Microarray Analysis of Normal and Abnormal Chick Ventricular Myocardial Development

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Summary

The left and right ventricle originate from distinct parts of the cardiac tube, and several genes are known to be differentially expressed in these compartments. The aims of this study were to determine developmental differences in gene expression between the left and right ventricle, and to assess the effect of altered hemodynamic loading. RNA was extracted from isolated left and right normal chick embryonic ventricles at embryonic day 6, 8, and 10, and from day 8 left atrial ligated hearts with hypoplastic left and dilated right ventricles. cRNA was hybridized to Affymetrix Chicken Genome array according to manufacturer protocols. Microarray analysis identified 302 transcripts that were differentially expressed between the left and right ventricle. Comparative analysis detected 91 genes that were different in left ventricles of ligated hearts compared to age-matched ventricles, while 66 were different in the right ones. A large number of the changes could be interpreted as a delay of normal maturation. The approach described in this study could be used as one of the measures to gauge success of surgical procedures for congenital heart disease and help in determining the optimal time frame for intervention to prevent onset of irreversible changes.

Keywords

Gene chip array; Chick embryo; Left atrial ligation; Hypoplastic left heart syndrome

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Introduction

The evolution of the cardiovascular system has permitted increases in organism growth, size and complexity beyond the metabollic constraints imposed by a diffusion-based system. With the development of an initially open circulatory system, metabolic rate and size in three dimensions could be dramatically increased, enabling the eventual emergence of chordates. The heart is therefore the first adult organ to start functioning during embryogenesis (Kirby 2007).

Development of the myocardium starts during gastrulation with the precardiac mesoderm, organized into cardiac crescent (Kirby 2007). Anteriorly localized cells would normally give rise to the left ventricle, while posteriorly located ones would mostly contribute to the atrial tissues; however, their fates are not firmly set and respond to local environment cues. There is also the second, by some called secondary or anterior, heart field, which gives rise to the right ventricle and the outflow tract (Kelly and Buckingham 2002). As the cells migrate towards the midline, they start to organize themselves into two tubular structures with future endocardium on the inside and myocardium on the outside. In the midline, the fusion process starts first with the endocardial tubes, and later on with myocardium. The primitive cardiac tube is initially straight, and is composed of myocardium, acellular cardiac jelly, and endocardium. It consists of the portion that will become the future left ventricle; the presumptive atria are located at the inflow (posterior) end. The first contractions commence in the ventricular segment already during the second day of incubation in the chick (Kamino *et al.* 1981), and soon the circulation of blood starts.

The crucial process for further morphogenesis and septation of the heart is termed cardiac looping. In a strict sense, the initially straight tube is thus transformed into a cardiac loop, and at that period begins expansion of atrial and ventricular chambers, which acquire their molecular identity. The left and right ventricle become morphologically distinguishable during this "ballooning" period, and the muscular portion of the interventricular septum (compact in mammals, trabeculated in the chick) starts to form between them (Sedmera *et al.* 2000). The original ("primitive", slowly conducting and contracting) myocardium flanks the atrial and ventricular segments, forming sinus venosus at the inflow (venous) end, atrioventricular canal between the atria and ventricle, and myocardial outflow tract at the outflow (arterial) end. The process of cardiac looping was well described from the morphological point of view by Manner (2000), and the molecular fingerprints of cardiac chambers are continuously updated by the Moorman lab (Sedmera 2005, Rana *et al.* 2007, Aanhaanen *et al.* 2009).

Formation of trabeculae in the ventricular chambers is the morphological hallmark of their differentiation and distinguishes these segments from the rest of the heart loop, where the myocardium is organized into a circular, multilayered tube. Ventricular trabeculae express different patterns of genes, such as connexin40 (Becker *et al.* 1998, Miquerol *et al.* 2004) that contributes to its higher conduction velocity. It is believed that the primary reason for trabeculation is the necessity to provide sufficient nutrition for increased myocardial mass in the (initial) absence of coronary circulation (Minot 1901, Tota *et al.* 1983). Indeed, the ventricles of some adult lower vertebrates are organized in such a way (Tota *et al.* 1983),

even though there are coronary arteries present in different parts of those hearts (Sedmera *et al.* 2003). Such trabeculated hearts work well as volume pumps, but are not suited for generating higher pressure necessary for sustained aerobic performance. Not surprisingly, a different morphological form of the ventricle, dependent primarily on the thick, vascularized outer compact layer, is found in large, fast-swimming fish species (Ostadal and Schiebler 1971, Agnisola and Tota 1994).

The left and right ventricle originate from distinct parts of the cardiac tube, and several genes are known to be differentially expressed in these compartments (Di Lisi *et al.* 2000, Moorman and Christoffels 2003). The two chambers differ both morphologically and physiologically after birth, and careful consideration of these differences is essential in planning of surgical procedures in complex forms of congenital heart disease such as hypoplastic left heart syndrome. It is, however, unknown to what extent these differences are pre-programmed from the onset, and how much is due to adaptations to different hemodynamic loading. Due to advances in ultrasound imaging, it is possible to study the function of the trabeculated heart not only in model species (chick, mouse), but also in humans. This leads to increased interest in its normal and abnormal function as a prelude to development of embryonic heart failure (Sedmera 2011).

The goals of this study were to determine developmental differences in gene expression between left and right ventricle, and to assess the differential effect of altered hemodynamic loading on left and right ventricle. In contrast to previous studies focusing on single genes or group of factors, we chose the gene chip array approach for a wide coverage of the transcriptome of the developing heart.

Material and Methods

Eggs were incubated in a force draft incubator at 37.5 °C and high humidity until embryonic day (E) 6, 8, or 10 (Hamurger-Hamilton stages 29, 34, and 36). Embryos were removed from the eggs and dissected in RNAlater (Ambion); the left and right ventricles were separated by microdissection (Sedmera *et al.* 1997, Fig. 1) and aliquoted into sterile 2 ml Eppendorf tubes with RNAlater. Only embryos that appeared externally normal and were of the appropriate stage were used; dysmorphic or growth-retarded embryos were discarded.

Left atrial ligation (LAL)

Partial ligation of the left atrial appendage was performed *in ovo* at E4 (Stage 24), as described (Sedmera *et al.* 1999). Reduction of left atrial volume lead to decreased blood flow across the interatrial septum, resulting in decreased preload of the left and increased preload of the right ventricle. Tissues from Stage 34 (E8) embryos with established phenotype (Fig. 1) were carefully dissected, collected and stored in RNAlater, and then pooled for RNA isolation.

Each sample for hybridization contained a minimum of 6 to 12 separate biological specimens. RNA was purified using RNeasy columns (Qiagen), converted into biotin-labeled cRNA targets (Invitrogen SuperScript cDNA synthesis kit and Enzo Bioarray Hi Yield kit), and then hybridized to Affymetrix Chicken Genome arrays (4 µg per

hybridization) according to manufacturer protocols. All samples were hybridized in technical duplicates.

Gene hybridization intensities were normalized by gcRMA algorithm (Wu *et al.* 2005) and detection calls generated by MAS5 algorithm, both implemented with ArrayQuest (Argraves *et al.* 2005). Data were analyzed by pairwise comparison using the following differential expression criteria: fold change >4; p<0.05 (Student's unpaired t-test), and 100 % 'presence' call for either set of technical replicates. Hierarchical clustering of genes was performed with dChip software using a centroid linkage model with distance metric. The microarray protocol adhered to MIAME standards. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE37070 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE37070). Functional enrichment of biological processes was conducted with the DAVID functional annotation tool (Huang da *et al.* 2009a,b). Biological processes with p-values <0.005 were considered significant.

Results

Developmental differences between left and right ventricle

Microarray analysis identified 302 transcripts that were differentially expressed between the left and right ventricle at developmental stages E6, E8 and E10 (Fig. 2). Overall, the number of genes differentially expressed between left and right ventricle at a given stage increased progressively with embryonic age, suggesting an increasing functional divergence of the ventricles as developmental advanced. There were 103 genes different at E6, 99 at E8, and 150 at E10. Of the entire 302, 255 were different at only one stage, 44 were different at 2 stages, and only 3 were different at all three stages by our statistical criteria. Thus, the genes different at each stage were largely independent, non-ovelapping groups. These observations are consistent with use of distinct transcriptional programs during advancing ventricular differentiation.

Examination of developmental profiles of the 302 genes different between left and right ventricles revealed several interesting patterns. For example, there was a fair number of genes that were progressively down-regulated with advancing development, especially, and sooner in the left ventricle (Fig. 2, Box #1). On the other hand, there was a small cluster of genes that were selectively upregulated in the right ventricle, and more strongly so with advancing development (Fig. 2, Box #2). It is also appreciable that the changes in the left ventricle often preceded those in the right ventricle by approximately 2 days.

Expression data for all 302 genes satisfying the differential expression criteria are included in Supplemental Table 1 (in on-line version only). Analysis of biological processes associated with the 302 genes confirmed that heart development and morphogenesis genes were significantly enriched in this group (Table 1). Other notable significant processes were cardiac cell differentiation, positive regulation of phosphorylation, extracellular region, and transforming growth factor-beta-related.

Changes under experimentally altered loading conditions

We next analyzed the microarray data to identify genes significantly different in left and right ventricles at E8 as a consequence of LAL. Comparative analysis detected 91 genes that were different in left ventricles of ligated hearts compared to age-matched ventricles (Supplemental Table 2, in on-line version only), while 66 were different in the right ventricles compared to age-matched ventricles (Supplemental Table 3, in on-line version only). Review of these genes showed that 21 were shared between the groups affected in left and right ventricles. Analysis of biological processes associated with these genes revealed that secretion (p=0.0005), extracellular region (p=0.0006), and signal peptide (p=0.002) were significantly enriched among genes affected in left ventricle. However, a similar analysis of genes affected in right ventricle did not detect any significantly enriched biological processes (p<0.005), although the category of signal peptide gave the highest enrichment score (p=0.03).

Developmental expression patterns of the genes affected in E8 left and right ventricles by LAL are shown in Figure 3 (left ventricle, left panel; right ventricle, right panel). Collectively, a large number of the changes in expression could be interpreted as a delay of normal maturation, i.e., the gene expression pattern in the ligated hearts is reminiscent of the earlier developmental stage. In the left ventricle, there were 53 transcripts (58 %) showing this pattern, and 47 in the right ventricle (71 %). Some of these genes showed a lack of normal developmental upregulation (blue in the experimental group, similar to the earlier control stage, but red in same age and older controls; Fig. 3, yellow shading), while others showed a lack of downregulation (red in the experimental group, similar to the earlier control stage, but blue in same age and older controls; Fig. 3, green shading). In the overloaded right ventricle, the majority of differentially expressed genes (70 %) exhibited this signature of defective up- or downregulation, suggestive of an overall delay in the embryonic program.

Discussion

The developmental approach in experimental and clinical cardiology is of crucial importance. Epidemiological studies have shown that the risk factors of serious cardiovascular diseases, such as atherosclerosis and ischemic heart disease, are present already during the early phases of ontogenetic development. It follows that experimental studies of the pathogenetic mechanisms of these disturbances must shift to the early ontogenetic period. Therefore, the interest of theoretical and clinical cardiologists in the developmental approach keeps increasing. Recent advances of molecular biology and the developing possibilities of prenatal cardiology accelerated this trend substantially.

Congenital cardiovascular anomalies remain the single largest cause of infant mortality from congenital defects in industrialized countries. In case of patients operated for hypoplastic left heart syndrome using Norwood palliation ending in complete Fontan (Sedmera *et al.* 2005), the major problem of those surviving into adolescence is gradual intractable failure of the single right ventricle. Many hypotheses about the possible origin of this failure have been proposed, including ischemic injury during repeated surgical procedures. It has been suggested previously that the right ventricle is not suitable as a long-term systemic pump

because of its different geometry (Sedmera *et al.* 2005). Our study adds to this data by showing that 1) there are significant differences in gene expression patterns between the left and right ventricle already at pre-septation stage, which increase with further development, and 2) hemodynamic perturbation resulting in development of hypoplastic left heart syndrome has profound effects on gene expression patterns, which are suggestive of developmental delay. This is not suprising in the underloaded left ventricle, where decreased expression of growth factors and contractile proteins was shown previously by immunohistochemistry (Sedmera *et al.* 1999, 2002). It is interesting to note that in agreement with our previous morphological and cell proliferation data showing that the right ventricle compensates volume overload by dilation rather than increased myocyte proliferation (Sedmera *et al.* 1999, 2002), this molecular signature could be interpreted also as a sign embryonic heart failure. Ventricular expression of "fetal gene program" including specific isoforms of contractile proteins (Hirzel *et al.* 1985) or re-expression of atrial natriuretic peptide (Izumo *et al.* 1988) is a recognized hallmark in adult hypertrophy or heart failure. We noted previously using ultrasound biomicroscopy that the right atrioventricular valve in LAL hearts shows regurgitation (deAlmeida *et al.* 2007), similar to pediatric cardiology scenarios. Further studies into other models of embryonic heart failure with documented valve regurgitation, such as conotruncal banding (McQuinn *et al.* 2007) could confirm this hypothesis.

The results of this experiment are currently subject to verification by RT-PCR, *in situ* hybridization and immunohistochemistry (in case where antibody is available). One interesting set of candidate genes are extracellular matrix proteins, which could be associated with endocardial fibroelastosis. Edocardial fibroelastosis occurs often in association with human congenital cardiovascular anomalies (Forfar *et al.* 1964), most notably hypoplastic left heart syndrome, aortic stenosis, aortic atresia or patent ductus arteriosus. The endocardial thickening is believed to be caused by persistent and increased wall tension in the ventricles, possibly secondary to damaged myocardium.

One crucial question is the potential reversibility of changes in myocardial architecture induced by hemodynamic alterations. Our earlier study showed that experimental reversal of altered hemodynamics in the left atrial ligation by means of right atrial clipping (deAlmeida *et al.* 2007) results in a rapid increase in myocyte proliferation in the hypoplastic left ventricle and augmentation of myocardial volume. Currently, the infants with hypoplastic left heart syndrome are operated after birth, but some studies (Foker *et al.* 1999) suggest that corrective surgery (biventricular repair) has a higher likelihood of success if performed early after birth, where the growth of myocardium is still based upon cell proliferation. Several clinical studies (Maxwell *et al.* 1991, Allan *et al.* 1995, Tworetzky *et al.* 2004, Makikallio *et al.* 2006, McElhinney *et al.* 2009) have reported results of prenatal intervention aimed at prevention of development of the hypoplastic phenotype with variable success. While balloon dilation of severely stenotic aortic valve carries good chance of success with relatively reasonable risk, perforation of prematurely closed foramen ovale did not bring the desired effects despite good technical success (Marshall *et al.* 2004). The developmental gene chip array approach described in our study could be used as one of the measures to

gauge success from the molecular perspective and could help in determining the optimal time frame for intervention prior to onset of irreversible changes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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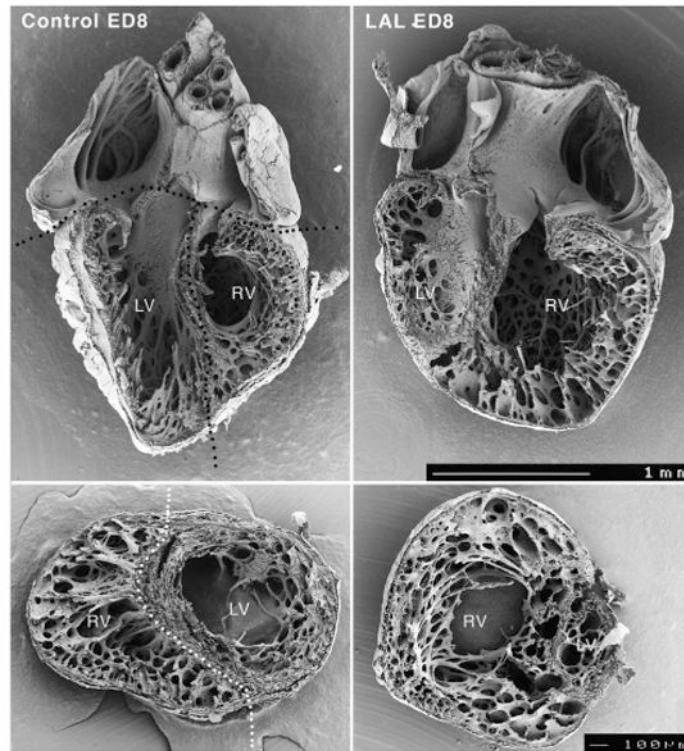
Fig. 1.
Phenotype of experimental left heart hypoplasia in scanning electron microscopy. **A.** control slice at mid-ventricular level illustrating dissection into left (LV) and right (RV) ventricle used for RNA extraction. **B.** Slice from heart with severe left ventricular hypoplasia showing also dilation of the right ventricle. Scale bar 100 μm.
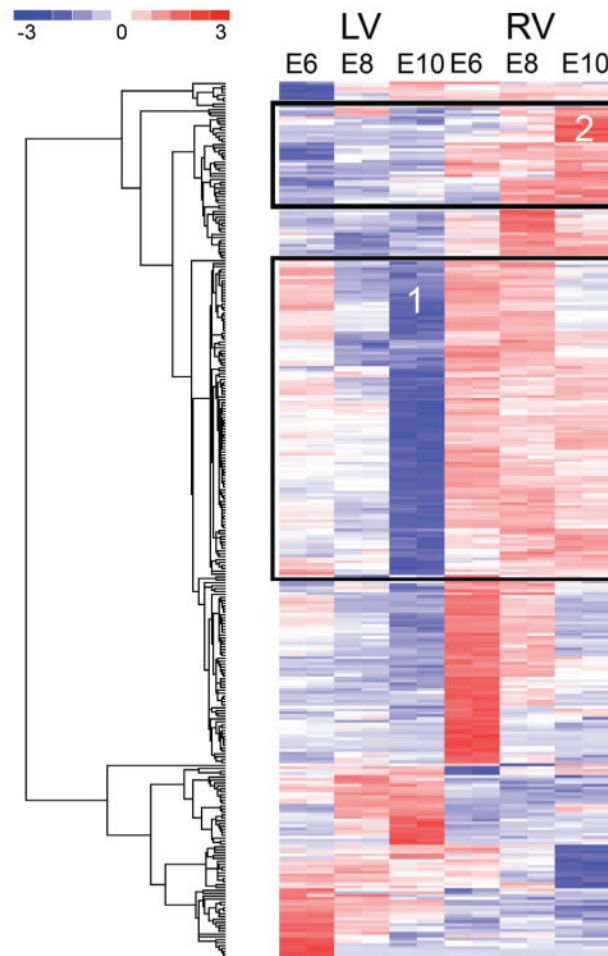
Fig. 2.
Heat map of longitudinal changes in gene expression between left and right ventricle. Boxes 1 and 2 show genes upregulated in the right ventricle and downregulated in the left ventricle (blue) with advancing development, respectively. Detailed description of individual transcripts is provided in Supplemental Table 1 (in on-line version only). Hierarchical clustering of expression patterns is shown in dendrogram (left). Colorimetric scaling of the standardized gene expression values (z-scores) ranging from low (blue) to high (red) is shown in the legend (upper left). E6-E8, embryonic days, LV, left ventricle, RV, right ventricle.
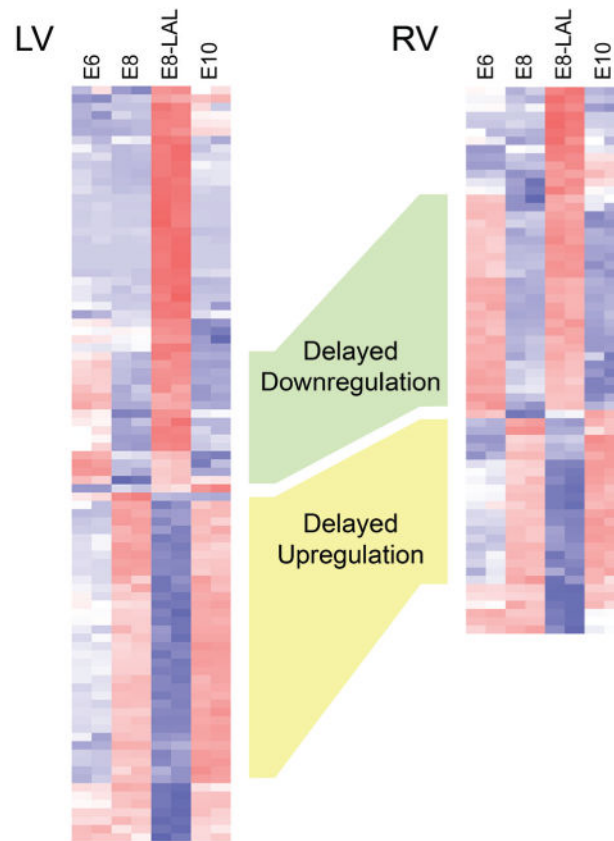
Fig. 3.
Heat map of changes in gene expression patterns in chick experimental left heart hypoplasia at embryonic day 8. For developmental perspective and interpretation, expression levels of particular genes at stages 48 hours before and after are added. Comparing the color (blue for decrease, red for increase), it is obvious that the changes indicate shift towards earlier expression pattern in both ventricles in the majority of differentially expressed transcripts. E6-E8, embryonic days, LV, left ventricle, RV, right ventricle, H, experimental (hypoplastic) group. Detailed description of all differentially expressed transcripts is provided in Supplemental Table 2 (in on-line version only).

Table 1

Functional categories enriched for 302 genes differentially expressed between left and right ventricles at E6, E8 and E10 developmental stages.

Biological Process	Process ID[1]	Count[2]	P-Value[3]
Appendage development	GO:0048736	10	<0.0001
Heart development	GO:0007507	11	<0.0001
Embryonic morphogenesis	GO:0048598	12	<0.0001
Heart morphogenesis	GO:0003007	6	0.0001
Growth factor activity	GO:0008083	8	0.0003
Growth	GO:0040007	7	0.0004
Cell-cell signaling	GO:0007267	8	0.0006
Sensory organ development	GO:0007423	8	0.0008
Palate development	GO:0060021	4	0.0011
Positive regulation of cellular protein metabolic process	GO:0032270	6	0.0012
Cardiac cell differentiation	GO:0035051	4	0.0016
Skeletal system development	GO:0001501	8	0.0021
Positive regulation of phosphorylation	GO:0042327	5	0.0021
Forebrain development	GO:0030900	6	0.0028
Extracellular region	GO:0005576	18	0.0034
Tube development	GO:0035295	7	0.0047
Transforming growth factor-beta-related	IPR:015615	4	0.0048

[1] Biological process ID from the Gene Ontology or Interpro database.

[2] Number of unique genes associated with the biological process functional tag.

[3] P-value for biological process enrichment.