpm for 10 minutes at 4°C. Soluble proteins in the supernatant were transferred to fresh tubes and 1µg of antibody or normal rabbit or mouse IgG was added to the sample. Following over-night incubation of cardiac lysates with antibodies at 4°C, protein-G coupled magnetic beads (Dynabeads, Life Technologies) were added and samples were incubated on a shaker for 3 hours at 4°C. Beads were washed 3 times with ice-cold 1x PBS supplemented with 0.2% NP-40, and bound immunocomplexes and associated proteins were analyzed by immunoblot analyses.

Immunoblots of SDS-acrylamide gels or SDS-agarose gels were performed as previously described(77, 78). Briefly, protein samples lysed into sample buffer were boiled for 2 min and loaded onto 4-20% polyacrylamide gradient gels (BioRad) or SDS-agarose gels. Protein samples were run at ~120V for 1h and transferred onto nitrocellulose membranes using the wet transfer method. Protein loading levels and successful transfer onto membranes was checked via Ponceau stain and blocked in either 5% milk/TBST or 5% BSA/TBST. Membranes were then probed using primary antibodies diluted in blocking solution. Following overnight incubation at 4°C, membranes were washed three times for 5min each with TBST solution, and incubated with appropriate secondary antibodies, diluted into blocking solution for 1h at room temperature. After washing of membranes five times for 10min in TBST, membranes were developed using
SuperSignal West Pico Plus chemiluminescent reagent. Densitometry was performed as previously described(79). If not stated otherwise, reported sample sizes (N) represent biologic replicates.

**Prediction of enrichment of transcription factors bindings in stretch-responsive genes**

oPOSSUM analyses(30) were performed to identify enriched transcription factors within the 5kb flanking the genes identified by stretch in vitro and in the RVs of HLHS patients. We examined the genes upregulated in EMCMs exposed to cyclic stretch in vitro as compared to static control with false discovery rate (FDR) < 0.05 and up over 1.2-fold in mRNA-Seq data(22). In order to identify transcription binding site enrichment in HLHS patient RVs as compared to control RVs, oPOSSUM analysis was performed on the upregulated genes identified by microarray analysis with FDR < 0.05(31).

**Statistics**

For the miRNA-Seq, the edgeR algorithm implemented under the Bioconductor suite was used to obtain significance (p-values, q-values)(64). Observed protein changes with a p-value of <0.2 were considered significant(74). Unless otherwise specified, significance was determined in Excel (Microsoft) or Prism (GraphPad Software, version 7) by performing unpaired two-tailed t-Test analysis, with a p<0.05 considered as significant.

**Study approval**

The human tissue was collected under a protocol approved by the University of Miami IRB.

The mouse experiments were performed under an IACUC approved protocol at UCSD.

The sheep experiments were performed under approved IACUC protocol at UCSF.
Author contributions: S.L., I.B., and V.N. designed and performed the experiments along with preparing the manuscript. L.L., K.C. and L.H. performed some of the experiments. R.S., R.M., and J.C. del A. performed and analyzed the DMFM experiments. M.R. collected the HLHS and control patient RV tissue. J.L. performed and analyzed the miRNA qPCR-array data on the human RV tissue. N.D. performed the echocardiograms. E.B. helped to analyze data and revise the manuscript. K.P. helped analyze the echocardiographic data. R.K. and J.F. performed the sheep shunt and control surgeries and provided the sheep RV tissue. M.G. performed the iTRAQ experiment.

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References


Figure 1. Perturbations in biomechanical stretch in utero can cause HLHS. A. Drawing of a normal fetal heart. During diastolic filling, cardiomyocytes are stretched; and, they shorten during systolic contraction. B. in utero stenosis (narrowing) of the foramen ovale, mitral valve, and/or aortic valve, which are represented by the green crosses, results in impaired filling or emptying of the left ventricle (LV). Disruption of LV diastolic filling causes the LV cardiomyocytes to experience of decreased stretch as compared normal hearts. Decreased cyclic stretch impairs the proliferation and growth of cardiomyocytes. As result of the attenuation of stretch-mediated stimulation of ventricular cardiomyocytes, the patients have impaired LV growth. This perturbation in biomechanical loading results in children being born with Hypoplastic Left Heart Syndrome. C. In HLHS hearts, the patient is born with a diminutive LV that is not large enough to pump enough blood to support the patient’s body.
Figure 2. miR-486 levels are increased in HLHS patient RVs, and shunted sheep right ventricles (RVs). A. In newborn HLHS patients, the RV cardiomyocytes experience increased stretch since the RV is facing increased volume and pressure loading. Based upon qPCR array data, miR-486 levels are up 4.9-fold (corresponding to average control cQ=9.46 vs average HLHS cQ=7.17) in HLHS RVs (p=0.08 as determined by one-tailed t-Test). Full qPCR array dataset is included in supplemental data. B. Sheep with significant pulmonary overcirculation represent a useful in vivo model of increased ventricular stretch. A large unrestrictive aortopulmonary shunt is surgically implanted in late gestation fetal life. After birth, the presence of this shunt continues to expose the right ventricle to systemic-level afterload. As a result of this increased afterload, the RV dilates(66) and ventricular cardiomyocytes experience increased stretch. Shunted sheep RVs have 60% more (corresponding to average control cQ=7.52 vs average shunt RV cQ=-8.2) miR-486 as compared to sham operated RVs (p=0.049 as determined by one-tailed t-Test).
Figure 3. miR-486 increases the contractile function of cardiomyocytes in vitro. A. Instantaneous phase-contrast snapshots of confluent beating EMCMs live-stained with wheat germ agglutinin (WGA) Alexa-Fluor 488 conjugate. Left panel: scramble control. Right panel: miR-486 mimic treated cardiomyocytes. Vectors represent the cellular displacement field \( \vec{u}(x, y, t) \) measured by tracking the motion of WGA speckles. Color mapping represents the absolute value of the divergence of the displacement field, \( |\nabla \cdot \vec{u}(x, y, t)| \), which is a non-dimensional quantity that represents the relative change of cell area due to contractility. Its spatial average \( D(t) = \frac{1}{L_x L_y} \int_0^{L_x} \int_0^{L_y} |\nabla \cdot \vec{u}(x, y, t)| dx dy \), provides a temporal tracing of cellular contractility for each sample. B. Measured tracings of contractility \( D(t) \) of scramble control (red, N=14) and miR-486 mimic treated cardiomyocytes (blue, N=19). Thick lines represent the average for each group while the shaded ribbons span the SEM. C. miR-486 treated cardiomyocytes generate higher peak contractility \( D_{\text{peak}} \) as compared to scramble controls (0.014 ± 0.001 vs. 0.019 ± 0.001 s, \( p = 0.003 \) as determined by t-Test). D. miR-486 treated cardiomyocytes generate higher time-averaged contractility \( D_{\text{mean}} \) as compared to scramble controls (1.9 × 10^{-3} ± 2 × 10^{-4} vs. 2.9 × 10^{-3} ± 2 × 10^{-4} \( p = 0.002 \) as determined by t-Test). E and F. The scramble control and miR-486 cardiomyocytes have similar beating periods \( T_{\text{cycle}} \) (1.11 ± 0.06 s vs. 1.21 ± 0.13 s, \( p = 0.51 \) as determined by t-Test) and contraction time \( T_{\text{contract}} \) (0.27 ± 0.01 s vs. 0.29 ± 0.01, \( p = 0.31 \) as determined by t-Test).
Figure 4. miR-486 is sufficient to increase left ventricular growth and cardiomyocyte proliferation in neonatal mice. Echocardiograms were performed on neonatal mice three days after they were treated with miR-486 mimic or scramble control. A. Representative M-mode echocardiography images of scramble BlockIT control (CTL) or miR-486 treated neonatal mouse hearts used to calculate cardiac parameters (in B-G). B. Fractional shortening (FS) was not changed between neonatal mice treated with either BlockIT (CTL) or mir-486. C-D. LV internal dimension at the end of diastole (LVIDd; C) or in systole (LVIDs; D) were increased in the miR-486 mice by 16.9% (1.52mm vs 1.3mm; ** p<0.01 as determined by t-Test) and 24.6% (0.71 mm vs 0.57 mm; ** p<0.01 as determined by t-Test), respectively. E-F. LV posterior wall thicknesses (LVPWd; E) and interventricular septal thicknesses (IVSd; F) during diastole were unchanged between BlockIT (CTL) and mir-486 treated neonatal mice. N=7 control, N=9 miR-486 treated. G. End-diastolic diameter/posterior wall dimension (EDD/PWD) was increased by 20.7% in miR-486 mice (3.61 vs 2.99 p<0.005 as determined by t-Test). H-I. Cardiomyocyte proliferation was quantified from cardiac sections of miR-486 or scramble control (CTL) treated mice using phospho-Histone H3 (highlighted by arrows; DAPI and F-actin/phalloidin as counterstain; H). Quantification of the ratio of phospho-Histone H3 (S10) positive versus DAPI stained nuclei (I). miR-486 hearts exhibited a 2.48-fold increase in cardiomyocyte proliferation (** p<0.01 as determined by t-Test, N=3).
Figure 5. miR-486 is sufficient to increase Stat1 levels \textit{in vivo} and \textit{in vitro}. A. Volcano plot showing the results of mass-spectrometry proteome comparison of proteins from miR-486 mimic treated hearts as compared to scrambled BlockIT controls. Stat1 was one of the most upregulated proteins. Sarcomeric proteins highlighted by asterisks were found dysregulated only in iTRAQ analysis, but not in immunoblots (Supplemental Figure 1C and 1D). B. Pathway enrichment analysis of significantly changed proteins. C. Immunoblot analysis of heart lysates from miR-486 or BlockIT (CTL) treated neonatal mice demonstrates that total Stat1 levels are increased in miR-486. Of note, miR-486 hearts did not demonstrate alterations in total Jak1 or Stat3 levels. D-E. Isolated cardiomyocytes transfected with miR-486 mimic have significantly higher total normalized Stat1 (1.74 ± 0.1249, N=3, p=0.006 as determined by t-Test) and p-Stat1 Ser727 levels (2.425 ± 0.3063, N=3, p=0.035 as determined by t-Test) as compared to scramble control treated cells (BlockIT). Protein levels were normalized to GAPDH. F. Pie chart showing that 37.6% of genes upregulated in cardiomyocytes exposed to cyclic stretch \textit{in vitro} are predicted have Stat1 binding sites (left panel), and pie chart showing that 36.1% of genes upregulated in the RVs of HLHS patients as compared to control RVs are predicted to have Stat1 binding sites (right panel).
Figure 6. miR-486 modulates Stat1 levels by targeting Tgf-β/Smad signaling. A-B. Cardiomyocytes transfected with miR-486 have significantly less Smad2 (0.71 ± 0.08, N=3, p=0.036 as determined by t-Test) and Smad3 levels (0.69 ± 0.06121, N=3, p=0.044 as determined by t-Test) as compared to scrambled controls (BlockIT). Smad levels were normalized to actin. C. The TGF-β2 treated cells have 53.3% less (corresponding to average control cQ=0.22 vs average TGF-β2 treated EMCM cQ=1.32) miR-486 as compared to untreated cells exposed to cyclic stretch (p<0.05 as determined by t-Test). D-E. Cardiomyocytes treated with TGFβ2 have significantly decreased p-Stat1 S727 levels (0.44 ± 0.06, N=3, p=0.0099 as determined by t-Test) as compared to untreated controls (BlockIT). Total Stat1 levels were not significantly changed (0.87 ± 0.08, N=3, p=0.4347 as determined by t-Test). Stat1 and p-Stat1 levels were normalized to actin.
Figure 7. A. miR-486 increases Srf and Gata-4 expression levels in transfected cardiomyocytes after 3 days. Quantification of blots is shown in the right panels. Srf and Gata-4 levels were normalized to actin. B-C. Co-immunoprecipitation experiments using either antibodies directed against Gata-4 (B) or SRF (C) demonstrate association with Stat1 in cardiac protein extracts. Normal mouse or rabbit IgG was used as negative controls. (In= input, Pellet= bound immunocomplexes) D. Outline of proposed mechanism by which stretch increases miR-486 and left ventricular growth. Diastolic filling stretches ventricular cardiomyocytes. This stretch results in increased expression of Srf, which in turn augments expression of miR-486. miR-486 forms a positive feedback loop that increases Srf/Gata-4, and a negative feedback loop with Tgf-β/Smad signaling. Decreased Tgf-β/Smad signaling releases its repression of Stat1. Stat1 co-activates Gata4/Srf target genes; thereby, increasing cardiomyocyte proliferation and ventricular growth.
Tables

Table 1. Results of miRNA-Seq identifying stretch-responsive miRNAs from embryonic mouse cardiomyocytes exposed to cyclic stretch in vitro. miRNA-Seq was performed on RNA from embryonic mouse cardiomyocytes exposed to cyclic stretch of 16% at 1 Hz for 24 h as compared to static controls (N=3 for both groups). Using a false discovery rate (FDR) of < 0.05, 11 miRNAs were found upregulated by stretch, including miRNA486, while 23 miRNAs were downregulated.

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<td>2.44e-003</td>
<td>4.9e-002</td>
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<tr>
<td>mmu-miR-145</td>
<td>-3.01</td>
<td>-10.5</td>
<td>7.34e-012</td>
<td>1.69e-009</td>
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<td>1.63e-012</td>
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<td>5.77e-015</td>
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<td>2.41e-004</td>
<td>1.3e-002</td>
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<tr>
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<td>-8.38</td>
<td>-21.8</td>
<td>5.8e-005</td>
<td>5e-003</td>
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<tr>
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<td>-3.2e+008</td>
<td>-35.9</td>
<td>2.53e-003</td>
<td>5e-002</td>
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</tbody>
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Table 2
Top 10 most upregulated and downregulated proteins identified in the cardiac proteome analysis of proteins from miR-486 mimic treated hearts as compared to scrambled BlockIT control hearts.

<table>
<thead>
<tr>
<th>Upregulated proteins</th>
<th>Downregulated proteins</th>
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<tr>
<td>Protein ID</td>
<td>log2 fold</td>
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<tr>
<td>Aldh2</td>
<td>1.042477815</td>
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<tr>
<td>Gc (vitamin D binding prot.)</td>
<td>0.805601181</td>
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<td>Stat1</td>
<td>0.750934031</td>
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<tr>
<td>Fga</td>
<td>0.72233378</td>
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<tr>
<td>Ech1</td>
<td>0.681382714</td>
</tr>
<tr>
<td>Gsn</td>
<td>0.628990039</td>
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<tr>
<td>Dmrt1</td>
<td>0.575275028</td>
</tr>
<tr>
<td>Cox5b</td>
<td>0.525098214</td>
</tr>
<tr>
<td>Fus</td>
<td>0.494168768</td>
</tr>
<tr>
<td>Cox5a</td>
<td>0.464175535</td>
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