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Gene expression in pediatric heart disease with emphasis on conotruncal defects

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Abstract

Developmental abnormalities of the heart are the underlying cause of many congenital heart malformations. The embryological development of the integrated cardiovascular tissue is the result of multiple tissue and cell-to-cell interactions involving temporal and spatial events under genetic control. Recent technological advances, like microarray analysis of gene expression, are providing new tools to aid in deciphering the complex networks of gene expression that regulate cardiac development. Here, we review our current understanding of the genetics of congenital heart disorders with emphasis on gene expression studies and report preliminary data from infants with conotruncal defects. We report our microarray analysis showing over- and underexpression of individual genes and gene network interactions from dysplastic pulmonic tissue from two infants with tetralogy of Fallot compared with normal pulmonic tissue from an unaffected control infant.

Keywords

Congenital heart defects; Pediatric cardiology; Gene expression; Microarray; Conotruncal defects; Cardiac development

1. Introduction

Recent advances in molecular genetics have been propelled by the development of a genetic map of highly informative polymorphisms, gene expression data, and efficient rapid molecular genetic methods that have increased our ability to identify disease causing genes. These advances are now being applied to the genetics of congenital cardiovascular disease and cardiogenesis [1,2]. Congenital heart disease occurs in approximately 0.5 to 1% of all newborns but subtle ventricular septal defects may occur in as high as 5% of all neonates. Congenital heart defects represent a significant proportion of birth defects and account for the majority of morbidity and mortality related to birth defects. The origin of most

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congenital heart disease is thought to be multifactorial implying both anomalous expression of genes and epigenetic factors. The origin of some cases of congenital heart disease may be directly related to chromosome anomalies or defects in a single gene such as those controlling laterality. A cascade of gene activation must occur to develop left and right sides of the embryo and therefore the heart. Anomalous expression of genes (e.g., *Nodal*, *Pitx2*) appears to produce heterotaxia or errors in laterality and congenital heart defects [3]. Similarly, rapid change in connective tissue gene expression has been reported (e.g., porcine procollagen and tropoelastin) in normal pulmonary arteries immediately after birth as the vasculature remodels [4].

Patients with conotruncal defects and pulmonary atresia with abnormal development of their pulmonary vasculature present with a complex set of problems. These patients are diverse and provide a constant challenge in terms of management and outcomes. The group consists of a heterogeneous population that presents in many different forms, including: pulmonary atresia with intact ventricular septum, with and without coronary sinusoids; pulmonary atresia with ventricular septal defects (VSD); pulmonary atresia, ventricular septal defect, or tetralogy of Fallot (TOF) and multiple aortopulmonary collaterals; tetralogy of Fallot with pulmonary atresia and/or hypoplasia of the pulmonary arteries; and complex single ventricle anatomies with pulmonary atresia. In addition, other conotruncal abnormalities associated with abnormal pulmonary artery development include transposition of the great arteries with ventricular septal defect and pulmonic or subpulmonic stenosis, and truncus arteriosus. Inadequate development of pulmonary arteries may occur as an isolated anomaly, but more commonly occurs in conjunction with other cardiac and extra cardiac defects.

The variable development of the pulmonary vasculature requires individualized treatment strategies and has a profound effect on outcomes in these patients. Initial therapies are directed towards encouraging growth of pulmonary vasculature, with the goal of eventual successful biventricular repair or definitive palliation with complete cavopulmonary connection. Abnormal development of the pulmonary vasculature is characterized by heterogeneity in terms of the anatomy of affected patients, but consistent in terms of the challenging nature of its management.

2. Genes and cardiovascular development

The heart is the first organ to form during embryogenesis and its circulatory function is critical early on for viability of the embryo. Developmental abnormalities of the heart are widely recognized as the underlying cause of many congenital heart malformations. The development of the integrated cardiovascular tissue is the result of multiple tissue and cell to cell interactions involving temporal and spatial events under genetic control.

The genes for a number of syndromes with congenital heart defects as important manifestations have now been identified and cloned. The knowledge gained by studying the affected gene products has led to significant insights into the disease process and glimpses into the complicated genetic organization of cardiac development. Some of these genetic disorders include: Holt–Oram syndrome, atrial and ventricular septal defects, supra-valvular aortic stenosis, and Williams, DiGeorge, Ellis–van Creveld, Noonan, Alagille, and Marfan

syndromes. Further disorders include conotruncal anomalies, total anomalous pulmonary venous return, and cardiac myxomas. Various genetic studies, including cytogenetic and linkage analysis, have established loci but gene defects in several of these syndromes have not been clearly elucidated. In addition, a wide variety of genes have been identified in murine, chicken, *Drosophila*, and zebra fish that modify cardiac development and are supported by application of various techniques (e.g., “knock out”). For example, the *Rae 28* gene is a mammalian homologue of the *Drosophila* gene, polyhomeotic, known to maintain gene transcription states probably by regulating chromatin structure. Homozygous *Rae 28* deficient mice display cardiac anomalies similar to congenital heart disease in humans. The *Rae 28* deficient mouse embryos show expression of the cardiac selector gene, *Nkx2.5*, but expression is not sustained later in development. This impaired expression of *Nkx2.5* proved to have a crucial effect on cardiac morphogenesis [5] and will be addressed below.

As indicated earlier, some of the genes involved in normal cardiogenesis include transcription factors (e.g., *NKX2.5*, *GATA6*, *GATA4*, *HAND1*, *HAND2*, and *NFATC*), which can regulate the expression of genes in a tissue specific and quantitative manner, as well as soluble factors including bone morphogenic proteins (acts as a positive facilitator of nodal induction and left–right asymmetry), transforming growth factor beta isoforms and fibroblast growth factor isoforms (may play a role in cardiac hypoplasia) [6–8]. These genes identified in lower animals also participate in human cardiogenesis. For example, Gln170ter, Thr178Met, and Gln198ter mutations in the transcription factor, *NKX2.5*, in humans cause atrial septal defects and conduction disease [9], while *HAND* genes are essential for normal cardiac and extra embryonic development and are expressed in adult heart but are downregulated in cardiomyopathies [10]. Recently, novel frameshift mutations of the *NKX2.5* gene (e.g., 7 bp deletion in exon 1 at nucleotides +215 resulting in a truncated protein of 172 amino acids) were identified in two families [11]. The mutations cosegregated with atrial septal defects and/or atrioventricular conductive abnormalities, as an autosomal dominant trait. This report suggests that an expanded population of subjects could now be examined for mutations within this transcription factor and its upstream and downstream regulatory elements.

Additionally, the gene *TBX5*, which causes Holt–Oram syndrome, an autosomal dominant disorder with clinical features of atrial septal defects, ventricular septal defects, conduction abnormalities, and upper limb anomalies, is localized to 12q24.1. This gene is a member of the T box gene family and mutations interfere with its binding to target DNA sites. These target sites are present upstream of several cardiac-expressed genes including cardiac alpha actin, cardiac myosin heavy chain alpha and beta, myosin light chain 1A and IB, and *NKX2.5* which are associated with congenital cardiac defects [12]. Similarly, congenital long QT syndrome (LQTS), a genetically heterogeneous arrhythmogenic disorder, is caused by mutations in at least five different genes encoding cardiac ion channels [13]. This study of 95 patients with LQTS identified a single nucleotide polymorphism (SNP) in one of the ion channel genes, *KCNQ1*, in 6 patients. This SNP changed the coding sequence from glycine to serine at position 643 (G643S) which is mostly associated with a milder phenotype often precipitated by hypokalemia and bradyarrhythmias. Voltage clamp experiments designed to characterize the cellular phenotype caused by the SNP revealed that, in the presence of G643S, *KCNQ1* forms functional homomultimeric channels that

have a significantly smaller current than the wild type ion channels. Co-expression studies suggested that the polypeptide with the G643S substitution had a weaker dominant-negative effect on the heteromultimeric complexes. Thus, a common polymorphism in the *KCNQ1* potassium channel could be a molecular basis for weaker current through the cardiac circuitry and measurable in potential gene carriers of this arrhythmic syndrome. This study adds further support to the growing evidence that gene polymorphisms such as SNPs can play a role in causation of a cardiovascular condition and can be used to identify at-risk candidates for genotype/phenotype correlations, diagnosis, prognosis, and genetic counseling of at-risk individuals.

The mapping of SNPs in human genomes has generated much interest in both the academic and industrial biomedical research community. In conjunction with SNP mapping, researchers have shown that haplotypes possess considerably more potential than the traditional single SNP approach in disease gene mapping and in understanding of complex diseases and linkage disequilibrium. The use of in silico methods for haplotype reconstruction has attracted much attention and will play a role in human haplotype block structure of grouped genes (e.g., genes on 22q11.2) and in candidate gene studies of congenital heart disease in the future. Several computer assisted statistical approaches (e.g., HAPLOTYPER) to estimate haplotype phases of a large number of SNPs are in use including maximum likelihood estimates.

3. Expression of cardiac genes

When searching computer databases such as the NCBI GENE database for “cardiac and development” or “heart and development”, 149 loci in humans were identified. Table 1 shows a list of candidate human genes for cardiac development. Data from the Human Genome Project indicate that the human genome contains 30,000–40,000 genes. Within individual cell types, only a small percentage of these genes, perhaps 10–15%, is actually expressed. The expression of certain genes will create cellular patterns in development of tissue and organ pathology. Gene expression generally refers to a multiplicity of processes that lead to cellular development of functional protein through regulated steps involving gene transcription, messenger RNA turnover and stability, protein translation, production and turnover, and post-translational modifications. Gene expression can be investigated in the human heart and gene expression profiling patterns should play an important role in confirming candidate genes for cardiogenesis as well as identification of other potential genes in the study of normal and abnormal tissue of individuals with specific congenital heart defects.

A substantial amount of information has been generated using expression profiling methods in tissues and cells from cardiovascular systems. Several different types of experimental design have been utilized in reported studies of the cardiovascular system from the healthy to diseased state. The effects of drugs such as isoproterenol or angiotensin-converting enzyme inhibitor, captopril, on cardiovascular gene expression have been examined using subtractive hybridization and high throughput sequencing of cDNA libraries [14]. Microarray analysis is also being used to identify key genes involved in heart failure [15]. However, there is a paucity of data in causative gene expression studies in congenital heart

disease. Microarray expression profiling is beginning to be applied to congenital heart defects and is generating information which will lead to many new insights into molecular disease mechanisms. Kaynak et al. [16] were able to develop a “global molecular portrait” of the normal and defective heart. They were able to identify sets of genes which appeared to contribute to specific malformations including septal defects, as well as, genes that were regionally expressed (i.e., chamber specific) within the heart.

Velculesa et al. [17] identified 9449 unique genes in cardiomyocytes searching several data sets while Dempsey et al. [18] estimated that 20,000–27,000 genes are expressed in the cardiovascular system. These data were substantiated using Affymetrix arrays of mRNA from the adult human left ventricle [19]. Arteriosclerosis, dilated cardiomyopathy, hypertension, and myocardial infarction are the cardiovascular indications most commonly studied by high throughput RNA expression profiling. For example, global gene expression profiling of cardiovascular disease has been performed particularly in end-stage dilated cardiomyopathy using human cardiovascular-based cDNA microarrays containing 10,848 nonredundant elements.

4. Genetic basis of congenital heart disease

Approximately 30% of subjects with congenital heart defects (CHD) have associated extra cardiac malformations with chromosome anomalies (trisomy, deletions, duplications) representing the most striking association (120 times more frequent than in control subjects) [20]. Mendelian disorders and syndromic associations are also commonly found in patients with CHD. The pathogenetic classification of congenital cardiovascular malformations proposed by Clark [21,22] is appropriate to consider when examining links between genetic mechanisms and causation. According to Clark’s classification, there are six causative mechanisms delineated: ectomesenchymal tissue migration abnormalities (causing conotruncal malformations and aortic arch anomalies); intracardiac blood flow defects (causing septal defects and left or right heart obstructive malformations); cell death abnormalities (causing septal defects and valve abnormalities); extra cellular matrix abnormalities (causing atrioventricular canal defects); abnormal targeted growth (causing partial or total anomalous pulmonary venous return and cor triatriatum); and abnormal situs and looping (causing left–right positioning problems). Therefore, the search for specific genes involved in cardiogenesis is a complex process. However, recent advances in molecular genetic technology such as micro-array genome-wide expression, whereby thousands of genes can be studied simultaneously, and access to gene data generated from the Human Genome Project can lead to future haplotype disequilibrium and linkage studies pertinent to identifying causative genes for congenital heart disease.

Evidence to date supports the following partial list of genes for ventricular growth: *CXC*, *NT-3*, *TEF-1*, *WT-1*, *NRG1-4*, *ERB2*, *NKX2.5*, *TBX5*, *ERB4*, *FOG2*; for aortic growth: *HEY2*, *END1*, *EDNRB*, *ELN*, *FBN1*, *FBN2*; and for valvular formation: *NFATC*, *NOS3*, *PTPN11*, *TGFB*, *BMP*. Table 2 shows a list of syndromes, clinical features, and putative cardiac genes. Some heart malformations are single such as ventricular septal defect (VSD), which is the most common form of CHD and present in 33% of all affected individuals. It occurs in 12 of 10,000 births and is thought to be multifactorial in origin [20]. However,

there are several types of VSD (perimembranous, muscular, posterior, subarterial) and each type may involve a different mechanism of morphogenesis. For example, subjects with Down syndrome or trisomy 21 have thousands of genes in excess, or syndromes involving a single gene mutation (e.g., *TBX5* in Holt–Oram syndrome (discussed elsewhere in this issue) may present with different types of VSDs.

In addition, there is a nonrandom association between VSDs and DiGeorge syndrome (discussed elsewhere in this issue), a relatively common disorder with a prevalence of 1 per 2000 births and due to a microdeletion of chromosome 22q11. Individuals with this syndrome present with variable phenotypic findings including cardiac malformations, defective cellular immunity, thymic anomalies, cleft palate, craniofacial defects, and hypocalcemia. The most frequent cardiac defect in this syndrome is conotruncal anomaly which includes tetralogy of Fallot (TOF), pulmonary atresia with VSD, truncus arteriosus, and interrupted aortic arch. Septation of the outflow tract (conotruncus) with separation of the aorta and pulmonary trunk is an especially complex process.

Conotruncal defects account for 15% of CHD and are the result of abnormal ventricular outflow tract morphogenesis or defective bronchial arch development. These malformations are due to ectomesenchymal tissue migration abnormalities. Pulmonary atresia with VSD or TOF with pulmonary atresia present in a high frequency of genetic syndromes (25–30%) and most commonly in 22q11 deletion. A patient with pulmonary atresia with VSD and other major cardiovascular defects such as aorta-pulmonary collateral arteries has a 35% probability of having a 22q11 deletion. However, other genes (e.g., *PTPN11*, *JAG1*) not located on chromosome 22 are also thought to play a role in pulmonary artery stenosis and VSD [23].

Right-sided obstructive lesions include pulmonary valve stenosis and pulmonary artery stenosis. Isolated pulmonary valve stenosis represents 7.8% of all cardiovascular malformations with a total prevalence of 3.8 cases per 10,000 live births [24], Noonan syndrome is the most common genetic syndrome associated with this cardiac defect [25] and the valve is usually dysplastic with fibrous thickening (discussed elsewhere in this issue). Pulmonary artery stenosis is seen without other types of congenital heart defects. However, the majority of cases may be associated with noncardiac anomalies as seen in Alagille syndrome and Williams syndrome [20].

A public domain computer genome database for chromosome 22 lists 121 genes localized to 22q11.2, the chromosome band deleted in 90% of patients with DiGeorge syndrome. A critical region on 22q11.2 appears to encompass at least nine candidate genes found over a 480-kb interval [26]. Recently, a high density SNP map of the 96 kb region containing the entire human DiGeorge syndrome critical region 2 at chromosome 22q11.2 was generated from the Japanese population [27]. A total of 102 SNPs were isolated (9 SNPs from the 5′ flanking region, 3 in the 5′ untranslated region, 2 in the coding regions, 77 in introns, 7 in the 3′ untranslated region, and 4 in the 3′ flanking region). The comparison of their SNP data from the dbSNP database from NCBI showed that 80 (78.4%) were considered novel. The ratio of transition to transversion change was 3.08:1. Their high resolution map will be

useful in analyzing gene scans in patients with congenital heart disease specifically in the syndromic (22q11.2 deletion) vs. nonsyndromic subjects.

5. Specific genes and known syndromes with cardiac abnormalities

Congenital cardiovascular malformations are the most frequent birth defect and as indicated earlier can occur as an isolated event but can be found in constellation with other defects involving multiple organs. The defects may be a primary effect of gene mutations or secondary to altered cardiac physiology. One important gene for cardiac development and pulmonary atresia and congenital heart disease is *PTPN11*. Mutations in the *PTPN11* gene have been reported in patients with Noonan syndrome, an autosomal dominant disorder characterized by dysmorphic facial features, proportionate short stature, and heart disease, most commonly pulmonic stenosis and hypertrophic cardiomyopathy [28]. Webbed neck, chest deformity, cryptorchidism, mental retardation, and bleeding diathesis are frequently found. The condition is relatively common with an incidence as high as 1 in 2500 live births but with genetic heterogeneity. It has been mapped to chromosome 12q24.1.

Missense mutations in *PTPN11*, a gene coding the nonreceptor protein tyrosine phosphatase, SHP-2 containing two Src homology 2 (SH2) domains have been reported in 50% of Noonan syndrome subjects [28]. Energy based structural analysis of two SH2 mutants shows a gain of function change supporting that the pathogenesis of Noonan syndrome may arise from excess SHP-2 activity. Two unrelated subjects showed the same de novo missense mutation in exon 13 (S502T) while others had mutations in exon 3 (Y63C). No mutations were seen in matched controls [29]. Other mutations (e.g., Y279C) in the *PTPN11* gene have been reported in subjects with LEOPARD syndrome with multiple lentigines, congenital cardiac abnormalities, ocular hypertelorism, and growth retardation [30]. More recently, 119 unrelated Noonan syndrome patients were studied and 54 of the 119 individuals had *PTPN11* mutations [28]. All mutations were missense. The vast majority of mutations were located at or around the interacting surfaces of N-SH2 and PTP domains. Pulmonic stenosis was more prevalent among the Noonan syndrome subjects with this mutation than in those without the mutation indicating a key gene in development of pulmonic stenosis. The A922G mutation in exon 8 was the most common mutation. Thus, different mutations of the *PTPN11* gene can lead to different phenotypic findings including congenital heart defects such as pulmonary atresia.

Alagille syndrome is another autosomal dominantly inherited disorder and characterized by developmental abnormalities of the liver, heart, skeleton, eyes, face, pancreas, and kidneys. The most common cardiovascular defect in this syndrome is peripheral pulmonic stenosis. It is one of the most common genetic causes of chronic liver disease in childhood with an estimated frequency of 1/70,000 live births. Mutations of the *JAG1* gene on chromosome 20p were reported to cause Alagille syndrome [31]. The gene consists of 26 exons and spans 36 kb encoding a 5.5 kb message. *JAG1* encodes a ligand in the Notch intercellular signaling pathway. In one study, 35 intragenic mutations included 9 nonsense mutations (26%), 2 missense mutations (6%), 11 small deletions (31%), 8 small insertions (23%), and 1 complex rearrangement (3%), all leading to frameshifts and 4 splice-site mutations (11%) [32]. Furthermore, in a study of 105 patients with Alagille syndrome, mutations were found

across the coding sequence of 12 exons of *JAG1* gene in 63 subjects or 60%. The spectrum of mutations were seen with 52/63 (83%) noted to produce protein truncation, 7/63 (11%) were missense, 1/63 (2%) were splice site, and 3/63 (5%) had total gene deletions demonstrable by fluorescence in situ hybridization (FISH) [33].

The spectrum of mutations suggests that mechanisms other than haplo-insufficiency cause the Alagille syndrome phenotype. *JAG1* is expressed in the developing heart and associated vascular structures in a pattern that correlates with congenital cardiovascular defects seen in Alagille syndrome suggesting an important role for *JAG1* and Notch signaling in early mammalian cardiac development [34]. More than 90% of individuals with *JAG1* mutations have cardiac muscular defects with bronchial pulmonary artery stenosis being the most common abnormality [35]. Interestingly, some cases of isolated tetralogy of Fallot or pulmonic stenosis have been reported with mutations of the *JAG1* gene [36].

A third important condition is DiGeorge or velo-cardio-facial syndrome generally due to a 22q11.2 deletion. As stated earlier, the congenital heart defect in this syndrome usually includes conotruncal anomalies. This deletion is usually undetectable with routine chromosome studies and requires FISH analysis of metaphase chromosomes using a 22q11.2 probe. The typical deletion is about 1.5 to 3 Mb in size and contains more than 20 genes. Although several genes in this chromosome region are important for consideration as candidate genes for causing DiGeorge syndrome, *TBX1* appears to be the most likely candidate gene. Mouse studies have shown that *TBX1* is required for development of pharyngeal arches and pouches including septation of the outflow tract of the heart as predicted by the DiGeorge clinical phenotype. Mutations of this gene in mice cause multiple cardiovascular defects and disrupt neural crest and cranial nerve migratory pathways [37].

Mice generated to be hemizygous for a 1.5 Mb deletion corresponding to that on 22q11 exhibited significant perinatal lethality, conotruncal anomalies, and parathyroid defects. The conotruncal defects are partially rescued by a bacterial artificial chromosome containing the human *TBX1* gene [38]. Mutation analysis of *TBX1* in patients who have DiGeorge syndrome or velo-cardio-facial phenotype with or without a detectable deletion of 22q11.2 by FISH should provide insight into the role of *TBX1* in the etiology of these disorders either as a form of haplo-insufficiency or mutations. Recently, Garg et al. [39] analyzed the *TBX1* gene in 105 patients who did not have deletions or rearrangements of 22q11.2 by FISH analysis but had clinical features of DiGeorge or velo-cardio-facial syndrome or had cardiovascular defects associated with 22q11.2 deletion. They identified eight common polymorphisms and 10 rare variants (3 were deletions, 1 was an insertion, 1 was a duplication, and 5 were single nucleotide changes). In contrast, the majority of mutations in a related gene, *TBX5*, in Holt–Oram syndrome were predicted to produce haplo-insufficiency by nonsense or frameshift mutations in the T box region. In cases where parental DNA was available, most rare variants were transmitted to the patient from an unaffected parent.

6. Microarray gene expression in nonsyndromic congenital heart disease

The first genome-wide cDNA array analysis of congenital heart malformations was performed by Kaynak et al. [16] and they attempted to elucidate complex cardiac phenotypes by comparing gene expression patterns. They proposed that as a consequence of the heart malformations, abnormal hemodynamic features occur and cause an adaptation process of the heart (e.g., right ventricular pressure overload hypertrophy). They studied tissue collected during cardiac surgery and examined gene expression profiles from patients with tetralogy of Fallot (TOF), ventricular septal defect (VSD), atrial septal defect, and right ventricular hypertrophy (RVH). They also presented data on chamber-specific gene expression by using differential expression analysis and real-time PCR using microarrays composed of 12,657 well annotated genes. Characteristic expression patterns were obtained from a linear model analysis for the comparison of normal atria and ventricle as well as atrial and ventricular tissue from individuals with congenital heart malformations (e.g., TOF or VSD). To provide an overview of the molecular signatures, they clustered all known genes that appeared significantly different in their statistical analysis of the genome-wide microarray procedure. They reported that about 25% of congenital heart disease associated genes were not chamber-specific in the normal heart. Inspection of gene expression data from TOF and right ventricular hypertrophy showed similarities but an opposite expression dynamic was found when compared with ventricular septal defect. They also found that a large number of genes characteristic of the molecular signature for VSD were not differentially expressed in any other tissue analyzed. Comparison of the expression profile of genes characteristic for TOF and RVH provided the possibility of subtracting both from each other which may identify genes specific for either TOF or RVH.

In comparison of gene expression patterns from the atria and ventricle, well known chamber specific genes were found such as atrial and ventricular myosin light chains. However, additional diverse and previously unknown chamber-specific genes were identified for muscle contraction, extracellular components, cell growth and differentiation, and energy metabolism. Possibly due to less force, the atria were characterized by higher expression of genes encoding proteins associated with extracellular matrix or actin modulation, such as *CST3* and *PCOLCE* [16]. The translation factor, *EEF1A* and the DNA helicase, *REQL4* were highly significantly upregulated in the atrial tissue. The *KCNIP2* gene, which is a potential target for ventricular tachycardia was more highly expressed in the atria than in the ventricle. Gene expression that was higher in the ventricular tissue than in the atria belonged to three major functional classes: cytoskeleton–contractile, metabolism–energy turnover, and cell cycle–growth.

Kaynak et al. [16] concluded that two distinct molecular patterns of gene expression emerged for TOF and RVH. Besides genes involved in the cell cycle, a characteristic gene expression pattern for TOF was identified with upregulation of ribosomal protein genes. Their expression data revealed a TOF specific dysregulation of pathways leading to abnormal cardiac development such as upregulation of *SNIP*, *A2BP1*, and *KIAA1437* genes. Genes found to be downregulated included *STK33*, *BRDGI*, and *TEKT2*. In RVH, they reported a hypertrophy-specific gene expression pattern of genes mainly involved in stress response, cell proliferation, and metabolism.

As seen in TOF, several ribosomal protein genes were differentially expressed but downregulated in VSD in the studies performed by Kaynak et al. [16]. Other genes expressed in VSD included ion transporters (solute and potassium channels) cell proliferation, differentiation during embryogenesis, and apoptosis such as *AMD1*, *RIPK3*, *EGLN1*, and *SIAHBPI*. Global analysis of VSD gene expression patterns showed a general transcriptional downregulation compared with TOF.

Preliminarily, we have performed microarray analysis using total RNA from two infants (a 7-month-old male and a 4-month-old female) with conotruncal defects and abnormal pulmonary vascular development. To identify specific gene expression patterns, we compared expression of the abnormal septal tissue (dysplastic pulmonic valve) from the affected infants to that of normal septal tissue from a control infant at a comparable age (two months) and without a heart defect. Both affected infants were nonsyndromic and had tetralogy of Fallot [obstruction of right ventricular outflow (pulmonary stenosis), VSD, dextroposition of the aorta with septal override, and RVH] without a known cause of their cardiac defect (e.g., no chromosome 22q deletion was found by FISH analysis). The control infant had no anomalies and no evidence of infection. Tissue specimens were collected after human subjects committee review and consent from each patient at the time of surgery. The tissues were immediately placed in RNeasy lysis buffer (Qiagen, Crawfordsville, IN) and flash frozen in liquid nitrogen. The tissues were pulverized with a mortar and pestle in liquid nitrogen and transferred to a dounce homogenizer in TriReagent (Molecular Research Center, Cincinnati, OH). Total RNA was purified according to the manufacturer's instructions.

Genome wide microarrays (human 1 cDNA microarrays) were obtained from Agilent Technologies. The Human 1 cDNA microarrays were developed from Incyte's Unigene 1 and human drug target clone set. Hybridization followed the microarray manufacturer's suggestions. The Human 1 Microarray contained more than 12,800 well characterized expressed human sequences. Fig. 1 shows a close up of a representative array generated using amplified RNA (aRNA) from one of our nonsyndromic infants with tetralogy of Fallot and normal chromosome studies compared to our unaffected control infant. The aRNA from the dysplastic pulmonic valve was labeled with Alexa 647 (red) and aRNA from the normal pulmonic tissue from the control infant was labeled with Alexa 555 (green).

Several genes known to be important to cardiac development were identified through statistical analysis and highlighted from the microarray gene expression profile. Of particular interest were two genes, *JAG1* and *LFNG*, which are members of the Notch signaling pathway. The Notch pathway plays a critical role in pattern formation in embryogenesis and is particularly important for proper development of the fetal heart. Fig. 2 is a representative scatterplot generated from a portion of the microarray. *JAG1* is identified showing its expression to be easily detectable in RNA isolated from the control infant but the signal was below the threshold of detection in the infant with a dysplastic pulmonic tissue.

The microarray results were verified by using reverse transcription-PCR (Fig. 3A,B). Primers from exon 26 and primers spanning exons 17–21 were used to verify that expression from *JAG1* could not be detected in the RNA isolated from the infants with cardiac defects. A very faint band was detected in our affected infant 1 using primers spanning exons 17–21.

Clearly, there is a drastic reduction in expression of *JAG1* in the infants with defects. Reduced expression of *JAG1* is known to cause heart defects including TOF. The loss of expression of *JAG1* might be due to a microdeletion which was undetectable with traditional chromosome studies. Therefore, PCR analysis using genomic DNA as a template was undertaken with primers from exon 26 and a detectable DNA band was produced indicating that at least one intact copy of this portion of the *JAG1* gene was present (Fig. 4). In addition, further studies have shown up and downregulation of gene families that are known (or unknown) to be involved in cardiac development.

There were a total of 703 genes underexpressed at least five-fold in both infants with cardiac defects relative to the control infant and 259 genes were overexpressed relative to the control. The control male infant may show more gene expression compared to the female infant with TOF due to the presence of Y chromosome genes; however, the overall effect would be minimal due to the small number of genes involved. In addition, all of the over- and underexpressed genes identified were shared by both male and female infants with TOF. Table 3 lists functional categories with the number of genes that were identified as over- and under-expressed (categories defined by Ingenuity Pathways Analysis, Ingenuity Systems, Mountain View, CA). Some of the differences in gene expression were undoubtedly due to individual variation but further analysis of interconnected networks reveals some striking concurrences. Fig. 5 shows a diagram of a so-called “network” of interacting gene products which collectively influence the expression of multiple genes and their associated networks. Interestingly, in this particular group of interacting genes, nearly all genes were underexpressed (at least five-fold less than the control infant, colored symbols) in the two infants with cardiac defects (see Fig. 5). Clearly, a large deficit of gene expression (or specific gene expression pattern) was present in the two infants with cardiac defects which collectively resulted in the failure of proper cardiac development.

Table 4 lists several genes known to be associated with cardiac development which were not expressed in our subjects with cardiac defects but were easily detected in the RNA from our control subject. *JAG1* and *LFNG* are particularly interesting because they are part of the Notch signaling pathway which influences embryonic cardiac development and *JAG1* is the cause of Alagille syndrome, a malformation syndrome with congenital heart defects. Table 5 lists several genes which are known to have a role in cardiac development which were overexpressed in our infants with cardiac defects compared to the control subject.

Analysis of gene expression in our two nonsyndromic infants with TOF revealed the loss of expression of a number of genes that are known to be important for embryological heart development. Loss of expression of these genes is undoubtedly significant, particularly *JAG1* and *LFNG* since they are part of the same regulatory pathway. The entire coding sequence of *JAG1* was sequenced in each of the infants to determine if any known or unknown mutations might account for the loss of RNA. The coding sequence contained no mutations, nor deletions (N. Spinner, personal communication). We also sequenced 500 bp of the promoter region immediately upstream of the gene start codon which also revealed nothing unusual in the sequence. Taken together, these observations suggest that there is not a structural defect in *JAG1* in these two infants and that the source of loss of expression lies elsewhere, not in the *JAG1* gene but in its relationship with other single genes or family of

genes. Fig. 5 supports this contention by illustrating a large number of interacting genes with dramatically reduced expression.

Reduction or loss of expression of individual transcription factors may not result in serious consequences. However, when a sufficient number of upstream genes have low levels of expression with a cumulative reduction in a specific set of genes then failure of key downstream gene expression may occur resulting in improper organ formation. This occurrence may be very difficult to discern but could possibly manifest as the failure of a single key gene from an interconnected network. As an example, *TP73L*, a transcription factor, may impact on *JAG1* expression as illustrated in Fig. 5. *TP73L* is downregulated in both of our infants with cardiac defects, possibly due to the failure of *TP73L* to be adequately activated transcriptionally. Low levels of *TP73L* may lead to failure of gene expression initiation of the *JAG1* gene resulting in abnormal cardiac development. Additional studies are needed to clarify the relationship of the underexpressed and overexpressed genes identified in causing the dysplastic pulmonic tissue seen in our infants with TOF. Studies are in progress to examine abnormal cardiac tissue from patients with known genetic lesions (e.g., 22q deletion in velo-cardio-facial syndrome) to identify similarities and differences in gene expression patterns between syndromic and nonsyndromic congenital heart defects.

In addition, we compared our list of genes overexpressed and underexpressed from our microarrays with the genes identified by Kaynak et al. [16] as being overexpressed or underexpressed in their subjects with TOF. There were 20 genes in common that were overexpressed relative to controls (e.g., *MYOD1*, *TP53*, *MYC*, *LMNA*, *P2RX1-3*, and *P2RX 5*) and 17 (e.g., *TBP*, *RFC2-4*, *ITGA3*, *ITGA6*, and *ITGB1*) that were underexpressed relative to controls in both experiments. These genes were underexpressed in both tissues examined (myocardium examined by Kaynak et al. [16] and dysplastic pulmonic tissue used in our study). It is not clear how a common set of genes can participate in the embryological formation of cardiac structures. It is important to mention that the tissue studied from our infants was acquired postnatally while these cardiac defects originated prenatally during the first 16 weeks post-conception. The ability to analyze complex processes like organogenesis is rapidly increasing in sophistication with new analytical tools like microarrays coupled with statistical approaches designed to elucidate interconnected networks. Characterizing gene expression patterns in specific tissues and at specific times should yield important new insights into the molecular mechanisms involved in cardiac development.

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Fig. 1.

Microarray expression using 12,814 genes of which 61% were active or detectable in normal pulmonary tissue from the control male infant and 56% were detected from dysplastic pulmonary tissue from each of the two infants (male and female) with TOF. RNA from normal pulmonary tissue from the unaffected control infant was labeled with Alexa 555 (green) and RNA from dysplastic pulmonary tissue from the affected infant 1 was labeled with Alexa 647 (red). Selected cardiac genes are illustrated. Red spots represent genes overexpressed in the dysplastic pulmonary tissue or underexpressed in normal pulmonary tissue

(which is unlikely), green spots represent genes overexpressed in the normal pulmonic tissue (which is unlikely) or underexpressed in abnormal pulmonic tissue (e.g., *JAG1*, *LFNG*), and yellow spots (vast majority) represent genes with similar expression in normal and abnormal tissues (e.g., *FBNI*, *ELN*, *ZIC3*).

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Fig. 2.

Representative scatterplot of a subset of probes (including the location of the *JAG1* gene) from the Human 1 microarray slide (Agilent Technologies, Palo Alto, CA) in Fig. 1. Lines indicate fold change in intensity for individual spots representing genes compared between the two (control and affected) infants. There is a trend for the genes to be underexpressed in the affected infants (closer to the axis of the unaffected control infant).

Fig. 3.

Reverse transcription-PCR (RT-PCR). Brain cDNA was obtained from Stratagene (La Jolla, CA). Total RNA, isolated from normal and abnormal pulmonic tissues from the unaffected control infant and infants 1 and 2 with tetralogy of Fallot, respectively, was treated with DNase and reverse transcribed (RT-PCR). (A) PCR was performed using specific primers for *JAG1* (exon 26) and *GAPDH* as a control probe. *GAPDH* expression is noted from all three infants but *JAG1* gene expression was detected only from brain cDNA and normal pulmonary artery from our control infant. No expression was observed from the dysplastic pulmonic tissue from our two infants with tetralogy of Fallot which is in agreement with our microarray gene expression data. (B) RT-PCR was performed using specific primers for *JAG1* (spanning exons 17–21). A faint band can be detected only in infant 1.

Fig. 4.

PCR using primers specific for exon 26 of the *JAG1* gene and genomic DNA (isolated from the thymus of our two infants with tetralogy of Fallot) as the template clearly demonstrates the presence of the *JAG1* gene. Exon 26 corresponds to the 3' region of gene examined in the expression studies. A section of the promoter was also PCR amplified and sequenced supplying further evidence that the *JAG1* gene region was not deleted.

Fig. 5. Interacting network of genes identified using Ingenuity Pathways Analysis Software. Symbol shape represents functional categories for each gene product. Lines between gene symbols indicate interactions. The type of interaction is indicated with a letter (e.g., B = binding) as noted in the legend. Filled or colored symbols were expressed at least five-fold less in both infants with cardiac defects compared to the normal control infant. These data were generated using Ingenuity Pathways Analysis, a web-delivered application that enables the discovery, visualization, and exploration of potentially relevant gene interaction networks. A detailed description of Ingenuity Pathways Analysis can be found at www.Ingenuity.com.

Table 1

NCBI GENE database (limited to human) searched using keywords “(cardiac or heart) and development” and 149 genes found

Gene symbol	Chromosome	Name
<i>ABCA1</i>	9q31.1	ATP-binding cassette
<i>ABCB1</i>	7q21.1	ABCB1 ATP-binding cassette
<i>ACE</i>	17q23	ACE angiotensin I
<i>ACTC</i>	15q11–q14	Actin, alpha, cardiac muscle
<i>ADAM10</i>	15q22	Disintegrin and metalloproteinase domain
<i>ADM</i>	11p15.4	Adrenomedullin
<i>AGT</i>	1q42–43	Angiotensinogen (serine (or cysteine) proteinase inhibitor
<i>AGTR1</i>	3q21–q25	Angiotensin II receptor, type 1
<i>AGTR2</i>	Xq22–q23	Angiotensin II receptor, type 2
<i>ANGPT1</i>	8q22.3–q23	Angiopoietin 1
<i>APOE</i>	19q13.2	Apolipoprotein E
<i>ARVCF</i>	22q11.21	Armadillo repeat protein
<i>ATP2A2</i>	12q23–q24.1	ATPase, Ca ⁺⁺ transporting, cardiac muscle, slow twitch 2
<i>BBS1</i>	11q13.1	Bardet–Biedl syndrome 1
<i>BBS7</i>	4q27	Bardet–Biedl syndrome 7
<i>BCL2L1</i>	20q11.21	BCL2-like 1
<i>BIN1</i>	2q14	Bridging integrator 1
<i>BMP10</i>	2p13.3	Bone morphogenetic protein 10
<i>BVES</i>	6q21	Blood vessel epicardial substance
<i>CACNA1H</i>	16p13.3	Calcium channel, voltage-dependent, alpha 1H subunit
<i>CASQ2</i>	1p13.3–p11	Calsequestrin
<i>CCL2</i>	17q11.2–q21.1	Chemokine (C–C motif) ligand 2
<i>CCND1</i>	11q13	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)
<i>CD36</i>	7q11.2	CD36 antigen (collagen type I receptor)
<i>CDK5RAP3</i>	17q21.32	CDK5 regulatory subunit associated protein 3
<i>CECR</i>	22pter–q11	Cat eye syndrome chromosome region
<i>COL1A2</i>	7q22.1	Collagen, type I, alpha 2
<i>CSRP3</i>	11p15.1	Cysteine and glycine-rich protein 3 (cardiac LIM protein)
<i>CTF1</i>	16p11.2–p11.1	Cardiotrophin 1
<i>CUGBP2</i>	10p13	CUG triplet repeat, RNA binding protein 2
<i>CXCL16</i>	17p13	CXC chemokine ligand 16
<i>CYP2D6</i>	22q13.1	Cytochrome P450, family 2, subfamily D, polypeptide 6

Gene symbol	Chromosome	Name
<i>DES</i>	2q35	Desmin
<i>DLG1</i>	3q29	Presynaptic protein SAP97
<i>DVL1</i>	1p36	Dishevelled 1
<i>DVL3</i>	3q27	Dishevelled 3
<i>EBAF</i>	1q42.1	Transforming growth factor, beta-4
<i>ECE2</i>	3	Endothelin converting enzyme 2
<i>ECGF1</i>	22q13.33	Endothelial cell growth factor 1 (platelet-derived)
<i>EDNRB</i>	13q22	Endothelin receptor type B
<i>EEF1A2</i>	20q13.3	Elongation factor 1-alpha 2
<i>ENO2</i>	12p13	Enolase 2 (gamma, neuronal)
<i>ESR1</i>	6q25.1	Estrogen receptor 1
<i>F3</i>	1p22–p21	Coagulation factor III (thromboplastin, tissue factor)
<i>F5</i>	1q23	Coagulation factor V (proaccelerin, labile factor)
<i>FBLN2</i>	3p25.1	Fibulin 2
<i>FGF2</i>	4q26–q27	Fibroblast growth factor 2 (basic)
<i>FGF4</i>	11q13.3	Fibroblast growth factor 4
<i>FGF12</i>	3q28	Fibroblast growth factor 12
<i>FOSB</i>	19q13.32	FBJ murine osteosarcoma viral oncogene homolog B
<i>GALR2</i>	17q25.3	Galanin receptor 2
<i>GATA4</i>	8p23.1–p22	GATA binding protein 4
<i>GJA1</i>	6q21–q23.2	Gap junction protein, alpha 1, 43kDa (connexin 43)
<i>GJA5</i>	1q21.1	Gap junction protein, alpha 5, 40kDa (connexin 40)
<i>GJA7</i>	17q21.31	Gap junction protein, alpha 7, 45kDa (connexin 45)
<i>GP1BA</i>	17pter–p12	Platelet glycoprotein Ib alpha polypeptide
<i>GSCL</i>	22q11.21	goosecoid-like
<i>HAND1</i>	5q33	Heart and neural crest derivatives expressed 1
<i>HAND2</i>	4q33	Heart and neural crest derivatives expressed 2
<i>HMOX1</i>	22q13.1	Heme oxygenase (decycling) 1
<i>HOP</i>	4q11–q12	Homeodomain-only protein
<i>HSPA1A</i>	6p21.3	Heat shock 70 kDa protein 1A
<i>HTR2A</i>	13q14–q21	5-Hydroxytryptamine (serotonin) receptor 2A
<i>IGF1</i>	12q22–q23	Insulin-like growth factor 1
<i>IGFBP1</i>	7p13–p12	Insulin-like growth factor binding protein 1
<i>IGF1R</i>	15q26.3	Insulin-like growth factor 1 receptor

Gene symbol	Chromosome	Name
<i>IL2</i>	4q26–q27	Interleukin 2
<i>IL-4</i>	5q31.1	Interleukin 4
<i>IL-10</i>	1q31–q32	Interleukin 10
<i>INHBA</i>	7p15–p13	Inhibin, beta A (activin A, activin AB alpha polypeptide)
<i>IRX4</i>	5p15.3	Iroquois homeobox protein 4
<i>JAG1</i>	20p12.1–p11.23	Jagged 1
<i>JARID2</i>	6p24–p23	Jumonji, AT rich interactive domain 2 protein
<i>KCNK3</i>	2p23	Potassium channel, subfamily K, member 3
<i>KIT</i>	4q11–q12	Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog
<i>LIM</i>	4q22	LIM protein
<i>LMNA</i>	1q21.2–q21.3	Lamin A/C
<i>LOX</i>	5q23.2	Lysyl oxidase
<i>MAPK12</i>	22q13.33	Mitogen-activated protein kinase 12
<i>MB</i>	22q13.1	Myoglobin
<i>MKKS</i>	20p12	McKusick–Kaufman syndrome
<i>MMP1</i>	11q22.3	Matrix metalloproteinase 1
<i>MMP2</i>	16q13–q21	Matrix metalloproteinase 2
<i>MMP3</i>	11q22.3	Matrix metalloproteinase 3
<i>MTHFR</i>	1p36.3	5,10-Methylenetetrahydrofolate reductase
<i>MTR</i>	1q43	5-Methyltetrahydrofolate-homocysteine methyltransferase
<i>MYBPC2</i>	11p11.2	Myosin binding protein C
<i>MYBPC3</i>	11p11.2	Myosin binding protein C, cardiac
<i>MYH6</i>	14q12	Myosin, heavy polypeptide 6, cardiac muscle, alpha
<i>MYH7</i>	14q12	Myosin, heavy polypeptide 7, cardiac muscle, beta
<i>MYH8</i>	17p13.1	Myosin, heavy polypeptide 8, skeletal muscle, perinatal
<i>MYL2</i>	12q23–q24.3	Myosin, light polypeptide 2, regulatory, cardiac, slow
<i>NCOA6</i>	20q11	Nuclear receptor coactivator 6
<i>NFATC4</i>	14q11.2	Nuclear factor of activated T cells
<i>NKX2-5</i>	5q34	NK2 transcription factor related, locus 5
<i>NOS3</i>	7q36	Nitric oxide synthase 3
<i>PDCD1</i>	2q37.3	Programmed cell death 1
<i>PDLIM7</i>	5q35.3	PDZ and LIM domain 7
<i>PITX2</i>	4q25–q27	Paired-like homeodomain transcription factor 2
<i>PKD2</i>	4q21–q23	Polycystic kidney disease 2 (autosomal dominant)

Gene symbol	Chromosome	Name
<i>PLCE1</i>	10q23	Phospholipase C, epsilon 1
<i>PON1</i>	7q21.3	Paraoxonase 1
<i>PON2</i>	7q21.3	Paraoxonase 2
<i>POPDC3</i>	6q21	Popeye domain containing 3
<i>POU6F1</i>	12q13.13	POU domain, class 6, transcription factor 1
<i>PPARA</i>	22q13.31	Peroxisome proliferative activated receptor, alpha
<i>PPARG</i>	3p25	Peroxisome proliferative activated receptor, gamma
<i>PTH</i>	11p15.3–p15.1	Parathyroid hormone
<i>PTH1H</i>	12p12.1–p11.2	Parathyroid hormone-like hormone
<i>PTPLA</i>	10p14–p13	Protein tyrosine phosphatase-like member A
<i>REN</i>	1q32	Renin
<i>RET</i>	10q11.2	Ret proto-oncogene
<i>ROR1</i>	1p32–p31	Receptor tyrosine kinase-like orphan receptor 1
<i>RYR3</i>	15q14–q15	Ryanodine receptor 3
<i>S100B</i>	21q22.3	S100 calcium binding protein, beta
<i>SCUBE1</i>	22q13	Signal peptide, CUB domain, EGF-like 1
<i>SCN1A</i>	2q24.3	Sodium channel, voltage-gated, type I, alpha
<i>SCN5A</i>	3p21	Sodium channel, voltage-gated, type V, alpha
<i>SFRP1</i>	8p12–p11.1	Secreted apoptosis-related protein 2
<i>SGCD</i>	5q33–q34	Sarcoglycan, delta (35kDa dystrophin-associated glycoprotein)
<i>SHH</i>	7q36	Sonic hedgehog homolog
<i>SHOX2</i>	3q25–q26.1	Short stature homeobox 2
<i>SOX6</i>	11p15.3	SRY-box containing gene 6
<i>SRI</i>	7q21.1	Sorcin
<i>TAZ</i>	Xq28	Tafazzin
<i>TBX1</i>	22q11.21	T-box 1
<i>TBX5</i>	12q24.1	T-box 5
<i>TCRB</i>	7q34	T cell receptor beta locus
<i>TDGF1</i>	3p21.31	Teratocarcinoma-derived growth factor 1
<i>TFAM</i>	10q21	Transcription factor A, mitochondrial
<i>TGFB1</i>	19q13.1	Transforming growth factor, beta 1
<i>THRAP2</i>	12q24.21	Thyroid hormone receptor associated protein 2

Gene symbol	Chromosome	Name
<i>THRB</i>	3p24.3	Thyroid hormone receptor, beta
<i>TIMP1</i>	Xp11.3–p11.23	Tissue inhibitor of metalloproteinase 1
<i>TIMP3</i>	22q12.3	Tissue inhibitor of metalloproteinase 1
<i>TNF</i>	6p21.3	Tumor necrosis factor (TNF superfamily, member 2)
<i>TNFRSF7</i>	12p13	Tumor necrosis factor receptor superfamily, member 7
<i>TNFRSF9</i>	1p36	Tumor necrosis factor receptor superfamily, member 9
<i>TNFSF5</i>	Xq26	Tumor necrosis factor (ligand) superfamily, member 5
<i>TNNI3</i>	19q13.4	Troponin I, cardiac
<i>TNNC1</i>	3p21.3–p14.3	Troponin C, slow
<i>TNNI3</i>	19q13.4	Troponin I, cardiac
<i>TNNT2</i>	1q32	Troponin T2, cardiac
<i>TP53</i>	17p13.1	Tumor protein p53
<i>TPM1</i>	15q22.1	Tropomyosin 1 (alpha)
<i>TTN</i>	2q31	Titin
<i>UTS2</i>	1p36	Urotensin 2
<i>VEGF</i>	6p12	Vascular endothelial growth factor
<i>WNT16</i>	7q31	Wingless-type MMTV integration site family, member 16

Table 2

Representative list of candidate genes for cardiac defects and human disorders

Symbol	Chromosomal location	Clinical features	Human disorder
<i>ANKRD2</i>	10	Cardiac hypertrophy	
<i>BMP1</i>	8p21	–	–
<i>CFC1</i>	2q21.1	Heterotaxy, transposition of the great arteries, double outlet right ventricle	
<i>CRELD1</i>	3p25.3	Atrioventricular septal defects	
<i>CXCR4</i>	2q21	–	–
<i>DVL1L1</i>	22q11.21	Developmental delay, cardiac defects (conotruncal), abnormal facies, thymic hypoplasia, cleft palate, hypocalcemia, chromosome 22q deletion	DiGeorge syndrome (velo-cardio-facial)
<i>EDN1</i>	6p24.1	–	–
<i>EDNRB</i>	13q22	–	Hirschsprung Disease
<i>EDR1</i>	12p13.31	–	–
<i>ERBB2</i>	17q21.1	–	–
<i>ERB4</i>	2q33.3–q34	–	–
<i>ELN</i>	7q11.23	Aortic root stenosis distal to the aortic valve, abnormal facies, developmental delay, hypercalciuria, hoarse voice	Williams syndrome
<i>EVC, EVC2</i>	4p16.1	Atrial septal defect, short stature, skeletal abnormalities, dental anomalies.	Ellis–van Creveld syndrome
<i>FBN1</i>	15q21.1	Mitral valve prolapse, aortic root dilatation, tall stature, lens dislocation	Marfan syndrome
<i>FBN2</i>	5q23–q31	Contractural arachnodactyly, crumpled ears, mitral valve prolapse	Beals syndrome
<i>FHL1</i>	Xq26	Heart failure	
<i>ZFPM2 (FOG2)</i>	8q23	Tetralogy of Fallot	
<i>FZD2</i>	17q21.1	–	–
<i>GATA4</i>	8p23.1p22	Atrial septal defects	–
<i>HAND1</i>	5q32	–	–
<i>HAND2</i>	4q33	–	–
<i>HEY2</i>	6q22.2–q22.33	–	–
<i>JAG1</i>	20p12	Peripheral pulmonary artery stenosis, abnormal facies, intrahepatic cholestasis, vertebral anomalies	Alagille syndrome
<i>MAPK1</i>	22q11.21	–	–
<i>NFATC1</i>	18q23	–	–
<i>NKX2.5</i>	5q34	Ostium secundum atrial septal defect (ASD), progressive atrioventricular conduction delay, tetralogy of Fallot	Familial ASD with atrioventricular block
<i>NODAL</i>	10	Situs ambiguous	–
<i>NRG1</i>	8p22–p11	–	–
<i>PITX2</i>	4q25–q26	Multiple cardiac anomalies	Riegers syndrome
<i>PTPN11</i>	12q24	Pulmonary artery stenosis, hypertrophic	Noonan syndrome

Symbol	Chromosomal location	Clinical features	Human disorder
		cardiomyopathy, sternal abnormalities, developmental delay, hypertelorism, hearing defect, abnormal facies	
<i>RANBP1</i>	22q11.21	–	DiGeorge syndrome
<i>TBX1</i>	22q11.21	Conotruncal anomaly	DiGeorge syndrome
<i>TBX5</i>	12q24.1	Atrial septal defect, ventricular septal defect, conduction abnormalities, upper limb anomalies	Holt–Oram syndrome
<i>TEAD1</i>	11p15.4	–	–
<i>TFAP2B</i>	6p21–p12	Patent ductus arteriosus	
<i>TGFB1</i>	19q13.1	–	–
<i>THRAP2 (PROSIT240)</i>	12q24.21	Transposition of the great arteries	
<i>TMP1</i>	15q22.1	Cardiomyopathy	Cardiomyopathy
<i>WT1</i>	11p13	–	Denys–Drash syndrome, WAGR syndrome, Wilms tumor, type 1
<i>ZIC3</i>	Xq26.2	Situs anomalies	X-linked visceral heterotaxy

Table 3

Genes at least *five-fold* over- or underexpressed in the infants with cardiac malformations relative to the control infant grouped into categories (only those with 10 genes per category are shown) assigned by Ingenuity Pathways Analysis^a

Category	Over (N=259)	Under (N=703)
Cellular growth and proliferation	37	110
Organismal development	39	103
Cancer	32	96
Cell death	16	92
Cell-to-cell signaling and interaction	29	80
Cellular development	25	69
Hematological system development and function	31	64
Immune response	25	63
Cellular movement	25	60
Tissue morphology	8	60
Immune and lymphatic system development and function	22	59
Tissue development	33	52
Cell cycle	8	50
Cell morphology	20	47
Organismal survival	0	40
Cellular function and maintenance	14	37
Nervous system development and Function	14	35
Hematological disease	14	34
Lipid metabolism	9	33
Gene expression	2	33
DNA replication, recombination, and repair	2	32
Organ development	17	29
Cell signaling	3	29
Cellular assembly and organization	22	27
Immune response	25	0
Vitamin and mineral metabolism	0	25
Reproductive system development and function	6	21
Immunological disease	5	20
Skeletal and muscular system development and function	13	18
Connective tissue development and function	13	18
Tumor morphology	9	18
Cardiovascular disease	6	17
Neurological disease	6	17

Category	Over (N=259)	Under (N=703)
Organismal functions	5	17
Cardiovascular system development and function	16	16
Embryonic development	15	6
Cellular compromise	12	12
Reproductive system disease	6	11
Endocrine system disorders	2	10

Not all genes were assigned to a category and some genes were included in more than one category. A total of 12,814 genes were examined.

^aBiological functions were assigned to each gene network by using the findings that have been extracted from the scientific literature and stored in the Ingenuity Pathways Knowledge Base (Ingenuity Systems, Inc., Mountain View, CA.).

Table 4

Genes and their descriptions related to cardiac development expressing *five-fold less* in dysplastic pulmonic tissue from infants with TOF compared to normal pulmonary artery tissue from the control infant

Gene	Description
<i>JAG1</i> * (JAGGED 1)	Ligand for multiple Notch receptors involved in the mediation of Notch signaling. May be involved in early and late stages of mammalian cardiovascular development. Jagged1 is the cause of Alagille syndrome which includes tetralogy of Fallot.
<i>LFNG</i> * (Lunatic Fringe Homologue)	Glycosyltransferase initiates the elongation of O-linked fucose residues attached to EGF-like repeats in the extracellular domain of Notch molecules. Decreases the binding of JAGGED 1 to NOTCH2 but not that of DELTA 1. By similarity, essential mediator of somite segmentation and patterning.
<i>GJA1</i> (Gap Junction protein, Connexin 43, Cx43)	Mouse embryos lacking Cx43 die at birth as a result of a failure in pulmonary gas exchange caused by a swelling and blockage of the right ventricular outflow tract from the heart. Reaume et al. [40] reported that Cx43 may play an essential role in heart development. In homozygous mice for Cx43 deficiency, the major abnormality was a delay in the normal looping of the ascending limb of the heart tube including the right ventricle and the outflow tract [41]. This predisposes to subsequent complex malformation of the subpulmonary outflow tract and tricuspid valve, leading to the heart defects described by Reaume et al. [40].
<i>HAND1</i>	Plays an essential role in cardiac morphogenesis. Could be required for ongoing expression of cardiac-specific genes in the adult.
<i>ERBB2</i>	Gene knockout studies have demonstrated that the ErbB2 receptor is essential for normal development of the heart ventricle [42]. Conditional mutant mice (mutated ErbB2 specific to the ventricular cardiomyocytes) developed severe dilated cardiomyopathy with cardiac dysfunction. Ozcelik et al. [42] inferred that signaling from the ErbB2 receptor, which is enriched in T-tubules in cardiomyocytes, is crucial for adult heart function.
<i>TH</i> (Tyrosine hydroxylase)	Zhou et al. [43] showed that inactivation of both tyrosine hydroxylase alleles by gene targeting in embryonic stem cells results in midgestational lethality. About 90% of mutant embryos died between embryonic days 11.5 and 15.5, apparently of cardiovascular failure.
<i>ENG</i> (Endoglin)	Eng $-/-$ embryos exhibited an absence of vascular organization and the persistence of an immature perineural vascular plexus, indicating a failure of endothelial remodeling in Eng $-/-$ embryos. At embryonic day 10.5, the cardiac tube did not complete rotation and was associated with a serosanguinous pericardial effusion. By embryonic day 10.5, the major vessels including the dorsal aorta, intersomitic vessels, branchial arches, and carotid arteries were atretic and disorganized in Eng $-/-$ embryos [44].

Table 5

Genes and their descriptions related to cardiac development expressing *five-fold greater* in dysplastic pulmonic tissue from infants with TOF compared to normal pulmonary artery tissue from the control infant

Gene	Description
<i>DSH2</i> (Disheveled 2) ^a	Wnt pathway component. Mice with Dsh2 null mutations had cardiovascular outflow tract defects, including double outlet right ventricle, transposition of the great arteries, and persistent truncus arteriosus [45].
<i>FZD3</i> ^a (Frizzled homologue 3)	Wnt pathway component. The WNTs are a family of secreted glycoproteins that have been shown to be involved in a variety of developmental processes in many organisms and recently reported to impact the cardiovascular system [46].
<i>AGTR2</i> (Angiotensin II receptor, type 1)	Angiotensin receptor null mice have large ventricular septum defects [47].
<i>TNS</i> (Cardiac muscle tensin)	Structural protein, component of focal adhesions.
<i>ACTB</i> (Actin, beta)	Cytoskeletal structural protein.

^a Genes from a common developmental pathway.