CorSalud 2019 Jul-Sep;11(3):233-240

CorSalud

Cuban Society of Cardiology

Review Article

Cardiocentro Ernesto Che Guevara Santa Clara Cuba

Advances in the knowledge of the molecular and cellular bases of congenital heart diseases. Part 1 of 2: Cardiac morphogenesis

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Este artículo también está disponible en español

ARTICLE INFORMATION

Received: October 11, 2018
Accepted: November 23, 2018

Competing interests
The authors declare no competing interests

Abbreviations
DNA: deoxyribonucleic acid
TF: transcription factors
FGF: fibroblast growth factor

ABSTRACT

Congenital heart diseases are the most common congenital defect in humans. Many studies indicate that the cardiac development is tightly regulated by different cell signaling pathways and genetically controlled morphological events. The identification of new genes involved in the cardiogenesis process is very useful in order to know the molecular and cellular mechanisms by which the broad phenotypic spectrum of congenital heart disease is generated. An updated bibliographic review was carried out, with the aim of identifying the most recent advances in the knowledge of the molecular and cellular bases of congenital heart disease. This knowledge allows a more effective classification of these congenital defects and a future optimization of the individualized treatment for each patient, in addition to offering possible specific and susceptible points of intervention that would allow the prevention of some of these more frequent congenital defects in humans.
Keywords: Congenital heart defects, Morphogenesis, Single nucleotide polymorphism, Transcription factors, DNA methylation, Signal transduction

Avances en el conocimiento de las bases moleculares y celulares de las cardiopatías congénitas. Parte 1 de 2: Morfogénesis cardíaca

RESUMEN

Las cardiopatías congénitas son los defectos congénitos más frecuentes en humanos. Muchos estudios indican que el desarrollo cardíaco está estrechamente regulado por diferentes vías de señalización celular y eventos morfológicos, genéticamente controlados. La identificación de nuevos genes que intervienen en el proceso de cardiogénesis es de gran utilidad para conocer los mecanismos moleculares y celulares por el que se genera el amplio espectro fenotípico de las cardiopatías congénitas. Se realizó una revisión bibliográfica, con el objetivo de identificar los avances más recientes en el conocimiento de las bases moleculares y celulares de las cardiopatías congénitas; lo que permite una clasificación más efectiva de estos defectos congénitos y una futura optimización del tratamiento individualizado para cada paciente, además de ofrecer posibles puntos específicos y susceptibles de intervención que posibilitarían la prevención de algunos de los defectos congénitos más frecuentes en los seres humanos.
Palabras clave: Cardiopatías congénitas, Morfogénesis, Polimorfismos de un simple nucleótido, Factores de transcripción, Metilación de ADN, Vías de transducción de señales

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INTRODUCTION

A congenital defect is defined as any alteration of anatomical structure

visible on clinical examination of the newborn, or after birth, when the functional defect of an anatomically affected internal organ is perceived[1].

Congenital heart diseases are all congenital cardiac defects that occur as a consequence of morphological alterations during the organogenesis process. Most are well tolerated during intrauterine life and manifest after birth, when contact with the maternal circulation is lost.

There are different classifications, based on clinical criteria (cyanotic and non-cyanotic), genetic (syndromic and non-syndromic), prognosis (critical, potentially critical and non-critical) and depending on the presence or absence of extracardiac congenital defects (isolated and associated). The *Registro Cubano de Malformaciones Congénitas* (RECUMAC) Cuban Registry of Congenital Malformations classifies them as simple and complex, while the classification proposed by Hoffman and Kaplan is based on the severity of the congenital defect[1-3].

Congenital heart disease is the most common congenital defect, affecting approximately 6 to 11 infants per 1000 live births. Between 20% and 30% of these heart diseases are serious and their increase has been reported in recent studies, specifically septal defects without relevant variations in the prevalence of the most serious, which is related to a higher diagnostic level[4].

The causes are unknown in a large number of cases, although there is evidence that genetic factors play a decisive role in approximately 8% of cases and environmental agents are involved in 1-2% of them, the genesis of the rest (90%) is multifactorial and results from a complex interrelation between genetic predisposition, epigenetic susceptibility, parental environment and lifestyle.

Among the genetic syndromes that occur with congenital heart disease are described monogenic (3-5%), chromosomal (5-8%), microdeletions and some mutations in the mitochondrial deoxyribonucleic acid (DNA) molecule. Gene imbalances detected by conventional karyotype or molecular cytogenetic studies explain between 9% and 13% of all cases of congenital heart disease in the neonatal period[5-7].

The development of next-generation sequencing technologies (NGS) has emerged as a powerful tool to study the sequence of the entire human exome through the technique called WES (Whole-exome sequencing) or sequencing of specific exons in monogenic diseases of interest. These two molecular genetic methodologies have revolutionized the understanding of the molecular bases of many congenital defects, such as congenital heart disease, by providing more detailed information on gene variations than those provided by extensive genomic association studies in the single nucleotide polymorphisms (SNP) study[8-12].

Several studies indicate that cardiac development is closely regulated by a series of cell signalling pathways that modulate cell proliferation, migration and differentiation throughout developmental phases. Mutations in different genes involved in these intercellular communication pathways underlie the basis of different types of congenital heart disease[13-17].

METHOD

A review of the national and international medical literature published in Spanish and English languages was performed; the information was collected through search engines such as Google academic, and Medline/PubMed, Bireme (Scielo, Lilacs) and Cochrane medical library databases in October 2018, with the objective of identifying the advances in the knowledge of the molecular and cellular basis of congenital heart disease. A review of the literature was carried out based on chronological and thematic criteria, selecting those with the highest level of updating and relevance.

MOLECULAR AND CELLULAR BASES OF CARDIAC MORPHOGENESIS

Heart muscle cells or cardiomyocytes are specified early in development from a pool of mesodermal progenitors located at the anterior wall of the lateral plate mesoderm. The heart and great vessels are formed from the mesenchymal cells of the cardiogenic area, the first sign being the appearance of endothelial bands called “angioblastic cords” in the cardiogenic mesoderm during the third week of gestation. These cords are channeled to form paired longitudinal ducts, covered with endothelium, called "endocardial cardiac tubes"; which, as the lateral embryonic folding takes place, migrate to the embryonic midline, approach each other and fuse to

form the primitive heart tube, which by then already has two layers of tissue: an inner endothelial tissue and an outer layer of myocardial cells[5-7].

The fusion of the cardiac tubes begins at the cephalic end of the developing heart and progresses in a caudal direction. The heart begins to beat at the end of the third week (between 22 and 23 days), with blood flow beginning in the fourth week. The electrical and contractile phenomena that occur in the myocardium are controlled by genes that encode transmembrane proteins responsible for transporting ions through the cell membrane. These ion channels form macromolecular complexes that include a pore-forming main unit of sodium and potassium channels, and auxiliary proteins that regulate it[5-7].

This primitive heart undergoes torsion and clockwise rotation around the fourth week of gestation, positioning the atria above the ventricles. Meanwhile, the arteries of the aortic arch begin to emerge from the outflow tract. Around the fifth and sixth weeks of embryonic development, the cardiac septa are formed to divide the heart into four chambers and the outflow tract or ductus arteriosus is divided into the pulmonary artery and the aorta, thus resulting in pulmonary and systemic circulation division. Later an intensive valve remodeling occurs along with the growth of the ventricles to complete the maturation of the heart. The establishment of left-right symmetry is key for normal heart development[18].

Primitive atrium and ventricles muscle is continuous. The atrium acts as a transient cardiac pacemaker, until the venous sinus assumes that function. The sinus node appears during the fifth week of development, initially in the venous sinus wall, but then it is incorporated into the right atrium wall, near the entrance of the superior vena cava. Along with cells of the atrio-ventricular region (AV) they form the node and AV fascicle, located just above the endocardial bearings.

The fibers emerging from the AV fascicle migrate from the atrium to the ventricle (dividing into two branches), right and left, that spread throughout the ventricular myocardium.

The cardiovascular system is the first organic system that reaches a functional state, this early cardiac development is necessary because the rapid growth of the developing embryo cannot meet its nutritional and oxygen needs, only through the diffusion process[18].

Although the heart of different vertebrate species can have between two and four chambers, the different stages of the morphogenesis process are highly conserved at the molecular and cellular level. Morphogens are diffusible molecules that specify the type of cell generated in a specific anatomical location and direct cell migration and its processes to their final destination, among which are distinguished retinoic acid, transforming growth factor-beta, bone morphogenic proteins, the Hedgehog intercellular communication pathway and the Wnt family of proteins[2,19].

Retinoic acid is the bioactive form of vitamin A, which at the molecular level binds to its intracellular receptors and activates them, regulating the "sequence down" gene expression. Hox genes are crucial targets of these acid receptors in the morphogenesis process. The transforming *growth factor-beta superfamily* is made up of more than thirty members, among which are the *transforming growth factor-beta* (*TGFB*) and bone morphogenetic proteins (BMP). The TGFB superfamily plays a pivotal role in embryonic development, cell differentiation and organogenesis. Phylogenetic evidence suggests that this is one of the oldest intercellular signaling pathways, (it is estimated that it emerged about 1.3 billion years ago), before the evolutionary divergence of arthropods and vertebrates. Studies in vertebrates show that at least three members of the BMP family are expressed in cardiogenesis. BMP-2 is expressed in the lateral endoderm, while BMP-4 and 7 are expressed in the immediate ectoderm adjacent to the precardiac mesoderm. The BMPs, together with the fibroblast growth factors (FGF), promote cardiogenic induction and limit the size of the heart field, confirming the importance of this intercellular signaling pathway in the morphogenesis process[2,3,13,18]. Intercellular signaling is the process by which cells exchange chemical messages that modulate intracellular functioning and give rise to specific responses aimed at promoting the adaptation of the entire organism in a changing environment. The union of an extracellular signaling molecule (ligand) with its receptor in a target cell, triggers a specific response consisting of a series of mutual activating or inhibiting molecular events.

Intercellular signaling systems are essential in controlling gene expression and protein function. These systems will be the ones to control where, when, how much and for how long the ribonucleic acid (RNA) molecules are expressed. In the case of

proteins, they also control changes in location, protein traffic within a cell, cellular degradation, and the functional interactions they set up. Consistent with this decisive role in the functioning of organisms, it is estimated that more than 20% of the genes in the human genome encode proteins involved in signal transduction[20]. The Wnt intercellular signaling pathway is an intricate network of signals whose functions have also been maintained through the evolution of species. The Wnt family comprises a group of signaling genes that has a very important function in organogenesis. This group of genes determines the internal structure of an organ or growth arrest when the organ has reached its appropriate size. Other functions that involve these genes include cell polarity, cell restructuring and axial development. The term Wnt derives from the contraction of wingless and integrase-1 (Int-1). The first was coined from the polarity segment gene whose absence generates a wingless-fruit fly (Drosophila melanogaster)-phenotype, and the second gave the name to a mouse cell line transformed by Moloney virus that caused breast tumor[2, 18].

For their part, the more than 12 genes that make up the Hedgehog signaling pathway form a network, rather than a unidirectional pathway. The Hedgehog signal owes its name to the fact that the Hh gene coding was identified by mutagenesis studies in the Drosophila fly, where the mutant embryos showed a phenotype with disorganized spike-like exoskeleton tips similar to those of hedgehogs. Three members of this family of proteins have been identified in mammals: Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh) that participate in the morphogenesis process[19-21].

New strategies for the study of cardiogenesis have led to a drastic change in the way cardiac development is being approached, which has implied moving from sequential cuts of embryonic hearts of different animals, to the implementation of molecular cell-lineage tracing methods, when experimenting with transgenic models and retrospective clonal analysis. These technologies have given a more dynamic view of the development of the cardiovascular system and have made it possible to find the sometimes unsuspected origin of various anatomical structures from certain cell groups, as well as genes and their protein products[9-12,22.9-12,22]. Studies on mouse and chicken embryos have clarified the molecular basis of the cardiogenic process, thus demonstrating the presence of two basic helix-loop-helix (bHLH) genes in the primitive endocardial tubes and in more advanced stages of cardiac morphogenesis: HAND1 and HAND2 (heart and neural crest derivatives expressed transcript 1 and 2). The bHLH genes are a class of transcription factors (TF) that regulate the determination of cell fate and differentiation in many different tissues during embryonic development[3,6,14].

The formation of the cardiovascular system is strictly controlled by a regulatory network made up of many intercellular signalling pathways, TF, among others. Primordial cardiac cells begin to express characteristic myocardial genes, such as NKX2-5 and GATA4. The expression of the first of these TFs depends on the proteins in the underlying endoderm such as cerberus, BMP, FGF8 and FGF12[9,18,20].

There are more than 50 growth factors, also known as cytokines, comprising a large group of proteins that act as messengers, each of which binds with great specificity to a cell surface receptor protein, activating different intracellular signal transduction proteins. They act mainly on cell proliferation, although they also have other functions such as cell differentiation, survival and migration. Many of the signal transduction systems used by growth factors to transfer information to the nucleus and modulate gene transcription do so through TF activity. These TFs are protein products encoded by transcription genes. It is estimated that transcription regulation is based on at least 2000 of these protein factors encoded in the mammalian genome, which bind to a specific nucleotide sequence in the promoter/amplifier regions of target genes and regulate the speed of the process by which a gene is transcribed to a complementary sequence of ribonucleic acid (RNA), which starts the protein production process, thus playing an important role in the control of organogenesis. Moreover, specific micro-RNAs have a key role in in cardiac morphogenesis by coordinating the patterns and levels of TF expression[20].

Genes expressed in the cardiogenic plaque such as TBX1, TBX5, NKX2-5, GATA4, HAND2, CASP3, TFAP2, FGF12, LBX1, MYH11, CRKL, PDLIM3 and TXNRD2 code for TF that are expressed in early stages of heart development and are crucial for the activation of specific cardiac genes thus constituting the regulatory center of the cardiac morphogenesis network, which controls cardiac tube rotation, left-right symmetry and the formation of cardiac chambers. TBX5 and NKX2-5 genes directly bind to the promoter of the gene for cardiac-specific natriuretic

peptide precursor type A (Nppa) and thus activate this gene which is essential in cardiac development[23,24]. TBX genes belong to a gene family with a high degree of phylogenetic conservation. They have a common DNA binding domain called T-box, and code for TF involved in the regulation of cardiovascular morphogenesis, particularly in heart tube elongation within the anterior pole and the preservation of mesenchymal precursor cells for formation of a myocardialized and septated outflow tract. The reduction in TBX1 expression occurring in the hemicigotic deletion, often referred to as haploinsufficiency, significantly influences the initial gene expression involved in cardiac morphogenesis[25].

On the other hand, NKX2-5 (NK2 homeobox 5) and LBX1 belong to a group of genes that code for homeobox domain TF (a DNA sequence around 180 base pairs long found in different species), which have an important role in the regulation of tissue-specific genetic expression, essential for tissue differentiation and determination of temporal and spatial patterns during development. These TFs are critical for cardiac formation in Drosophila, since their absence prevents the formation of the loop and differentiation of the ventricles[19,20,23,25]. Other TF proteins are the so-called "zinc finger" as they are structured around this ion and contain finger-like protrusions. In this way, they remain fixed with a helix that recognizes a DNA sequence in the major groove, corresponding to promoters of specific genes; TF GATA4 belongs to this group.

The mutant model of this gene causes a defective anterior folding of the embryo; there is no fusion of the cardiac tube, and death occurs perhaps due to the importance of GATA4 in signaling for ventral migration[24,25].

Recent studies of exomic sequencing in cases with congenital heart disease have revealed genes encoding for proteins with two main types of molecular functions (those of chromatin binding and those forming the TF complex) which play a major role in the regulation of critical-cardiovascular development-genes. When analyzing variations in the number of «copies» by a comprehensive genomic study of 154 cases with interventricular communication An *et al*[23] identified de novo mutations in seven genes (PRKCB, HIRA, SOX2, ING2, TP63, BCL6 and PAX3) that are involved in the chromatin-binding pathway. TFs with confirmed effect on cardiogenesis include, LBX1, TBX1, EN1, SOX2 and PAX3. Among the types of mutations, they observed duplications in the PAX3 and LBX1 genes and deletions in CRKL, GP1BB, PDLIM3, TBX1 and TXNRD2. In addition to these two molecular functions, many of these genes encode proteins with important biological functions, such as the cardiac morphogenesis process and the regulation of cell proliferation, among others (**Table**).

PRKCB gene codes for protein kinase and HIRA does it for a histone involved in the cell-cycle regulation. SOX2 genes belong to the HMG (high mobility group) which contain an evolutionarily conserved sequence of 79 amino acids which give rise to three helical structures, one of which is immobilized and penetrates into the DNA minor groove, in the promoter region, causing a flexion that facilitates the approach of other proteins to the transcription start site. Whereas the PAX gene family consists of nine members (PAX1-9) which encode FT proteins with the same DNA-binding domain located towards the amino-terminal end (paired box domain). PAX3 is a key regulatory factor in controlling the migration of myogenic precursor cells[23,25].

Studies on the cellular bases of the cardiogenesis process reveal that there are several types of cells contributing to cardiac growth. First heart field cells (FHF) contribute only to the formation of the right ventricle and atrioventricular canal, while the atria, left ventricle and much of the outflow tract, come from mesenchymal precursors residing in the second heart field (SHF)[17,18,20].

Nodal/TGF signaling pathway has an important effect at early stages of differentiation of human embryonic stem cells in directing them to develop into different embryonic lineages. SMAD3 or SEMA3D gene is a key intracellular messenger regulating factor in the Nodal/TGF signaling pathway, playing important roles in embryonic and, particularly, cardiovascular system development[15,26,27].

Likewise, the Notch signaling pathway plays a fundamental role throughout the embryonic development period, as it is essential for the germ layers to give rise to the tissues that constitute a multicellular organism. This signaling pathway also determines the cellular specification, histogenesis and morphogenesis of different tissues in the embryonic development of vertebrates and invertebrates, and is key in the specification of cardiomyocytes and blood vessels. This pathway is evolutionarily conserved in the phylogenetic scale and owes its name to the phenotype that cause mutations in the gene encoding its receptor, characterized by recesses (Notch)

Table. Functions of some genes that participate in the cardiogenesis process[4,7,22-24].

Functions	Genes
Molecular Functions	
Chromatin binding	HIRA, SOX2, PRKCB, ING2,TP63, BCL6, PAX3
DNA-specific sequence binding	PAX3, EN1, SOX2, TBX1, TP63, LBX1, BCL6
Sequence-specific DNA binding transcription factor activity	HIRA, EN1, SOX2, PAX3, TBX1, TP63, LBX1, BCL6
Nucleic acid binding transcription factor activity	HIRA, EN1, SOX2, PAX3, TBX1, TP63, LBX1, BCL6
Cellular/Biological processes	
Cardiovascular morphogenesis	MYH11, TXNRD2, CRKL, FGF12, PDLIM3, TBX1, LBX1, CASP3, PRKCB, PAX3
Circulatory system development	MYH11,TXNRD2, CRKL, FGF12, PDLIM3, TBX1, LBX1, CASP3, PRKCB, PAX3
Regulation of cell proliferation	SOX2, CASP3, PAX3, BCL6, COMT, IGFBP7, LBX1, IL4R, TP63, TBX1, CHP2
Negative regulation of cell proliferation	SOX2, MED15, TBX1, TP63, LBX1, IL4R, BCL6

on the wing margins of Drosophila fly[13,28].

EPILOGUE

There is no exact genotype-phenotype correlation between molecular mechanisms and morphological defects of congenital heart disease. This happens because the proper formation of an anatomical structure often involves the right functioning of various cell signalling pathways that may involve the protein product of different genes. In this sense, different genetic pathways and mutations may be involved in the same congenital defect, or on the contrary, that due to the pleiotropic effect of mutations in one of the critical genes in the cardiogenesis process, different types of congenital heart disease arise; All of which will be summarized in the second part of this article where some aspects of genes and signaling pathways involved in the origin of congenital heart disease will be discussed.

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