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The influence of altered circulating blood volume on ventricular morphogenesis in the chick embryo: analysis by scanning electron microscopy

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SUMMARY

Cardiac morphogenesis represents a balance of myocardial differentiation, growth and remodelling, and congenital cardiovascular malformations likely reflect a range of similar final common pathways generated by numerous primary genetic and environmental abnormalities. In the present work, we analyzed the morphology of the developing ventricle in the chick embryo following experimental alterations in total circulating volume. In parallel experiments, we injected three different volumes of blood into a branch of the right vitelline vein at stages 22, 25 and 29 of Hamburger and Hamilton (n≥10 per group). The embryos were reincubated to stage 35 of Hamburger and Hamilton and then processed for morphological analysis using scanning electron microscopy. 39% of the surviving operated embryos contained ventricular malformations, including disordered trabeculation and patterns of either cardiac dilation or cardiac hyperplasia. Despite the high prevalence of disordered trabecular morphogenesis, we identified very few ventricular septal defects. Thus, while alterations in ventricular loading conditions may alter morphogenesis, cardiomyopathic phenotypes may be separated from defects in ventricular septation.

Key Words: Chick embryonic heart – Myocardium – Hemodynamic – SEM

INTRODUCTION

Myocardial architecture can be influenced both genetically (Sucov et al., 1994; Kastner et al., 1994; Jaber et al., 1996) and by epigenetic mechanisms (Clark et al., 1984; Pexieder et al., 1992).

Embryologists have long speculated about the role of hemodynamic function in the structural development of the cardiovascular system. The difficulty in establishing a causative link between form and function during heart development has been due in part to the limitations of the techniques available to quantify intracardiac blood flow. Descriptions of normal intracardiac flow patterns are somewhat contradictory. By direct observation of moving blood cells in the embryonic heart, two spiral blood cell streams were described (Jaffee, 1978). An important role was attributed to these spiral streams in the formation of the atrial, ventricular, and aortopulmonary septa. It was proposed that septa would arise between the separate streams due to reduced internal pressure (De Vries and Saunders, 1962; Jaffee, 1978). Furthermore, defects in septation were explained

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as a result of abnormal spiral streams (Jaffee, 1978). Unfortunately, these two streams turned out to be an optical illusion caused by the non concentric cardiac contraction pattern (Steding and Seidl, 1990). In addition, subsequent visualisation of blood flow with dye indicators failed to demonstrate the spiral blood streams described earlier, although specific intracardiac blood flow patterns during development were reported (Hogers et al., 1995).

The influence of hemodynamics on heart morphogenesis is now well recognised. Several studies have investigated the effect of altered blood flow on heart development by manipulations at both the arterial and the venous pole, or directly on the heart, or employing physical or chemical teratogenic factors.

The heart has considerable functional plasticity enabling compensation when functional demands are changed abruptly. In the presence of a chronic hemodynamic overload, the type of response depends on the age of the animal. The early embryonic myocardium alters myocardial mass via myocyte hyperplasia and cell death (Rakusan, 1984), in contrast to the adult myocardium, which alters myocardial mass by myocyte hypertrophy or cell death (Anversa et al., 1983; Rakusan, 1984; Bugaisky and Zak, 1986; Hamrell and Alpert, 1986). While the mechanisms responsible for producing hypertrophy in the adult heart have been addressed in numerous studies, little is known about the stimuli required for alterations in myocardial growth during heart development. The embryonic or fetal heart responds to altered load conditions in a different manner. Increased pressure load induced in the fetal lamb heart (Fishman et al., 1978), or in the chick embryonic heart (Clark et al., 1989) or in the fetal guinea pig heart (Saiki et al., 1997) results in cardiomyocyte hyperplasia rather than hypertrophy. Sedmera et al. (1999) reported a differential response of the chick embryonic heart to changes in pressure and volume loading. Thus, increased pressure load is a powerful growth stimulus for hyperplastic cardiomyocyte growth while increased volume load is gradually compensated in three steps: first, dilation; second, proliferation of trabeculae; third, thickening of the compact myocardium. Pressure load is a more powerful stimulus for heart growth than volume load (Zak, 1974).

With a view to studying the long-term effect of increased volume load in ventricular morphogenesis, an embryonic chicken model was developed. Surprisingly, the same alteration in circulating blood volume produced two distinct phenotypes: cardiac hyperplasia and cardiac dilation. As occurs in the clinical setting, both phenotypes represent an imbalance between cardiac load and function.

Materials and Methods

Fertilized White Shaver strain eggs (*Gallus domesticus*) were cleaned with a 50% alcohol-ether solution and incubated horizontally in an incubator (Memmert-UV) at 37-38°C and 75% relative humidity. We extracted 1.5 cm^3 of albumin at 36 to 48 hours of incubation and then reincubated the eggs to the desired developmental stage. The chick embryos were staged according to the age-determination criteria of Hamburger and Hamilton (1951). We studied the following 5 groups of embryos:

Control embryos

We first examined control embryos from stages 17 to 35 (n=5 per stage) in order to generate control data for comparison with the experimental embryos. When the desired stage had been reached, individual embryos were removed from the egg, cleaned in saline phosphate buffer (PBS) (0.1 M and pH=7.2-7.4) and staged. Embryos were fixed in two different ways, depending on the stage: stage 17 to 30 embryos were perfused via the dorsal aorta while stage 31 to 35 embryos were perfused via the umbilical vein. We used 1% sodium heparin in PBS diluted 1:1 to wash out the blood followed by a 2% glutaraldehyde mixture to fix the heart internally. The hearts were subsequently dissected and post-fixed by immersion in 2% glutaraldehyde for 24 hours at 4°C, washed in pre-cooled PBS and then dissected along orthogonal planes (transverse, coronal or sagittal). Following gross sectioning, the embryos were dehydrated in a graded series of acetone, desiccated in a critical point dryer (Hitachi Critical Point Dryer HCP-1), mounted in a viewer for SEM, sputtered with gold (Fine Coat Ion Sputter JFC-1100) and then imaged using SEM (JEOL JSM-35 CF).

Donor embryos

Day 12 to 16 chick embryos were utilized as blood donors in the following manner. Each egg shell was carefully opened to expose the extraembryonic membranes. A chorioallantoic vessel was then located, dissected, and then incised allowing blood to drain into the mouth of a sterile flask. We extracted between 500 and 1,500 µl of blood from each embryo, depending upon embryo stage. Aliquot of 500 µl of blood were conserved using 2 µl of 1% sodium heparin.

Experimental embryos

The embryo was exposed via a window in the shell and an incision in the extraembryonic membranes. We then injected a volume of donor blood corresponding to 7, 15 or 25% of the total circulating volume through a branch of the right

vitelline vein at stages 22, 25 and 29 HH (n>10 per group). The technique employed, described by Pérez-Miguelsanz et al. (1989), involves sterile injection of the blood into the bifurcation of a distal right vitelline vein. A glass capillary needle with diameter of 10 to 20 µm, obtained using a horizontal pipette puller (PUL-1 WPI), was calibrated and connected through a series of plastic tubes of increasing diameter to an insulin syringe. The time of perfusion was 5-15 minutes. The window in the shell was then sealed with parafilm. All embryos were reincubated to stage 35 HH and then analyzed as described for the control embryos.

The choice of injection via the right vitelline vein and not the left vein was based on our previous results (Puerta et al., 1987, 1989 and 1994; Pérez-Miguelsanz et al., 1989), in which right vitelline vein injection produced a higher rate of survival than injection via the left vitelline vein.

The three volumes of blood injected at each stage were calculated based on total circulating blood volume data from Rychter et al. (1955). We selected these volumes to standardise the volumes perfused by Pérez-Miguelsanz et al. (1989). The stages of intervention (22, 25 and 29 HH) were selected because between stages 21 to 31 HH the heart undergoes the greatest number of morphogenetic changes.

Heparin controls

The stimulating role of heparin in blood vessel neoformation in the chorioallantoic membrane is well known (Folkman, 1985; Ribatti et al., 1987). Accordinglly this reason and in order to eliminate the possible involvement of heparin in ventricular malformations, as well as observing the vasculature of the chorioallantoic membrane of all the embryos studied in ovo, we obtained the following control embryos. We injected, as described for the experimental embryos, 0.1, 0.2, and 0.3 µl of heparin diluted in 2.5 µl of tyrode through a branch of the right vitelline vein at stages 22, 25 and 29, respectively (n=5 per stage). The three amounts of heparin injected correspond approximately to the heparin diluted in the donor blood, which corresponded to 25% of the total circulating volume at each stage. All embryos were reincubated to stage 35 HH and then analyzed as described for the control embryos.

Tyrode controls

These embryos were injected as described for the experimental embryos, with 2.5 µl tyrode alone at stages 22, 25 and 29 (n=5 per stage). This amount of tyrode corresponds to the tyrode injected in the heparin controls. All embryos were reincubated to stage 35 HH and then analysed as described for the control embryos.

The thickness of the compact layer and ventricular cross sectional area were measured on SEM photographs. These measurements gave a general idea about heart shape and dimensions. Trabecular orientation and morphology were evaluated visually.

Statistical analysis of quantifiable data was performed by application of the χ^2 distribution. Results $P < 0.05$ were considered significant.

Results and Discussion

Methodological aspects

We chose to increase circulating blood volume using chick blood rather than a mineral solution in order not to introduce a change in hematocrit or oxygen-carrying capacity as a potential teratogenic factor. With this, the hemoglobin and hematocrit values of the blood injected are higher than those of the blood of operated embryos at the moment of intervention (Rychter et al., 1955). Furthermore, blood flow viscosity increases in a semilogarithmic way, depending on the hematocrit status (Meier, 1987), leading to a decrease in the tendency toward turbulent flow.

Chicken inmune system is not well developed until satge 45 HH (Le Douarin et al., 1984) so using of donnor chicken blood was not mistaken by immunologic mechanisms.

The peripheral injection site was chosen to limit the possibility of an acute direct mechanical effect on the heart.

Control embryos

The normal ventricular trabeculation and septation in chick embryos has recently been studied by Sedmera et al. (1997, 2000) and by Murillo-González et al. (1997). It is appopriate, however, to comment on several aspects of normal trabeculation in order to put the findings obtained with the experimental embryos in perspective.

By stage 35, the external morphology of the embryonic chick heart is similar to the "mature heart", and despite continued growth there are few important later morphological events. The cavities appear clearly defined by their corresponding septum. At this stage of development, the most trabeculae have been incorporated by compaction into the free ventricular walls and interventricular septum. It is important to note that myocardial wall thickness is not uniform. The adult avian left ventricular compact layer is about five times thicker than the right one. The pattern of trabeculation is ventricle-specific. In the left ventricle, it is composed of longitudinal, slightly spiraled ridges, stretched between the apex and the mitral orifice; the trabeculae are coarser and less branched, and the intertrabecular spaces larger and more oblong. In the right ventricle they are arranged in an anticlockwise

spiral, which begins at the cranial end of the muscular interventricular septum and courses first to the apex and then towards the conotruncus (Fig. 1A) (Sedmera et al., 1997; Murillo-González et al., 1997).

The morphology of tyrode– and heparin-injected embryos does not differ from that of normal embryos.

Experimental embryos

Between stages 21 to 31 HH, the ventricle undergoes extensive remodelling. Adequate loading is important for nomal heart morphogenesis and the development of typical myocardial patterns. Therefore, it is not surprising that alterations in mechanical load could lead to malformations or death of the embryo.

The mortality of the experimental embryos was 43% (Table 1). We have no data about these hearts because the dead embryos were discarded.

The incidence of ventricular malformations was 39% (Table 1), divided into four principal groups: disordered trabeculation, cardiac dilation, cardiac hyperplasia and ventricular septal defects (VSD). We noted double-outlet right ventricle (DORV) in one case.

Embryos operated on stage 25 showed a higher mortality and had the highest incidence of heart malformations as compared with embryos operated on stages 22 and 29. Thus, stage 25 is a critical period in heart chamber morphogenesis. Between stages 23 to 30 HH, the ventricle undergoes extensive remodeling, and it is therefore not surprising that alterations in mechanical load could lead to malformations or death of the embryo.

Application of the χ^2 distribution test, like the test of variable independence, revealed the absence of significant differences among the frequencies corresponding to the different variables considered (number of experimental embryos surviving, cardiac malformations, stage of intervention and increase in volume).

In the group that corresponding to disordered trabeculation, we observed both delayed and abnormal trabecular patterns. The delayed trabecular pattern was characterized by the presence of large trabecular cords and delayed trabecular compaction in the ventricular free wall and interventricular septum (Fig. 1B, C), the persistence of immature trabeculae with large interfascicular spaces, and wide spaces between the endocardium and myocardium containing abundant residual matrix fibrils. The group with altered trabeculation patterns was characterized by the presence of anomalous trabeculae, atypical trabecular morphology, and altered trabecular orientation in both ventricles (Fig. 1D).

The group of embryos with cardiac dilation showed an increased diameter of one or both ventricular chambers, with no comparable increase in wall thickness (Fig. 1E). In contrast, embryos with cardiac hyperplasia showed an increase in ventricular wall thickness in one or both ventricles, with no increase in cavity dimensions (Fig. 1F). In both groups we observed disordered trabeculation.

Here we use the term cardiac hyperplasia rather than cardiac hypertrophy, based on the following data. In the group of embryos with cardiac hyperplasia, the size of the myocytes (as appreciated under high power view in SEM) was not different from the controls. Furthermore, several studies have shown that the embryonic heart responds by cardiac hyperplasia rather than hypertrophy in the presence of a chronic hemodynamic overload (Fishman et al., 1978; Clark et al., 1989; Saiki et al., 1997; Sedmera et al., 1999).

Sedmera et al. (1999) have shown that increased volume load to the chick embryonic right ventricle is preferentially compensated by cardiac dilation and only later followed by trabecular proliferation and thickening of the compact myocardium. We observed similar modifications at ventricular level, but were unable to establish a chronological sequence of their appearance because all the hearts were studied at the same stage (35 HH).

Table 1.- Mortality and cardiac malformations.

Stage	↑ blood volume	Mortality/ total	Abnormal/ survivor	DT	CD	CH	VSD	DORV
	7%	5/19	3/14	2	1	0	0	0
22	15%	11/21	2/10	1	0	0	1	0
	25%	2/12	3/10	1	1	1	0	0
	7%	12/22	5/10	3	1	1	0	0
25	15%	19/31	9/12	7	1	1	0	0
	25%	11/21	5/10	3	2	0	0	0
	7%	4/16	5/12	1	3	0	1	0
29	15%	5/17	3/12	2	0	0	0	1
	25%	8/18	4/10	3	0	0	1	0
Totals		77/177	39/100	23	9	3	3	1

DT: disordered trabeculation; **CD**: cardiac dilation; **CH**: cardiac hyperplasia; **VSD**: ventricular septal defect; **DORV**: double outlet right ventricle.

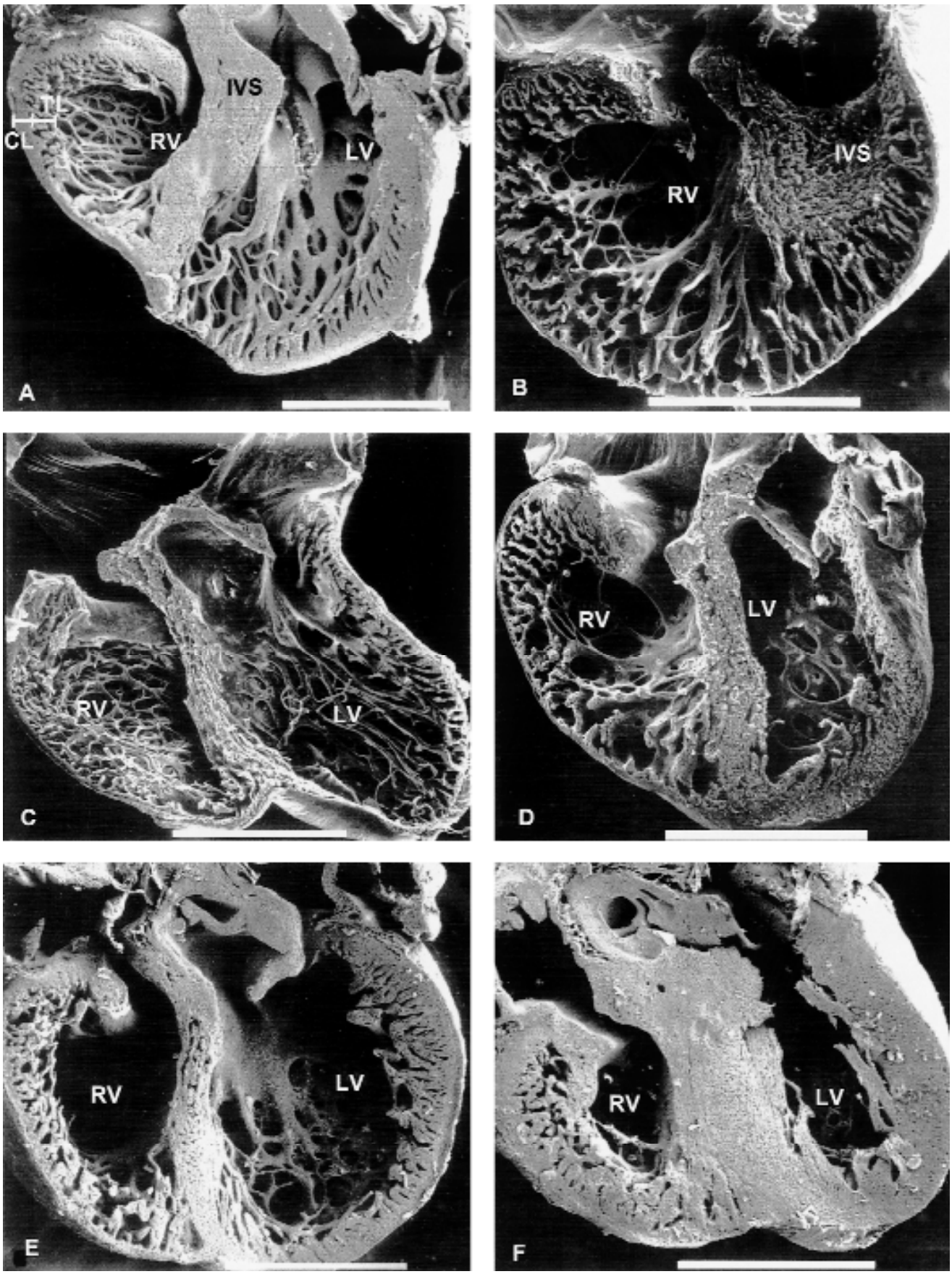

Fig. 1.- Scanning electron micrographs of frontal sections, dorsal portions and ventral views of stage 35 HH hearts. **A:** Control embryo. At the ventricular wall of the right ventricle the compact (CL) and trabecular layers (TL) are marked. **B:** Experimental embryo (↑ 25% at stage 29 HH). Note in right ventricle the presence of large trabecular cords and delayed trabecular compaction. **C:** Experimental embryo (↑ 25% at stage 22 HH). Note in both ventricles fine trabeculation, delayed trabecular compaction, shrinkage of compact layer and marked dilation. **D:** Experimental embryo (↑ 7% at stage 29 HH). Note an anomalous trabecular coarsening in both ventricles. **E:** Experimental embryo (↑ 15% at stage 25 HH). Cardiac dilation. **F:** Experimental embryo (↑ 15% at stage 25 HH). Cardiac hyperplasia. LV: left ventricle; RV: right ventricle; IVS: interventricular septum. Calibration bars =1000 µm.

The most frequently observed malformation following increased circulating blood volume was disordered trabeculation. During normal development, stage-specific intracardiac blood flow patterns occur and these are considered to play an important role in heart development (Hogers et al., 1995). The method used by us to increase intravascular volume probably altered intracardiac blood flow, similar to other hemodynamic alterations associated with cardiac malformations. Alterations in the relationship between intraventricular pressure and volume, myocardial wall stress and strain, and simultaneous myocardial morphogenesis likely influenced the rate and extent of trabecular remodelling. It is important to note, however, that the current study does not provide specific data on the direct forces necessary to alter trabecular morphogenesis.

In relation to cardiac dilation and cardiac hyperplasia, one unanswered question is why some embryos responded to increased circulating blood volume with dilation while others developed myocardial hyperplasia. Anomalies in the extracellular matrix have been noted in embryonic cardiomyopathies generated by an altered total circulating volume (Puerta et al., 1994). Puerta et al. (1989) used SEM and TEM on embryonic hearts following unilateral vitelline vein ligation. They observed alterations of trabecular patterns, as well as changes in the structure and assembly of ventricular myofibrils. We believe that the combination of an altered extracellular matrix and myofiber assembly may be due to altered cardiomyocyte cytodifferentiation, since these cells are partly responsible for extracellular matrix secretion.

It is possible that subtle alterations in the extracellular matrix or cell-to-cell connections might differentiate the two outcomes and future studies are needed to define these critical aspects of the developing myocardium.

Similar to other long-term hemodynamic interference studies (Jaffee, 1978; Clark et al., 1984; Hogers et al., 1997; Sedmera et al., 1999), we observed VSD and DORV. The frequency of VSD was 3%, which is considerably lower than the 56% recently observed by Hogers et al. (1997) in a left vitelline vein model and the 25% observed by Sedmera et al. (1999) in conotruncal banding and left atrial ligation models. The frequency of DORV was 1%.

Based on our data, we believe that some congenital cardiac malformations may result from modifications of intravascular volume during the period of heart morphogenesis. Despite the high prevalence of disordered trabecular morphogenesis, we identified very few ventricular septal defects. Thus, while alterations in ventricular loading conditions can alter morphogenesis, disorders in trabeculation may be separable from defects in ventricular septation. In most affected embryos, the primary morphologic lesion was disordered trabecular morphogenesis, cardiac dilation and/or hyperplasia. The literature contains several clinical cases describing cardiomyopathies associated with abnormalities at umbilical cord level (Heifetz, 1984; Byrne and Blanc 1985; Ricklan et al., 1988; Gnirs et al., 1988; Philippe, 1989) and at placental level (Fok et al., 1990) that could result in altered hemodynamics. Thus, successful morphogenesis of the embryonic myocardium depends upon both genetic programming and a proper mechanical and hemodynamic environment.

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