Congenital Heart Disease, Heterotaxia and Laterality

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Congenital heart disease occurs in about 0,8% of all newborns. Many cardiac malformations occur among relatives and have a polymorphic presentation. The origin of most congenital heart disease is thought to be multifactorial, implying both anomalous expression of genes and the influence of epigenetic factors. However, in a small number of cases, the origin of congenital heart disease has been directly related to chromosomal anomalies or to defects in a single gene. Curiously, defects in a single gene can explain a polymorphic presentation if the anomalous gene controls a basic embryonic process that affects different organs in time and space. Some of these genes appear to control the establishment of laterality.

The establishment of the left-right asymmetry starts at the Hensen node. Here, the initial embryonic symmetry is broken by cascades of gene activation that confer specific properties on the left and right sides of the embryo. Although there are variations between species, some basic patterns of gene expression *(Nodal, Pitx2)* appear to be maintained along the phylogenetic scale. Anomalous expression of these genes induces the heterotaxia syndrome, which usually courses with congenital heart disease. The development of heart malformations is illustrated with the mouse mutant *iv/iv*, which is a model for the heterotaxia syndrome and the associated congenital heart disease.

Key words: *Congenital heart disease. Heterotaxia. Left-right asymmetry.*

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Malformaciones cardíacas, heterotaxia y lateralidad

Las malformaciones cardíacas ocurren en aproximadamente el 0,8% de todos los nacidos vivos. Muchas de estas malformaciones se presentan en grupos familiares y muestran un tremendo polimorfismo. El origen de la mayor parte de las malformaciones cardíacas se desconoce, estableciéndose lo que se ha llamado un origen multifactorial. Aunque este término implica la expresión anómala de genes y la intervención de factores epigenéticos, el desarrollo de las malformaciones cardíacas se asocia en algunos casos a anomalías cromosómicas o a defectos de un único gen. Curiosamente, defectos de un único gen pueden explicar gran parte de las presentaciones polimórficas si este gen controla procesos embrionarios básicos que afectan, en tiempo y espacio diferentes, a distintos órganos. Algunos de estos genes parecen estar implicados en el establecimiento de la lateralidad embrionaria.

El establecimiento izquierda-derecha del eje embrionario comienza en el nódulo de Hensen donde se rompe la simetría inicial y se inducen cascadas de expresión génica que confieren a cada lado del embrión propiedades específicas. Aunque los desencadenantes de la ruptura inicial de la simetría varían entre las diferentes especies, existen patrones de expresión génica *(Nodal, Pitx2)* conservados a lo largo de la escala filogenética. La expresión anormal de estos genes induce la aparición del síndrome de heterotaxia, que se acompaña de malformaciones cardíacas. El desarrollo de estas malformaciones se ilustra con la mutante de ratón *iv/iv*, que constituye un modelo del síndrome de heterotaxia y las malformaciones cardíacas asociadas.

Palabras clave: *Malformaciones cardíacas. Heterotaxia. Asimetría izquierda-derecha.*

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INTRODUCTION

Cardiac malformations represent almost half of the malformations encountered at birth. Approximately 0.8% of all live births present this type of malformations.[1-3] The high incidence of congenital malformations of the heart has originated an intensive search to identify the factors involved in the development of these malformations. However, the results of this search have been disappointing and 90% of all the malformations continue to be of unknown origin. In these

ABBREVIATIONS

FISH: fluorescence *in situ* hybridization.
LRD: left-right dyneine.
Shh: sonic hedgehog.
Car: caronte.
BMP: bone morphogenetic proteins.
TGF: transforming growth factor.
CAVC: common atrioventricular canal.

cases we refer to a multifactorial origin, a term that indicates more about our ignorance of the topic rather than what we really understand. It seems logical, however, that the development of these malformations could be due as much to genetic as to environmental factors. In fact, it has been postulated that environmental factors, which act in genetically predisposed individuals, activate the anomalous expression of genes until the threshold of normality is breached, which is when they induce the development of a given malformation. The abnormal expression of all the genes involved would result in the production of a severe defect, often incompatible with life, while a disturbance in only part of these genes would cause much milder defects (or their absence). This would explain the presence of intermediate or subclinical forms that can be considered frustrated forms of the basic hereditary defect.[5-8] The variable phenotypical expression occurs in both family groups[3,4] and in animal models of cardiac malformations.[5,9,10]

CHROMOSOMAL ANOMALIES. SINGLE-GENE DEFECTS

Although our real knowledge of the origin of most of the cardiac malformations is fairly imprecise, it should not be overlooked that a genetic origin has been clearly established in a small number of cases. The relation between the presence of chromosomal anomalies and cardiac malformations is well known. These anomalies can be numerical, due to the absence of chromosomal disjunction, or structural, due to chromosomal breaks and loss of the broken fragment or its translocation to another chromosome. Among the numerical anomalies, trisomy 21 is associated in one-half of the cases with complex malformations, especially common atrioventricular canal and ventriculoarterial discordance.[2] The study of cases with partial trisomy of this chromosome has shown that the origin of the abnormal cardiac phenotype resides in band q22 of the long arm of this chromosome.[11] Trisomy 18 is accom-

panied by a large number of cases of atrial and ventricular septal defect, as well as valvular dysplasia in 100% of cases.[12] Trisomy 13 is associated with a high percentage of dextrocardia and tetrasomy of the short arm of chromosome 22 (cat´s eye syndrome) is associated with anomalous pulmonary venous return.[13] The same occurs in cases of failure of the disjunction of the sexual chromosomes, such as the Turner syndrome (which is associated with aortic coarctation and aortic stenosis) and Klinefelter syndrome (which is associated with tetralogy of Fallot and Ebstein anomaly).[14]

An important group of clinical syndromes that include cardiac malformations have been associated with specific deletions in different chromosomes. Deletion of the short arm of chromosome 5 (cat´s cry syndrome) or chromosome 4 (Wolf-Hirschhorn syndrome) are also accompanied by cardiac malformations. The development of new techniques like high resolution chromosomal banding and fluorescence *in situ* hybridization (FISH) has allowed the presence of minimal deletions in contiguous genes to be established, and has lead to the recognition of new syndromes like deletion of chromosome 22q11 (CATCH 22, velo-cardio-facial syndrome) and the Miller-Dieker (17p13.3) and Williams (17q11.23) syndromes, among others.[15]

The recognition of new syndromes does not directly explain the development of specific cardiac malformations or the severity of the syndrome. The fact that the search for anomalous genes now centers on increasingly smaller chromosomal segments has not yet made possible the massive identification of candidate genes. In fact, the mechanisms by which a gene or group of genes produce a specific syndrome vary widely. For example, it has been assumed that the loss of function of a dominant allele leads to specific syndromes. However, increased gene function, with the consequent increment in the amount of product of that gene (or anomalous production), can interfere with normal developmental mechanisms to produce a given syndrome.

Another alternative is that only the paternal or maternal allele of a certain gene is active in development (genomic impression). A defect in the maternal copy can be transmitted as an autosomal dominant defect, while the same defect in the paternal copy does not produce disturbances. Likewise, a defect of maternal origin can produce a certain syndrome, whereas the same defect of paternal origin produces a totally different syndrome, as occurs with the deletion of band q12 of chromosome 15 (15q12). In a similar way, the cardiac phenotype in Turner syndrome (45,X) seems to depend on the parental origin of the anomalous X chromosome.

Three percent of all cardiac malformations seem to be due to the action of a single gene. Within this group are included atrial septal defect associated with defects
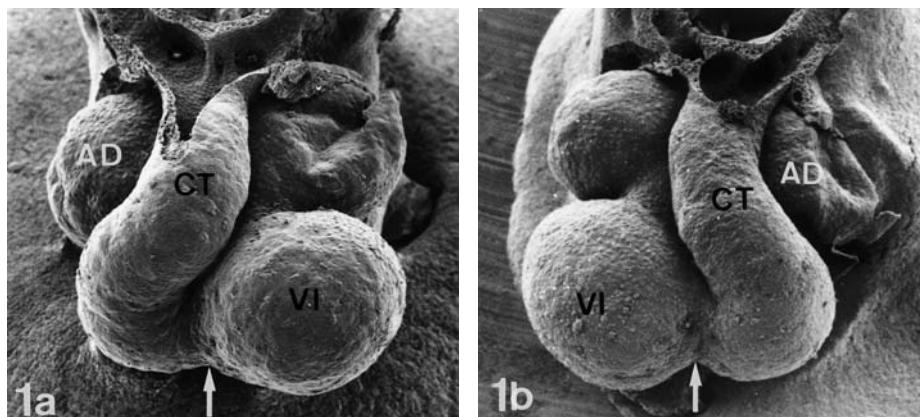
Fig. 1. Scanning electron microscopy. Mouse hearts, E10.5. *a)* situs solitus; *b)* situs inversus. Both hearts are morphologically normal and appear as mirror images. RA indicates right atrium; LV, left ventricle; CT, cono-trunk. The arrows in a and b indicate the interventricular furrow. a) b:×95. (Reproduced from Icardo, 1997.)

in cardiac conduction and hypertrophic subaortic stenosis.[4,8] The genetic origin is also clear in other anomalies like right ventricular dysplasia, some dilated cardiomyopathies, and complex atrioventricular septation defects, which seem to have an autosomal dominant transmission.[16-18] Likewise, the presence of cardiac malformations is frequent in coagulation disorders like von Willebrand syndrome or hemophilia.[8]

The existence of cardiac phenotypes originated by the loss of function of a single gene constitutes an attractive hypothesis for the study of cardiac development. In these cases, can the so-called multifactorial origin and polymorphic presentation be explained adequately? Recent evidence indicates that many polymorphic presentations are due to the action of a single gene. The concept of parsimony has been used to explain it. Throughout development, a single gene can control a basic morphogenetic process, such as the synthesis or degradation of a protein. That protein could be fundamental for the development of organs as different as the brain and kidney, so the gene has to be activated during embryogenesis at different times and in different places. Its deactivation would result in a series of defects in distant organs, and the severity of the presentation would depend on the capacity of each organ to supplement or compensate the genetic defect. In humans, various forms of defects with autosomal transmission and polymorphic presentation are apparently caused by deactivation of a single gene.[19] Some of these genes seem to be involved in establishing embryonal laterality. Their deactivation results in visceral spatial position anomalies and in a wide array of cardiac malformations.

SYMMETRY, ASYMMETRY, AND CARDIAC MALFORMATIONS

The design of the human body, like that of most vertebrates, has an evident bilateral symmetry with respect to the midline. However, this symmetry is not conserved inside the body since organ disposition is clearly asymmetrical. It is said that our body has a pseudobilateral symmetry. The visceral asymmetry is not limited to the thoracic and abdominal organs, it extends to the brain and nervous system organization. This is important in the functional specialization of the cerebral hemispheres and in behavioral aspects like the preferential use of one hand. The establishment of asymmetry in the nervous system seems to occur independently from that of the trunk, a question that we will not attempt to address in this article.

Among the first tasks of an embryo is to define the corporal plan, that is, to establish the primary embryonal axes. An anteroposterior or cephalocaudad axis is defined that will distinguish the cephalic and caudad ends, and a dorsoventral axis will distinguish the dorsal and ventral sides of the embryo. The left-right axis is automatically defined after these axes are formed.

The normal disposition of the heart and organs is called *situs solitus* (Figure 1). Although there is some confusion in the literature, *situs inversus* designates the perfect inversion of *situs solitus*, with the heart toward the right. Any different disposition is denominated heterotaxia or *situs ambiguus* (see below). The incidence of *situs inversus* is estimated at 1 in 10 000 births. The incidence of heterotaxia is generally much lower and usually accompanied by complex cardiovascular malformations.[20]

The relation between the presence of cardiac malformations and laterality defects has long been known.[21] An important number of heart diseases are accompanied by anomalies in the cardiac position, atrial isomerism, anomalous venous drainage, abnormalities in the form and position of the spleen, and anomalies in the position of the thoracic and/or abdominal viscera. Consequently, the asplenia-polysplenia syndrome has been described,[21-24] characterized basically by the tendency to visceral symmetry in organs that are normally asymmetrical. Another fundamental

aspect of this syndrome is its marked polymorphism, given that it is defined by the existence of a common cause with different final expressions.[25] The recognition of the existence of a single syndrome lead to a more general definition of heterotaxia, implying the presence of more or less complex anomalies in visceral and/or venous laterality. Heterotaxia also includes the absence of visceral asymmetry, a situation known as isomerism or sequence isomerism, which mainly involves the bronchi, lungs, and atria in the thorax.

Although the descriptions of heterotaxia were initially made in isolated cases, the study of large series has shown that in many cases there is a clear familial relation. The clearest example may be that of an Amish family with a high degree of consanguinity, in which various members had visceral *situs inversus* and cardiac malformations.[26] The study of this and other family groups[27-29] has revealed the presence of a genetic defect with an autosomal recessive, autosomal dominant, or even X chromosome-linked transmission.[30] Different degrees of heterotaxia are also observed in midline syndromes like the Meckel syndrome (*situs inversus* and polysplenia),[31] or Kartagener syndrome,[32] a primary ciliary anomaly characterized by bronchiectasia and, in 50% of cases, *situs inversus*. The latter syndrome is due to ciliary hypomotility caused by the absence of the external arms of microtubular dyneine.[33,34]

ANIMAL MODELS OF HETEROTAXIA

The existence of a mutant race of mice with the heterotaxia syndrome has opened new areas of investigation. The *iv/iv (inversus viscerum)* mutant strain has long been known,[35] but only recently was the existence of cardiac malformations in the embryonal products discovered.[36,37] Adult mice exhibit inversion of the cardiac site in 50% of the cases and a percentage close to 30% of visceral and/or venous heterotaxia. The heterotaxia includes anomalous venous return, portal vein in a ventral position, hepatic and pulmonary isomerism, atrial isomerism, polysplenia, and thoracoabdominal visceral discordance. In addition, the embryos have cardiac malformations in 45% of cases.[9,10,36,38,39] A careful characterization of these hearts has made it possible to recognize the existence of a typical malformation, the so-called bulboventricular loop, characterized by persistence of the sinus venosus, common atrioventricular canal (CAVC), and double outlet right ventricle (DORV).[9,10] This is the basic defect inherited, which normally courses with atrial isomerism. As occurs in human syndromes, the presentation is polymorphic, with simple atrial or ventricular septal defect occupying the opposite extreme of the phenotypical spectrum.[9,10,39] In addition, presentation is independent of sex. The same as in humans, the *iv* gene seems to exhibit complete dominance in such a way that, in ab-

sence of its function, the visceral site is determined randomly. The absence of this genetic control explains the different patterns of heterotaxia[35] as well as variations in the cardiac phenotypes.[40,41]

Another mutant strain of mouse, the so-called *legless* strain obtained by transgenic insertion, shows craniofacial and limb anomalies and *situs inversus* in 50% of cases.[42] The *inv/inv* mice, another mutant strain obtained by transgenic insertion,[43] show total *situs inversus* in 90% of cases, venous heterotaxia, anomalies of the right cardiac outflow tract and ventricular septal defect.[44]

The *iv* gene seems to be found 3 centimorgans from the gene of the heavy chain of immunoglobulin Igh-C in chromosome 12 of the mouse,[45,46] which is the equivalent of human chromosome 14. Interestingly, the transgenic insertion in the *legless* mouse is also located in chromosome 12, close to the site of the *iv* gene,[47] suggesting that the mutation could have affected the *iv locus*. While the *iv* and *lgl* mutations produce randomization of the visceral site, the mutated gene in the *inv* strain, which encodes inversin, is located on chromosome 4 and seems to direct the visceral site.[48] However, it could also be a mutation due to loss of function. The reason why genetic controls for the establishment of visceral site are located in such different positions is not known, but it suggests a close and complex regulation.

The identification of the different mutated genes in these strains of mice has clarified important aspects of their function. The modified gene in the *iv* and *lgl* strains encodes a dyneine associated with ciliary microtubules,[49] which is why it has come to be known as LRD (left-right dyneine). When this protein is deactivated in transgenic mice, the laterality anomalies found in *iv* and *lgl* mice are reproduced.[50] Other strains of mutant mice with alterations in the morphogenesis of the cilia [51,52] also present laterality anomalies. Curiously, the identification of inversin as the product of the mutated gene in the *inv/inv* strain does not explain any aspect of its function.

Many of these mutant mice strains do not show structural ciliary anomalies. This suggested that there was no relation with human Kartagener syndrome, where these structural anomalies do exist. However, patients with Kartagener syndrome also exhibit mutations in the dyneine proteins,[53] which suggests that many syndromes with laterality abnormalities have a common origin. More recent advances in molecular biology and new detection techniques have made it possible to prepare a complex picture, as yet incomplete, which includes the expression in cascade of a series of genes, the concurrent expression of other genes with the cascade, and ciliary activity in the node (of Hensen) or organizer during embryonal gastrulation stages. All these factors are involved in establishing normal laterality and their disruption causes late-

rality defects in both humans and animal models.

ESTABLISHMENT OF LATERALITY

In the initial stages of development, the embryo appears symmetrical with respect to the midline. Although a mild transitory asymmetry in the morphology of the Hensen nodule has been described in chick embryos,[54] the first clear evidence of morphological asymmetry emerges with the formation of the cardiac loop.[55] The heart, which at first is tubular and medial, invariably curves to form a loop to the right. Upon continuing embryonal development, the rest of the organs progressively acquire their characteristic asymmetrical distribution.

Conceptually, establishment of the left-right axis takes place in three phases.[56,57] In the first phase, the initial symmetry of the embryo disappears, specifying two unequal halves, one right and one left. The initial break in symmetry takes place during gastrulation, in relation with the nodule of Hensen. In a second phase, and as a consequence of the previous phase, numerous genes are expressed asymmetrically, to the left or to the right, thus giving identity to each embryonal side. Most of these genes encode signaling molecules that interact to establish signaling cascades. These cascades of asymmetrical expression start around the nodule and eventually end by establishing wide domains of asymmetrical gene expression in the lateral mesoderm. Finally, this gene expression translates into the normal asymmetrical morphology of the organs.

The factors involved in the initial break in symmetry are still largely unknown. In the mouse and, by extension, in mammals, the novel and attractive model of «nodal flow» has been proposed.[58-60] The cells of the node have a single cilium on their ventral surface. These cilia have a vortical movement that, in conjunction with other cells, produce a leftward flow of the perinodal fluid. It is postulated this flow causes an asymmetrical distribution of a postulated, but as yet not identified morphogen, which is responsible for initiating the path of left-right signaling. In fact, the flow of perinodal fluid to the left is very weak in *inv/inv* mice and does not take place in *iv/iv* mice due to the absence of ciliary motility. The ciliary flow could also be altered in other mutant strains of mouse characterized by the abnormal morphogenesis of nodal cilia,[50,58] or their absence.[51] The human syndromes with which site alterations are associated with primary ciliary anomalies[61,62] constitute an important support for this model.

However, the model of nodal flow may not be valid in other species. In the chick embryo, monociliary cells are irregularly distributed on the ventral and dorsal surface of the embryo, constituting only part of the cells of the nodule of Hensen.[63] Thus, the hypothesis of nodal flow cannot easily be applied to the chick embryo. In addition, it has been demonstrated that some genes are expressed asymmetrically before formation of the node.[64] It has thus been proposed that in birds and amphibians the break in symmetry originates in the tissues that surround the node.[65-68]

A hypothesis that is currently under study involves the cellular *gap* type junctions that are established in the tissues that surround the node. If one small molecule were capable of circulating through those junctions in single direction, molecules would accumulate on one side of the midline, thus disrupting symmetry and triggering a response of asymmetrical gene activation in the node.[67] In any case it seems that in all the species studied, the initial break in symmetry is related to the nodule of Hensen.[64] Parting from the node, in a second phase the specific gene cascades of laterality are activated, in such a way that the asymmetrical information is reinforced and transmitted to the lateral mesoderm (Figures 2 and 3). The derivatives of the lateral mesoderm will form the asymmetrical organs.

In the chick embryo it has been demonstrated that various signaling molecules show small domains of asymmetrical expression in the nodule of Hensen. Among these molecules are established regulatory loops that control the asymmetry of laterality (Figure 2). For example, the asymmetrical expression of *Sonic hedgehog (Shh)* on the left side of the node is essential for the correct development of laterality. At first, *Shh* is expressed symmetrically in the node but, due to negative signaling mediated by ActivinβB and *Bmp4*, its expression is repressed on the right side and remains confined to the left side. The asymmetrical expression of *Shh* on the left side of the node induces the asymmetrical expression of *Nodal* in the left lateral mesoderm.[67,69-76] *Nodal*, in turn, induces the expression of *Pitx2*.

The induction of *Nodal* by *Shh* is not direct, but mediated by an intermediate factor recently identified as *Caronte (Car)*.[64,77-79] *Car* is included in a group of molecules (the family of Cerberus/DAN) that act as antagonists of bone morphogenetic proteins (BMP). Various BMP are expressed in the lateral mesoderm in a lateral domain of *Nodal*. Since *Car* blocks the activity of these BMP, it has been proposed[77,78] that the function of *Car* is to antagonize the repressive effect of the BMP on *Nodal* expression (Figure 2). However, we have recently demonstrated that BMP signaling positively regulates *Nodal* expression.[80] BMP activity induces the expression of CFC *(Cripto/FRL-1/Cryptic)*, which is the only member of the family of EGF (epidermal growth factor)-CFC identified in chick embryo to date.[81,82] CFC is an essential extracellular factor for *Nodal* signaling.[83] Although more studies are needed to clearly establish the role that the BMP play in left-right specification, the existing discrepancies demons-
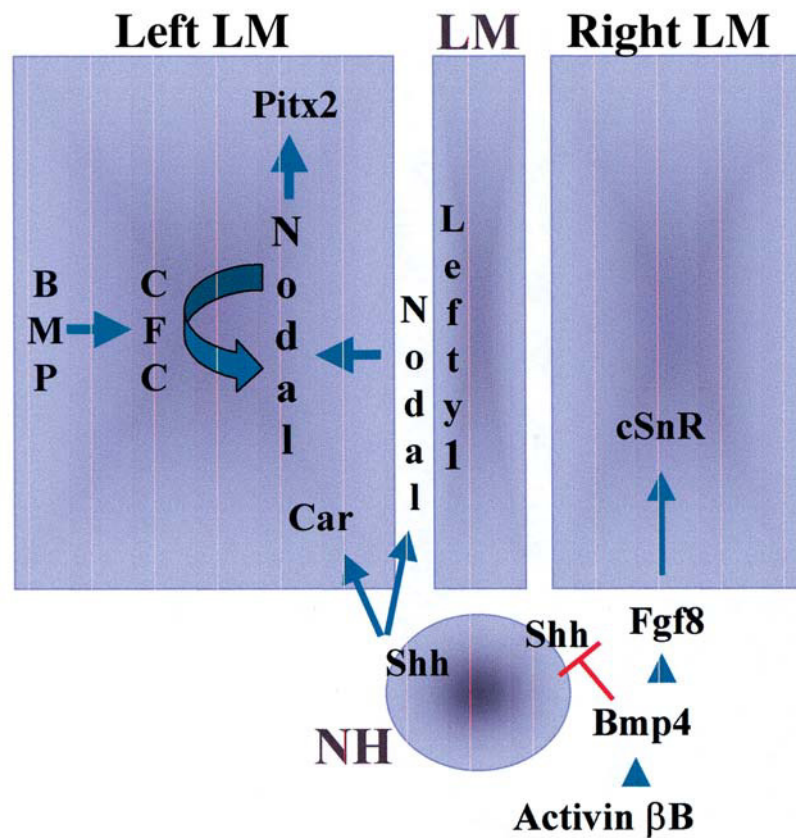
Fig. 2. In the chick embryo, the initially symmetrical expression of *Shh* in the nodule of Hensen (NH) is restricted to the right side by the activity of activinβ mediated by *Bmp*4. Shh induces the expression of Lefty1 in the left half of the midline as well as *Nodal* and *Car*. The expression of Nodal in the left lateral mesoderm (LLM) is facilitated by the expression of CFC under the control of *BMP*. On the side right, the activin pathway induces Fgf8, which in turn induces cSnR and prevents the expression of Nodal.

trate the complexity of the regulation established between the different molecular pathways that control laterality.

In the chick embryo, activation of the activin pathway on the side right of the nodule of Hensen results in the right expression of *Bmp4*. In turn, *Bmp4* inhibits the right expression of *Shh* and induces the expression of *Fgf8* which, in turn, induces the expression of the *Snail* (cSnR) transcription factor in the right lateral mesoderm. These activation sequences are specific to the right side.

Since specific signaling pathways are established on each embryonal side, it is important that the information on one side not pass to the opposite side. In this respect, the embryonal midline has a critical barrier role. The mutations that course with morphological or biochemical defects of the midline are accompanied by laterality disturbances. The expression of *Lefty1* on the midline (on the left half of the ground plate precursors; Figure 3) has been proposed as the molecule responsible for this barrier.[84] *Lefty1* is a member of the transforming growth factor-β (TGF-β) family and may carry out its function by blocking *Nodal*. Another member of

the same family, *Lefty2*, seems to control the temporal extension of the expression of *Nodal*.[84] An example that illustrates the importance of the midline in humans are the alterations of visceral site observed in Siamese twins. It has long been recognized that in twins joined by the trunk the twin on the right often presents visceral site alterations. This is interpreted as the influence of the left signaling cascade of the twin located on the left side over the twin on the right side.

We commented earlier that there seems to be a notable divergence between the different species in the beginning of left-right specification. However, very recent evidence indicates that the latter lateralization signals, as well as the pathways that modulate them, function similarly in humans. For example, mutations in the gene of the activin receptor[85] cause laterality disturbances and cardiac malformations. In particular, the pattern of expression of *Nodal* is markedly conserved in all the species studied to date, from the zebrafish to humans.[86] *Nodal* belongs to the TGF-β superfamily, is transitorily expressed in the left lateral mesoderm, and is considered a left determinant given that its expression correlates directly with the laterality
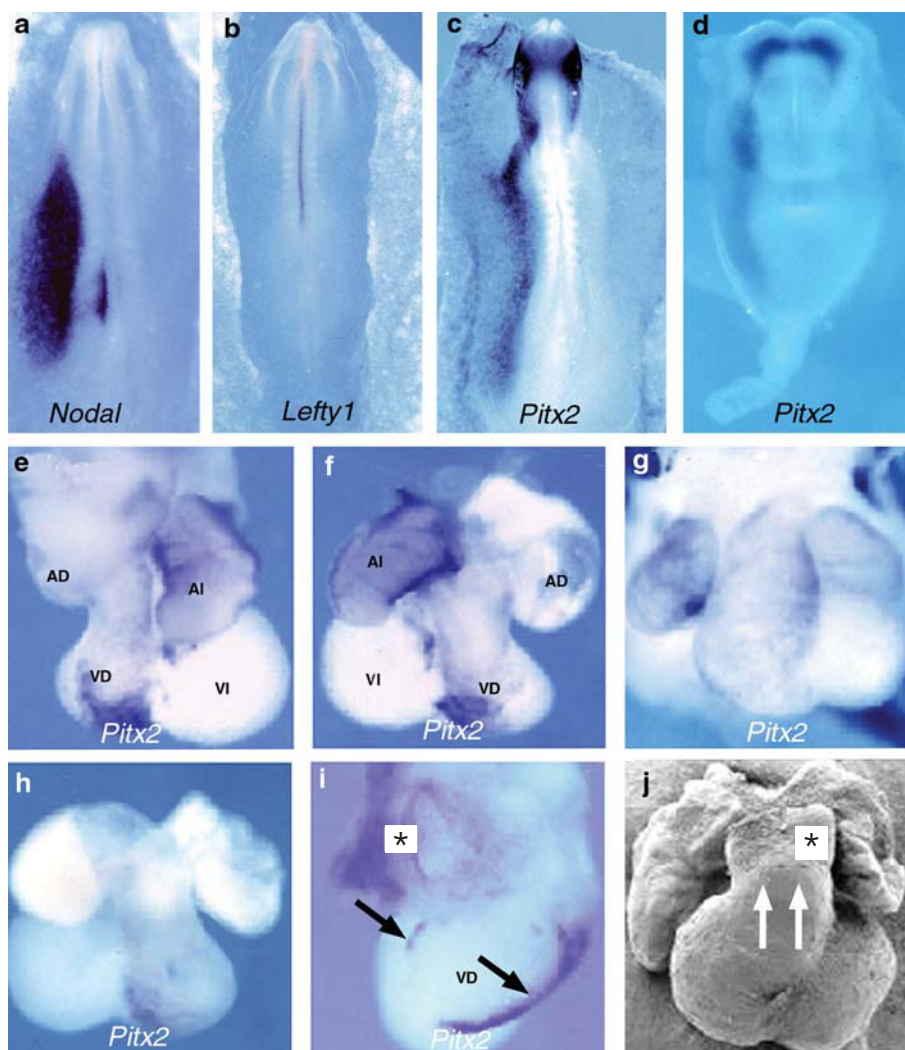
Fig. 3. a: Expression of Nodal in *a)* 4-somite chick embryo; *b)* expression of *Lefty*1 in a 4-somite chick embryo; c: expression of *Pitx2* in a 4-somite chick embryo; *d)* expression of *mPitx2* in a mouse embryo in E8.25; *e)* expression of *mPitx2* in the heart of an iv/iv mouse with right loop in E11.5; *f)* expression of *mPitx2* in the heart of an iv/iv mouse with left loop in E11.5; *g)* bilateral atrial expression of *mPitx2* in E11.5 with a loop to the right; *h)* absence of atrial expression of *mPitx2* in a heart with a loop to the left in E11.5; *i)* right view of the heart of an iv/iv mouse in E11.5 which shows expansion of the expression of *mPitx2* on the dorsal wall of the right ventricle; the right atrium (asterisk) is also positive; *j)* scanning electron microphotograph showing DORV (double date) in a heart that showed dorsal expression of mPitx2 in the right ventricle in E11.5. RA indicates right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle. (Figures e-j) reproduced from Campione et al, 2001.)

of the heart and other organs.[69,87] The conservation of the pattern of expression of *Nodal* and its target gene *Pitx2* (Figure 3) has meant that the stage in which they are expressed is denominated the left-right phylotypical stage of asymmetry.[88,89]

The third phase in the establishment of asymmetry is the translation of the previous gene expressions as the normal asymmetrical morphogenesis of the organs. *Pitx2* is the main target gene of *Nodal* to be identified to date.[90-94] *Pitx2* is expressed initially in a domain very similar to that of *Nodal*, but its expression continues while the asymmetrical morphogenesis of the viscera progresses, when the expression of *Nodal* has already been repressed. *Pitx2* is a transcription factor with a homeodomain of the *bicoid* type,[95] which has important functions during embryonal development in addition to its participation in asymmetry. *Pitx2* presents three isoforms denominated a, b and c. The asymmetrical expression of *Pitx2* corresponds exclusively to the *Pitx2c* isoform.[96,97] The study of transgenic mice in which the expression of *Pitx2* has been elimi-

nated or reduced (hypomorphic mutations)[98] indicates that the different organs have a variable sensitivity to the presence of *Pitx2*.

In order to understand the phenotypical variability in heterotaxia, it has suggested that the thresholds of *Pitx2* necessary for correct morphogenesis vary for each organ. In fact, different levels of expression in various segments of the lateral mesoderm could be related with thoracoabdominal discordance.[91,98] If this were to be the case, it could also be speculated that something similar could occur in different segments of the heart. This would help to explain the different cardiac phenotypes.

Pitx2 is initially expressed on the left side of the tubular heart and cardiac loop, and then is confined to the left atrium, anterior face of the ventricles, and left side of the outflow chamber (Figure 3). In the last phase, the expression of *Pitx2* is limited to the left atrium, until it ends up disappearing.[99] The patterns of expression of *Pitx2* are equivalent in the chick and mouse, and are inverted in mice with *situs inversus*. In malfor-
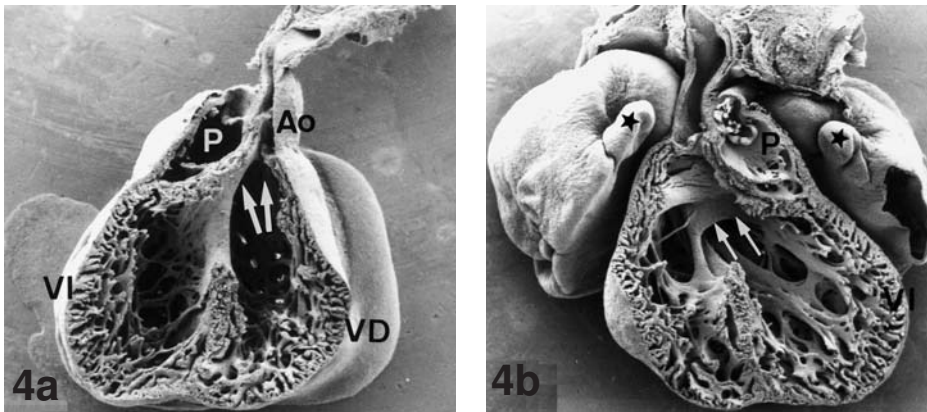
Fig. 4. Scanning electron microscopy showing the internal aspect of a heart (E18.5) with common atrioventricular canal and double outlet right ventricle. Situs solitus. The heart has been sectioned in the frontal plane and the ventral *a)* and dorsal *b)* halves are shown. The two ventricles communicate through a large ventricular septal defect that occupies approximately one-half of the ventricular height. The anterior leaflet (arrows in b) of the common atrioventricular valve is joined to the right side of the ventricular septum by a single papillary muscle. The two great vessels arise from the right ventricle by separate outflow chambers (arrows in a). A muscular band separates the origin of both chambers. The atria are symmetrical, even exhibiting symmetrical anterior prolongations (stars in b). Ao indicates aorta; P, pulmonary artery; a, b, ×45. (Reproduced from Icardo and Sánchez de Vega, 1991.)

med hearts, bilateral atrial expression (or the bilateral absence of expression) accompanies atrial isomerism, with expression also being able to exist on the posterior side of the right ventricle (Figure 3). In *iv/iv* mouse, this anomalous expression seems to be related directly with the development of double outlet right ventricle.[99] Thus, although there are clear indications that *Pitx2* intervenes directly in cardiac morphogenesis, the exact relation is unknown since the possible target genes have not yet been identified. Procollagen hydroxylysine could be one of these targets,[100] but its exact role is unknown.

CARDIAC MALFORMATIONS IN HETEROTAXIA

The organs reach their definitive form through a series of basic activities that include division and cell death, cellular emigration, cellular aggregation in tissues that specialize in different functions, the secretion of extracellular materials and, in the heart, the possible interaction of all these factors with hemodynamic forces. At present, it is not known which of these activities depend directly on *Pitx2* expression. To date, the only asymmetry detected at these levels is in the distribution of flectin, an extracellular glycoprotein that is expressed on the left side of the ventricle and on the right side of the outflow chamber during the formation of the cardiac loop. The differential expression of flectin is inverted in *iv/iv* mice, which is why it has been suggested that this protein is involved in the direction of the cardiac loop.[101]

As mentioned above, the *iv/iv* mutant is an excellent model for the study of the heterotaxia syndrome. Although we do not know how the patterns of gene expression are resolved in specific patterns of cellular conduct, the *iv/iv* mutant has also been shown to be a model for the study of the cardiac malformations found in heterotaxia.[9,37] The sequential study of cardiac development in *iv* mutants helps us to understand not only normal cardiac development, but also the mistakes in development that result in the production of malformations.[9,38-40]

The basic cardiac malformation or type corresponds to the so-called bulboventricular tube. Within the cardiac phenotypical syndrome there is, however, a regular combination consisting of CAVC and DORV (Figure 4).[102] CAVC is a complex malformation that includes atrial septal defect, ventricular septal defect, and common atrioventricular valve.[103-106]

In normal development, the two ventral and dorsal atrioventricular cushions fuse to form the so-called septal cushion. This mesenchymal mass constitutes the center of the developing heart, on which the *septum primum* that initially divides the atria, interventricular septum, and conal partition converge.[107] In malformed hearts, the first morphological deviations appear during the formation of the cardiac loop,[37] but they become clearer in stage E10.5,[40] being characterized by the presence of abnormal spatial relations between the different cardiac segments (Figures 5 and 6). The structure of the heart is normal although the endocardial cushions begin to show anomalies of position and form. In stages E11.5 and E12.5, these anomalies become more noticeable (Figure 7). The cushions appear hypoplastic, adopt a triangular form, and may be divided or present abnormal relations. In some cases, one of the lateral cushions is enormously enlarged. In stage E13.5, the atrioventricular cushions fuse in normal hearts (Figure 8). In malformed hearts they do not fuse and they remain separated by a wide space (Figure 9). At the same time, the *septum primum* does not contact
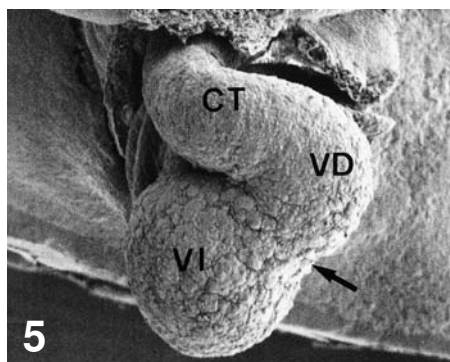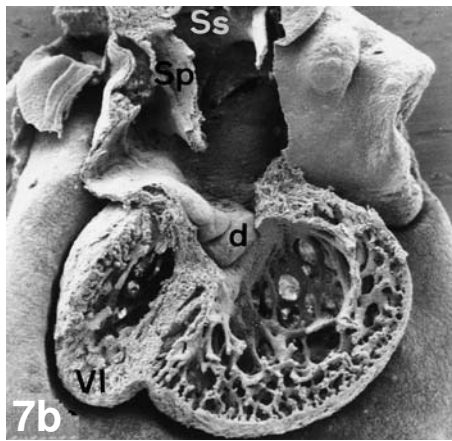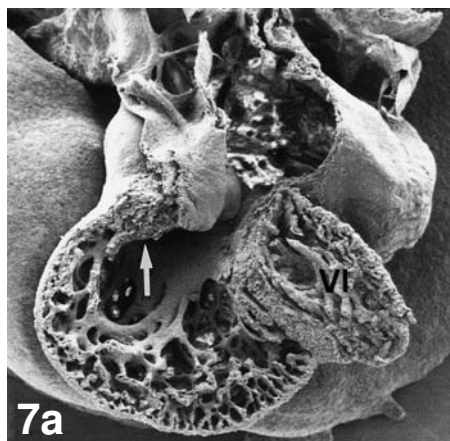
Fig. 5. E10.5. Situs inversus. This heart is displaced toward the side right of the embryo. The right ventricle (RV) and cono-trunk (CT) form a U with no clear separation. The right ventricle is cephalad with respect to left ventricle (LV). The arrow indicates the interventricular furrow. ×45.
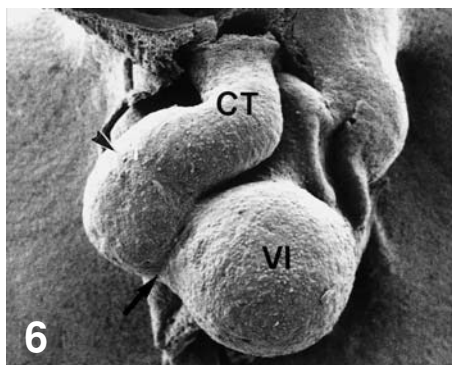
Fig. 6. E10.5. Situs solitus. The two ventricles do not maintain the characteristic lateral relation. The interventricular (arrow) and conoventricular (arrowhead) furrows appear abnormally marked. Compare the morphology of the hearts shown in Figures 5 and 6 with those that appear in Figure 1, which are in the same stage of development. ×110.

Figs. 5-9. Scanning electron microscopy showing some morphological aspects of normal and malformed hearts. (Reproduced from Icardo et al, 1995.)

Fig. 7. This photographs shows the ventral (a) and dorsal (b) halves of a malformed heart (E12.5), which has been sectioned in the frontal plane Situs inversus. The ventricles and interventricular septum are sectioned tangentially, indicating an abnormal spatial position. The right ventricle is much more anterior than the left ventricle. The septum primum (Sp) and septum secundum (Ss) appear in b. Note the abnormal spatial disposition of the atrial and ventricular septa. The dorsal cushion (in b) appears bifurcated. The two great arteries arise from the right ventricle (arrow in a). a and b: ×85.

the cushions and does not close the *foramen primum* (Figure 7). The interventricular septum, which has to contact the right side of the septal cushion, does not do so and is left in an intermediate position or even deviated to the left side. The septation of the cardiac outflow chamber, the cono-trunk, can be normal. However, the abnormal spatial disposition of the interventricular septum and undivided atrioventricular channel prevents the two partitions from contacting. The two ventricular outflow chambers do not become independent and originate abnormally. The later development of the atrioventricular cushions (dorsal, ventral, and lateral) will determine the final morphology of the common atrioventricular valve.

It is clear that the main anomaly in the development of CAVC in the heterotaxia syndrome is the non-fusion of the atrioventricular cushions. However, other structures, such as interatrial and interventricular septa, can exhibit an anomalous development, contributing in a variable way to the abnormal phenotype. The development of any organ is due to the close association established between the component parts. All the components must coincide in time and space for the organs to acquire their definitive form. When one or more of these components fail, the general morphogenetic mechanisms continue their course, but the organ in question has a deficiency that will be potentiated throughout development.

An important question that should be considered is whether all the cardiac phenotypes found in the heterotaxia syndrome can be explained by the action of a single gene. It is clear that in the initial stages of deve-
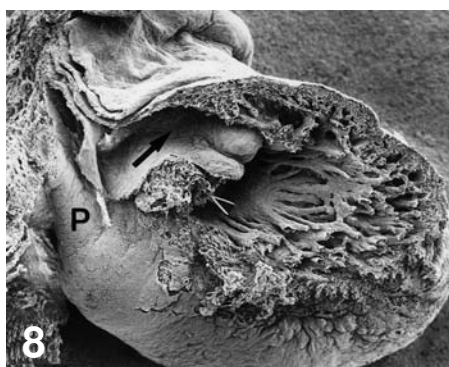
Fig. 8. Normal heart (E13.5). Situs solitus. The heart has been dissected in the parasagittal plane. The dissection plane passes through the left side of the atrioventricular region. The right half is observed after eliminating the left fragment. The atrioventricular cushions are fusing and the septum primum (arrow) continues into the septal cushion. The ventricular septal defect (arrowhead) remains open. P indicates pulmonary artery. ×90.
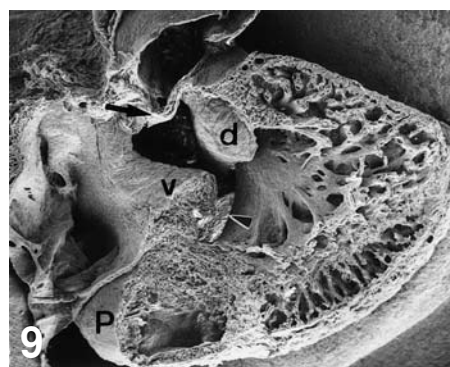
Fig. 9. Malformed heart (E.13.5). Situs solitus. The preparation is similar to that in Figure 8. The ventral (v) and dorsal (d) atrioventricular cushions have not fused. The septum primum (arrow) is normal, but does not contact the cushions. The arrowhead indicates the ventricular septal defect. P indicates pulmonary artery. ×90.

lopment anomalies of position and rotation of the cardiac loop take place. These anomalies are not corrected, but carried into later stages. The lack of alignment between the interatrial and interventricular septa can be explained by the loss of spatial signals in the primitive atrium and ventricle, and between these chambers and the atrioventricular canal. The same thing must occur in atrioventricular canal. Once the spatial signals are modified, control over the formation of the cushions is lost. This would explain both their abnormal position and variations in form and size.[40] In any case, the development of these malformations cannot simply be explained by spatial anomalies. Laterality anomalies should be considered among the basic morphogenetic mechanisms. For example, *iv/iv* hearts often show a lateral deviation of the interventricular septum with respect to the atrioventricular cushions. The growth of the interventricular septum is closely associated to the growth of the ventricles. The growth of the ventricles depends in great measure on the presence of active centers and cell proliferation. If these centers do not receive or produce suitable signals, or are located in an abnormal apposition, ventricular development will be abnormal and the septum will have an altered apposition. Hypothetical mechanisms of malformation, such as that which has just been described, are perfectly compatible with the action of a single gene. However, the possible influence of the *iv* gene on cell proliferation and other basic morphogenetic mechanisms is not known. It can be anticipated that some of these relations will be deciphered in the near future.

ACKNOWLEDGMENTS

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