Increased Ventricular Preload Is Compensated by Myocyte Proliferation in Normal and Hypoplastic Fetal Chick Left Ventricle

Angela deAlmeida, Tim McQuinn, David Sedmera

Abstract—Hemodynamics influence cardiac development, and alterations in blood flow may lead to impaired cardiac growth and malformations. The developing myocardium adapts to augmented workload by increasing cell number (hyperplasia). The aim of this study was to determine the influence of alterations in ventricular preload on fetal myocyte proliferation by manipulation of intracardiac shunting at the atrial level. We hypothesized that partial clipping of the right atrial appendage would increase the blood flow to the left ventricle and, in turn, lead to an increase in chamber volume and myocardial mass based on myocyte proliferation. Using an ex ovo culture setup, we performed partial right atrial clipping on embryonic day 8 chick embryos. Ultrasound imaging was performed before and after the surgery to assess the changes in left ventricular volume. Sampling after 24 hours was preceded by 2 hour of pulse-labeling with 5-bromodeoxyuridine. Ultrasound imaging showed that partial right atrial clipping led to a significant increase in left ventricular end-diastolic volume, demonstrating increased blood flow and preload. Anti–5-bromodeoxyuridine immunolabeling revealed a significant increase in myocyte proliferation in the left ventricle and atrium. No significant changes were found in the right heart structures. Increased left ventricular myocyte proliferation and myocardial mass after right atrial clipping was also observed in embryos with experimental left ventricular hypoplasia. These results demonstrate the ability of fetal myocardium to respond to increased preload by myocyte hyperplasia and support the rationale for prenatal surgical interventions in certain cases of congenital heart disease such as hypoplastic left heart syndrome. (Circ Res. 2007;100:1363-1370.)

Key Words: chick embryo ■ hemodynamics ■ fetal surgery ■ hypoplastic left heart syndrome

Hypoplastic left heart syndrome (HLHS) refers to a group of congenital cardiac anomalies characterized by underdevelopment of the left side of the heart. The anatomic abnormalities include underdevelopment of the left atrium and ventricle and hypoplastic or atretic mitral and aortic valves, and the right ventricle, rather than the left, forms the apex of the heart.1-2 HLHS affects more than 2000 infants a year in the United States alone and accounts for approximately 25% of cardiac deaths within the first year of life.3 The etiology of HLHS is largely unknown. There appears to be a genetic component,4 but the pattern of inheritance is multifactorial, as was demonstrated by a high incidence of heart defects in first-degree relatives of patients with HLHS.5,6 In particular, there is a strong association with other left ventricular outflow tract obstructive lesions, including bicuspid aortic valve.7 Another known association is with terminal deletion of the long arm of chromosome 11.8-9 Nevertheless, no single gene has been implicated to date, and no defined genetic animal models of HLHS exist. The most widely accepted hypothesis is that HLHS develops as a result of embryonic alterations in blood flow, such as premature narrowing of the foramen ovale10 and aortic stenosis.11 However, the primary cause of HLHS remains unclear, as hemodynamic alterations have been shown to lead to secondary mitral stenosis.12 Although the primary cause of HLHS is uncertain, hemodynamic alterations clearly result in the progressive severity of HLHS, as demonstrated by fetal echocardiography in the second and third trimester.13,14 Recent studies15-17 highlighted the possibility of later fetal development of HLHS that could explain the occurrence of neonatal cases who had normal routine 19-week fetal scan. From these reports, we learned that restrictive foramen ovale is not a primary cause in most HLHS cases; many cases (especially those amenable to fetal intervention) are caused so far by poorly characterized events between 19 to 23 weeks that worsen the flow across the aortic valve, and, perhaps most significantly, interventional improvement of this flow by means of balloon dilatation results in significant reversal.

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of left ventricular hypoplasia. From this perspective, it is noteworthy that in postnatal population, ≈20% of HLHS patients (excluding variants) present with both mitral and aortic valve patency;18,19 this percentage is likely higher in the fetal population, in whom stenosis did not progress yet to complete atresia.20

Without surgery, HLHS is uniformly fatal usually within hours or days of birth.21 Despite ongoing improvements,19 the most common palliative approach (Norwood) consists of 3 major cardiac procedures and leaves the patient with a single-ventricular (Fontan) circulation, with the right ventricle acting as the sole, systemic pump. Biventricular repair is the preferred approach because it restores the 2-ventricular physiology; however, it can only be performed in a very small subset of HLHS patients, in whom the left ventricle is small but potentially functional and both valves are patent.22,23 Therefore, prenatal intervention that would sufficiently rescue the left ventricle for possible restoration of 2-ventricular physiology would greatly improve the management of HLHS and afford patients a near-normal quality of life.

The chick embryo has a long and well-established history as a model system in biology and has contributed major concepts to many other disciplines.24 It is currently the only experimental prenatal model of HLHS, with adequate survival rates that allow for the long-term investigation of interventions. By left atrial ligation (LAL), a phenotype mimicking human HLHS is produced.2 Although not pathogenetically identical with most human cases, the resulting myocardial remodeling that is based on myocyte proliferation. Using an ex ovo culture setup, ultrasound imaging showed that this procedure indeed led to a significant increase in left ventricular end-diastolic volume, demonstrating increased blood flow and preload. We also demonstrated a significant increase in myocyte proliferation in the left ventricle and atrium, with no significant changes in the right heart structures in both normal and HLHS hearts. In addition, we noted increased left ventricular myocardial volume and improved myocyte differentiation in the hypoplastic left ventricle after clip. These results show that reversal of experimental left ventricular hypoplasia induced by LAL can be achieved with right atrial clamping, thereby redirecting flow by changing atrial compliance. Thus induced myocardial remodeling that is based on increased myocyte proliferation, leading to increased myocardial mass, demonstrates the viability of and rationale for prenatal surgical interventions in certain cases of congenital heart disease such as HLHS by exploiting the unique ability of the fetal myocardium to respond to hemodynamic load by myocyte hyperplasia.

**Materials and Methods**

For further experimental details on ex ovo culture setup, LAL, immunohistochemistry and image processing, myocardial volume estimation, and statistical analysis, which are all based on published protocols,12,27,28 see the online data supplement at http://circres.ahajournals.org.

**Right Atrial Clipping**

On embryonic day 8 (E8)/Hamburger–Hamilton stage 34,29 the vascularized chorioallantoic membrane was carefully pushed aside using blunt microsurgical forceps (WPI, Sarasota, Fla), and the amniotic cavity was carefully opened by tearing the membrane. The chest wall was still avascular. A fine silver neurosurgical microclip was then placed on the right atrium to reduce its volume and exclude part of it from circulation (Figure 1; for procedure details, see Movie 1 in the online data supplement). After visually verifying the position of the clip, the chorioallantoic membrane was returned to its original position, and the embryos promptly returned to the incubator. Control embryos were treated identically except for placement of the microclip. Surgery on embryos with HLHS was performed in an identical manner but in ovo. This latter technique necessitated coordinated action of 2 operators (D.S. and A.A.); the first placed the clip, while the other held the embryo in a suitable position. The experimental time line and numbers of embryos in each group are summarized in Table.

**Echocardiography of Chick Embryos**

Access to the embryo from a variety of imaging planes was made possible by the use of the ex ovo culture system. For imaging, we used a 55-MHz scanhead on the Vevo 660 ultrasound biomicroscopy system (VisualSonics LTD; Figure 1). The temperature of the embryos was maintained at 37.5±0.5°C during examination by using a combination of a Radnoti circulating water-jacketed incubator and a heat lamp. After preoperative imaging, the embryos were allowed to recover for 1 hour before clipping; after clipping, an additional 1 hour was allowed for recovery before postoperative imaging. For examples of echocardiographic images before and after surgery, see supplemental Movies 2 and 3. We found that optimal survival was achieved when both surgery and imaging time was limited to 5 minutes. For the quantitative estimation of left ventricular cavity volume, end-systolic and end-diastolic area and minor diameter (width; cavity cross-section was approximated to an ellipse; Figure 1) from 3 cardiac cycles acquired at 30 frames per second were measured by planimetry using bundled software by a single, nonblinded observer (D.S.). Blinding of the observer was not practical, because the silver microclip was visible in most of the postclip recordings that included the ventricles (Figure 1). All individual measured and calculated values can be found in supplemental Table I. The volume (V) was calculated using the formula $V = \frac{2}{3 \times \text{area} \times \text{width}}$, assuming geometry of a prolate ellipsoid with equal minor axes.30 Cycle length, determined visually as number of cycles during 10-second loops (300 frames) of B-mode recordings and confirmed by peak-to-peak contraction measurements in M-mode, was similar between the recordings using this protocol. It was impossible to obtain simultaneous ECG tracings with this setup, optimized for adult

**Results**

**Right Atrial Clipping Increases Blood Flow to the Left Heart Structures**

The results of echocardiographic examination before and after clipping showed an increase more than pclev values to
a varied degree in all 8 hearts examined (62% on average; Figure 1 and supplemental Table I). This increase was not attributable to a change in cycle length, as the basic functional parameters of the left ventricle established at baseline (heart rate, 181±10 bpm; ejection fraction, 63±3%) were not significantly changed; however, the stroke volume was increased, in accordance with the Frank–Starling relationship. Thus, right atrioplasty resulted in increased preload of the left ventricle. Despite the low survival of ex ovo LAL embryos to E8, 3 survivors were ultimately obtained, of which 2 had a
distinct HLHS phenotype and were subjected to right atrial clipping and echocardiographic measurements (supplemental Figures 1 and 2 and supplemental Movies 4 and 5). In both cases, the procedure led to an increase in left ventricular end-diastolic volume of a magnitude similar to normal embryos.

Increased Preload Is Compensated by Myocyte Hyperplasia

We were interested to see whether the increased volume loading would translate into myocardial remodeling. Cumulative 24-hour survival of embryos clipped ex ovo at E8/stage 34 was 28% (improving with learning curve; mortality most often caused by clip-related bleeding). Histological analysis of 5 E9/stage 35 survivors showed increased distension of the left ventricular cavity, and the position of the clip on the right atrium was ascertained (Figure 2). However, despite the left ventricular cavity being larger, the signs of decompensated dilation (globular shape of the heart, increased transverse to longitudinal diameter) were absent, indicating good compensation of increased volume loading. Similarly, no venous congestion or edema characteristic of embryonic heart failure was noted.

We next analyzed the proliferative structure of the myocardium to examine the cellular basis of the adaptive response of fetal myocardium to such hemodynamic alteration. First, we observed a dramatic decrease (drop to 48% of the nonclipped part, \(P = 0.005\) by paired t test) in proliferation in the excluded portion of the right atrium (Figure 2). Determination of the percentage of S-phase cells in matched areas of

![Figure 2.](http://circres.ahajournals.org/)

Heart morphology in normal E9/stage 35 embryos 24 hours after placement of the right atrial clip. A and B, Hematoxylin and eosin–stained sham-operated ex ovo heart and matched heart subjected to right atrioplasty. The clip was removed before processing, and its position on the right atrium is indicated by the arrows. Note the enlargement of the left ventricular cavity. C, Anti–5-bromodeoxyuridine immunostaining shows remarkable paucity of S-phase (red) nuclei in the part of the right atrium excluded from circulation. Quantification of the proliferative structure of the myocardium (D, bar chart) shows a significant increase in percentage of labeled myocytes in the left heart structures. Mean ± SEM; unpaired t test, \(^*P = 0.01\). E and F, Myocyte disorganization in the part of the right atrium excluded by clip for 24 hours (anti–sarcomeric actin staining, blue; nuclei in green). In the portion of the left atrium excluded by LAL for 96 hours (G and H), there is, in addition, decreased intensity of staining for the contractile proteins (anti-myosin, best seen in the pseudocolor image). Scale bars = 100 μm (C through H). LA indicates left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle.
different heart compartments showed a significant increase in the labeling index in the left atrium and ventricle, with no significant changes in the right heart structures (Figure 2). In control hearts, the labeling was higher in the right atrium than in the left, whereas, after clipping, this relationship was inverted. Myocyte proliferation in both left atrium (+42%, P<0.001) and left ventricle (+38%, P=0.009) was significantly higher than nonclipped, E9/stage 35 ex ovo controls.

Decreased Myocyte Proliferation in the Hypoplastic Left Ventricle Is Rescued by Increased Hemodynamic Loading

Because of the severe difficulties of survival until E8/stage 34 of the embryos subjected to LAL ex ovo, we modified the clipping technique to perform the surgery in ovo. After modifying the technique to gently lifting the embryo from the egg by its neck using a fine glass hook, we produced a group of 9 embryos with established left ventricular hypoplasia subjected to clip that survived for an additional 24 hours until E9 (stage 35). In a control group of 16 in ovo embryos with untreated HLHS hearts, the values were also greater than in "plain" HLHS embryos, a small, but not statistically significant decrease in left ventricular myocyte labeling was noted when compared with normal in ovo embryos. This finding was in agreement with our previous study.27 In HLHS embryos with right atrial hypoplasia, we noted the inversion of the right atrium>>left atrium proliferation gradient similar to that of the ex ovo group (Figure 2). Specifically, whereas, in “plain” HLHS embryos, the right atrial labeling index was 30% higher than in the left atrium, after clip, it was 5% lower; however, these changes were not statistically significant. The myocyte labeling index was significantly increased in the hypoplastic left ventricle, actually reaching normal values (Figure 3). In contrast to untreated HLHS hearts, the values were also greater than in the right ventricle (also described previously).27 There were no significant changes in the right ventricle (Figure 3). One of the embryos was excluded from the analysis, because it was moribund at the time of sampling (dilated vasculature, slow heart rate), and proliferation in all compartments was less than half of the group average. Semiquantitative evaluation of myocyte differentiation based on sarcomeric actin immuno-fluorescence showed recovery of staining to normal levels (98.5% of normal left ventricle). Inspection of the whole hearts showed (with the exception of 1 heart with a very severe phenotype) homogenous staining throughout the myocardium in contrast to a telltale decrease (~41.5%) in the hypoplastic left ventricle previously described.27 In contrast, no significant changes were noted between normal and normal+clip left ventricles. Last, the analysis of myocyte proliferation and differentiation in the ligated portion of the left atrium (excluded from circulation for 5 days) showed a decrease in proliferation (drop to 54% of values of the neighboring, nonexcluded part; P=0.03), as well as a decrease in differentiation indicated by reduced immunostaining (drop to 57.5%) for sarcomeric actin (Figure 2).

Increased Myocyte Proliferation in Rescued Hypoplastic Left Ventricle Translates Into Increase in Myocardial Volume

For a success of any fetal cardiac procedure, it is necessary to see the measured experimental parameter (myocyte proliferation) translated into an actual increase of myocardial mass. Figure 4 shows clearly that increased proliferation results in significantly increased ventricular myocardial volume and cell number in the hypoplastic but, interestingly, not in the normal left ventricle. If expressed as an absolute number of myocytes, the observed increase would be ≈2 400 000 myocytes.

Discussion

Echocardiography Provides Proof of Loading After Right Atrial Clip

In the clinical setting, a subgroup of HLHS patients with a premature closure of the foramen ovale has a particularly
poor prognosis postnatally. To alleviate this problem of blood flow restriction to the fetal left ventricle (that could contribute to phenotype progression and valve atresia), Marshall et al. attempted fetal balloon septoplasty. Despite a technically successful procedure, the transseptal flow to the left atrium was minimal, pointing out the need of an additional procedure to force the blood to the left heart structures. Although a stent that maintains the opening in the interatrial septum might serve this purpose in human patients (A. C. Marshall, personal communication), we have tested in our studies a right atrial volume reducing procedure that would redirect the blood toward the smaller, most likely less compliant left atrium. This approach was substantiated by the fact that in the chick model of HLHS, premature closure of the interatrial communication occurs rarely and never before E10 (stage 36). By using embryonic echocardiography, we were able to verify that right atrial clipping indeed succeeded in increasing the blood flow to the left ventricle. The same effect was noted also in 2 embryos with hypoplastic left ventricles that survived ex ovo until E8 (Table), suggesting that the smaller left ventricle has sufficient compliance to respond by an increase in end-diastolic volume. Histological examination at a later time point showed that this enhanced volume loading resulted in adaptive myocardial remodeling. For technical reasons (the shape not easily approximated to geometric solid, difficult delineation of actual lumen because of more extensive trabeculation), analysis of right ventricular volume could not be performed, but it is likely that the amount of blood flowing through the right atrioventricular valve was reduced by right atrial clipping. However, volume load and pressure load are linked. We are basing our terminology on original experimental embryonic cardiac manipulations of Clark and colleagues, who distinguish modified afterload (eg, in conotruncal banding model) and preload (in venous end manipulations, such as LAL or venous clip model). Indeed, because conotruncal banding increases ventricular end-diastolic volume, there is undoubtedly also volume load component, and, vice versa, left/right atrial clipping is accompanied by pressure changes. Unfortunately, direct pressure measurements in embryos are a nonsurvival procedure and probably impossible to achieve in highly mobile embryos at later fetal stages (the published data go only as far as E6/stage 29). Longitudinal examination of left ventricular systolic function (supplemental Table II) showed an increase in ejection fraction from 45% to 68% and average stroke volume from 0.52 to 0.79 mm$^3$ between E6 and E8, correlating with left ventricular wall compaction, completion of ventricular septation, and beginning of coronary perfusion.

**Increased Volume Loading Is Compensated by Fetal Myocyte Proliferation**

Previous studies have shown that increased pressure loading in the fetal heart is compensated for by myocyte hyperplasia. However, our own study of volume-overloaded preseptation right ventricle in the chick experimental model of HLHS cast some doubt on whether the same is true for volume overload stimulus, because minimal changes in the myocardial proliferative structure were noted in the dilated right ventricle. Here we show that at least for postseptation early fetal chick left ventricle, increased preload is a stimulus for increased myocardial growth. This apparent discrepancy could be explained by the different compliance and geometry between the left and right ventricle, making the right ventricle more prone to dilation rather than adaptive chamber wall thickening in response to increased hemodynamic loading. Along this line, the changes of myocyte proliferation in the right ventricle after clip were minimal and statistically not significant, and the sampling interval was probably too short to see any “shrinking.” Even in severe left ventricular hypoplasia, the right ventricular myocardial volume is increased only slightly, suggesting its high functional plasticity. Because apoptosis in developing myocardium is a rare event, and remodeling of myocardial architecture during this period is based on modulation of myocyte proliferation, we would expect the gradual involution of the dilated right ventricle to be based on a small decrease of proliferation over a longer time period (similar to mechanism of experimental left ventricular hypoplasia), which is consistent with our observations. Of note, it is likely that increased preload is actually a combination of volume and pressure increase; however, direct pressure measurements were impossible in this setup. Despite its statistical significance, the magnitude of changes in myocardial proliferation appears rather small; this is because of the necessity of the myocardium to maintain adequate pumping function even while continuing myocyte division. In addition, even minor changes in proliferation measured at a single time point are, over time, translated into appreciable differences in phenotype, and the role of proliferation can be confirmed using the method of radiolabel dilution.

Findings of decreased proliferation in both acutely and chronically excluded parts of the atria correlates with previously published data. The differentiation status of this in vivo unloaded myocardium varied, with no change in the relative amount of contractile protein apart from some myofibrillar disorganization in acutely (24 hours) and significantly less of both actin and myosin in chronically (4 to 5 days) excluded portions.

We found that both normal and hypoplastic left ventricular myocardium responded to increased volume loading in a similar fashion, ie, by increased myocyte proliferation. This is a significant finding, and gives hope to the idea that if a way is ascertained to hemodynamically reload the hypoplastic left ventricle prenatally, this chamber could reasonably be expected to respond by augmented growth and contractile differentiation. In addition, this increased cell cycling translated into increased myocyte numbers in the hypoplastic but not the normal left ventricle. Given the average cell cycle duration of less than 16 hours at that stage, this increase in myocardial mass is plausible, with an observed increase in cell cycling. Similarly, there was an improvement (to near-normal values) of differentiation status of myocytes in the reloaded hypoplastic left ventricle.

**Significance of Prenatal Cardiac Repair**

Our experience showed that to achieve optimal survival, careful “dosing” is necessary. Together with the learning curve, our survival rates improved over time, from 20% to...
80% of operated embryos. From clinical settings, it is known that the immature heart does not easily tolerate sudden hemodynamic changes and, hence, is among the reasons why surgical palliation for HLHS is performed in 3 steps. To gauge the effects of such interventions in practice, careful monitoring of the hemodynamic response to “dosed” ligation of the right atrium, either echocardiographically or by means of pressure measurements during cardiac catheterization would be necessary, with possible adjustments based on measured values. Such monitoring is now standard practice in postoperative follow up in pediatric cardiology.2

The limitations of this experimental study are several. The chick model, despite its 4-chambered heart and almost identical patterns of prenatal and postnatal circulation, is not an etiological model of the human condition (most often likely to be genetic in origin) but a hemodynamically created phenocopy. Its different size means that our findings should be ideally verified in a larger fetal mammalian model, such as sheep11 or pig.39 Nevertheless, it is a useful model of aberrant myocardial loading, highly relevant to understanding the biology of ventricular myocardium in a variety of congenital heart disease, of which HLHS is among the most significant. Furthermore, the majority of patients18,19 present with aortic and/or mitral atresia, which would necessitate modification of the interventional approach to account for this complication. Current clinical experience with prenatal balloon dilation of critical aortic stenosis suggests that careful patient selection, together with technical precision can result in favorable outcomes,15–17 making fetal interventions a viable alternative to postnatal palliation and our model timely.

Because the clipping of LAL embryos had to be performed in ovo, we had to rely on external visual inspection rather than more objective echocardiographic evaluation of the phenotype. We reached a complete agreement on the phenotype of further operated embryos between 2 observers with a cumulative experience with LAL exceeding 10 years, and correlation with histological examination (supplemental Figures 1 and 2) was excellent. Selective survival of “milder” phenotypes, which could provide an alternative explanation of observed phenotypic “rescue,” is also unlikely, because routine autopsy of all nonsurvivors did not show any overt difference in phenotypic severity between these groups.

We did not aim for survival past the set 24-hour interval, because we anticipated that the rather large silver microclip would interfere with chest wall closure and long-term survival. We are currently working on technical aspects of the procedure that would allow us to follow up the survival until hatching to compare the rates and address this issue.

In conclusion, we have shown that volume-displacing surgical procedures at the atrial level in the fetal heart can influence ventricular loading and that such an increase in ventricular preload in the early postseptation left ventricle is compensated by myocardial hyperplasia in both normal and hypoplastic left ventricle. Reversal of experimental left ventricular hypoplasia induced by LAL can be achieved with right atrial clipping, thereby redirecting flow by changing atrial compliance. The underlying mechanism of this myocardial remodeling is increased myocyte proliferation, leading to increased myocardial mass. These results provide hope that carefully designed human fetal procedures that seek to restore normal cardiac structure and function in the settings of severe congenital heart disease such as HLHS will be successful.

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Disclosures

None.

References


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ONLINE DATA SUPPLEMENT to deAlmeida et al., Increased ventricular preload is compensated by myocyte proliferation in normal and hypoplastic fetal chick left ventricle

Supplemental methods

Ex ovo culture setup

The method used followed the original protocol developed by Ono et al. with modifications described recently by our collaborators. After 48 hours of incubation at 37.5 °C in a forced-draft rocking egg incubator, the fertile eggs were cracked open under sterile conditions into hexagonal weight boats, which were then placed inside a large petri dish with sterile distilled water in the bottom to maintain humidity. The embryos (at approximately Hamburger-Hamilton Stage 12) were then reincubated at 37.5 °C in a standard tissue incubator without additional CO₂. Under these conditions, the embryos develop normally until ED 10, and can survive until ED 19 (of normal 21-day incubation period) with calcium supplementation.

Immunohistochemistry and image processing

After 24 h of re-incubation and two hours prior to sacrifice at ED 9 / stage 35, the embryos were labeled with 50µg of 5-Bromodeoxyuridine (Sigma, St. Louis, MO) in 200µl of Tyrode’s saline applied directly over the vascular bed. The embryos were fixed in Dent’s fixative (80% Methanol-20% DMSO, v/v), and processed into paraffin. Serial sections were cut at 8µm, and mounted on silane-coated slides. Histological analysis was performed using a triple staining protocol for sarcomeric actin (1:1000, Sigma) as a myocyte marker, BrdU (1:100, BD Biosciences) for S-phase nuclei, and DRAQ 5 (1:1000, Biostatus Limited, UK) for all nuclei (Figure 2). Triple-stained serial tissue
sections were examined on a Leica TCS SP2 AOBS confocal microscope. Fields from the middle of ventricular free wall were recorded using a 40x oil immersion lens at 1024x1024 pixel resolution. The final images were maximum intensity projections of five optical sections one micron apart. Cells were counted in Adobe Photoshop 8.0 (Adobe Systems Inc., San Jose, CA), where black dots were placed with the Pencil tool in separate layers over the nuclei, then transferred for automated counting to ImageJ (freeware, National Institutes of Health, Bethesda, MD). The percentage of BrdU-labeled myocyte nuclei of total myocyte nuclei was then calculated. Intensity of anti-sarcomeric actin immunolabeling was used to assess myocyte differentiation status. Relative measurements were performed on 8-bit, single channel confocal sections taken under 10x objective to minimize photobleaching using ImageJ. All the standard precautions (background subtraction, negative controls, all hearts in one block and stained at the same time) were performed to minimize variability, as described. Under these conditions, the background values ranged between 0-5, secondary control 10-20, and staining intensity 60-190 (on a scale from 0 to 255), indicating specificity of antibody staining and no over-saturation.

Myocardial volume estimation

Volume of the left ventricular myocardium was measured from serial histological sections (100 µm interval) using Cavalieri principle as described. Hematoxylin and eosin-stained sections were scanned as transparencies at 1200 dpi, and area occupied by the left ventricle at each section was delineated in Adobe Photoshop. After thresholding, the number of pixels occupied by the myocardium was measured in ImageJ. The total
volume was calculated as a product of mean area and ventricular length (determined from number of sections containing left ventricle). From these values, the number of myocytes was estimated by dividing with average volume of an individual myocyte. This value was calculated from average of 100 cells measured (control LV, hypoplastic LV, both with and without clip) using measurements of cell width and length. Since the shape of embryonic avian myocyte is best compared to prolate ellipsoid (in contrast to adult mammalian one, which is rod-shaped 7), the same formula used for left ventricular volume calculation from B-mode echocardiographic recordings was used. There was no difference in size among groups (P=NS), in agreement with our previous observations 4,6; therefore, the average value of 678 µm³ per myocyte was used.

Left Atrial Ligation (LAL)

Fertile white Leghorn chicken eggs were incubated blunt-end up in a rocking forced-draft 37.5°C incubator to Hamburger-Hamilton stage 24 (ED 4). The egg was positioned under a Leica SMX12.5 dissecting microscope with a 0.5x objective, where the eggshell and its membrane were removed to expose the embryo. The embryo was gently turned over using an L-shaped fine glass hook to expose the left side and microforceps (Dumont #5, Fine Science Tools) were used to make a slit-like opening in the thoracic wall. A loop of 10-0 nylon suture was then placed around the left atrial appendage and tightened 6. The embryo was gently repositioned to its original right-side-up position, the opening in the egg sealed with electrical tape, and the egg returned to the tissue incubator for re-incubation without further rocking until stage 34 (ED 8). Only embryos with a distinct phenotype of HLHS were used for the analysis of right atrial clip effects or as HLHS
controls. In total, 73 embryos were ligated, with 36 surviving until ED 8 (stage 34); of those, 18 presented with a distinct HLHS phenotype and were clipped, with average survival (improving with the learning curve) until sampling at stage 35 (ED 9) of 50%. Sixteen “plain” HLHS phenotype stage 35 embryos that served as a control for this group were survivors of 80 LAL embryos collected over the course of two months (average survival: 40%, only half of embryos had a distinct phenotype).

Statistical analysis
All data are shown as mean ± SEM. At minimum, five embryos per group were analyzed. Statistical comparison of differences between groups was performed using an unpaired two-tailed Student’s t-test after ANOVA. For comparison of regions within the same heart and ventricular volumes before and after surgery, a paired t-test was used. Results were considered significant at p<0.05.

Supplemental Table 1. Measured and calculated echocardiographic parameters of control ex ovo ED 8 / stage 34 embryos before and after right atrial clipping. Pre – before clipping, post – 1h after clipping; EDA, end-diastolic ventricular cross-sectional area in four-chamber view; EDW, end-diastolic width; ESA, end-systolic area; ESW, end-systolic width; HR, heart rate in beats per minute; EDV, end-diastolic volume; ESV, end-systolic volume; SV, stroke volume; CO, left ventricular output (mm³ per minute); LV EF, left ventricular ejection fraction.
Supplemental Table 2. Echocardiographically measured functional parameters of the chick embryonic left ventricle between day 4 and 8 of incubation.

Supplemental Figure 1. Histology (H&E on paraffin sections) compared with echocardiographic imaging. Short-axis views are matched with histological sections cut perpendicular to the long axis of the heart. Representative views of ED 8 / stage 34 sham and HLHS (LAL) hearts; the histological pictures are not from the same embryos as shown on the echo (those were further imaged in four-chamber view and then sectioned in the same plane, Supplemental Figure 2). Yellow dots on the echo pictures outline the ventricular chambers. Smallest division on the echo pictures equals 100 µm.

Supplemental Figure 2. Normal and LAL hearts at ED 8 / Stage 34. A. Normal four-chamber view showing complete septation and all cardiac chambers. Panel B shows Doppler flow across the right atrioventricular orifice. At this stage, active filling is the major component of the atrioventricular flow, and no regurgitation is present under physiological conditions. Panel C shows a heart subjected to left atrial ligation (LAL) ex ovo at Stage 24 with left ventricular hypoplasia. The abnormal valve morphology is clearly visible. D. Systolic regurgitation across the abnormal tricuspid valve is indicated on the Doppler image with red arrow. Mitral and aortic regurgitation was noted as well on this pre-clip recording. The left ventricle on panel C appears distended, since the right atrial appendage was clipped and the heart re-imaged by echocardiography; sampling for histology followed 2 h later since the embryo became moribund and hemodynamically unstable. a, active phase of ventricular filling, LA, left atrium, LV, left ventricle, p,
passive phase of ventricular filling, Pu, pulmonary artery, RA, right atrium, RV, right ventricle. Scale bar 1 mm for histological sections and 100 µm smallest division in echo pictures.

Supplemental movies

Supplemental Movie 1. Video recording of ex ovo placement of a silver microclip on the right atrial appendage in embryonic ED 8 / stage 34 chick embryo.

Supplemental Movie 2. Sample echocardiographic loop from an exteriorized embryonic ED 8 / stage 34 chick embryonic heart. Note the action of the mitral and aortic valve as well as clear delineation of the endocardial contour of the left ventricle.

Supplemental Movie 3. Echocardiographic imaging showing a silver microclip placed over the right atrium.


Supplemental Movie 5. Echocardiographic imaging of ED 8 heart with LAL-induced left ventricular hypoplasia in short axis view.

References to Online Data Supplement – Methods section


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<th>ESA (mm²)</th>
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Supplemental Table 1.
Echocardiographic data of embryonic day 8 / Stage 34 chick embryos.
Supplemental Table 2. Functional parameters of the developing left ventricle measured by echocardiography. Values are mean ± SD. These stages correspond to embryonic days 4, 6, and 8, respectively.

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Supplemental Figure 1
Supplemental Figure 2