

## Genetics of Cardiac Septation Defects and Their Pre-Implantation Diagnosis

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### Summary

Cardiac septation defects are among the most common birth defects in humans. The frequency of these defects reflects the complexity of cardiogenesis, which involves such processes as cell proliferation, migration, differentiation, and morphogenetic interactions.

Major advances in the understanding of the underlying genetic etiologies of cardiac septation defects have provided insight into the genetic pathways involved. These genetic factors are most often transcription factors involved in the early stages of cardiogenesis. The ability to modify these genes in animal models is providing a better understanding of the role of these genes in common pathways leading to diverse forms of cardiac defects. Ultimately, our understanding of these basic processes should lead to molecular-based treatment and prevention options for those individuals most at risk for such birth defects.

**Key Words:** Heart; congenital; genetics; septation defect; transcription factor.

### 1. Introduction

Congenital heart malformations (CHMs) occur in more than 0.5 to 1% of live births (*1*) and are among the most common birth defects in newborns. Atrial septal defects (ASDs) and ventricular septal defects (VSDs) account for the greatest proportion of CHMs (*2*). Serial echocardiography of large newborn populations has revealed that VSDs are present at birth in approx 5% of all newborns (*3*), but most VSDs close spontaneously before adolescence (*4*). In recent years, our understanding of the etiology of certain CHMs has increased significantly. Great strides have been made in the identification of genes responsible for both syndromic and nonsyndromic forms of CHM, and their role in cardiogenesis. By identifying human gene mutations associated with syndromes affecting cardiac chamber septation, in vitro and in vivo mod-

els of abnormal cardiogenesis can be created, and investigators can define genetic interactions that regulate cardiogenesis.

To understand how gene mutations cause syndromic and nonsyndromic forms of septal defects, one must delineate the process of septal formation during cardiogenesis. Cardiogenesis requires a complex series of events, including proliferation, migration, differentiation, and morphogenetic interactions involving cells from several embryonic origins. The susceptibility of the heart to developmental anomalies reflects the complexity of these embryonic processes. Among the most sensitive cardiac structures are the atrial and ventricular septa. Separation of the four cardiac chambers involves more than mere formation of simple partitions between the chambers. After formation of the primary heart tube, this linear tube loops to the right, creating inflow and outflow limbs. The primitive chambers become recognizable by the constrictions that demarcate the sinus venosus, common atrial chamber, atrioventricular (AV) canal, ventricular chamber, and conotruncus. Initially, endocardial cushions form within the common AV junction. Simultaneously, the myocardium of the atrial and ventricular chambers develop trabeculations of endocardium-covered myocardium that extend into the lumen of the heart tube. Ultimately, all of the septa fuse with the AV cushions, and these cushions divide the AV canal into left and right canals. Although many genes that regulate septogenesis remain to be identified, several genes have been shown to play key roles in septation.

Historically, recurrence risk assessment for CHM in individuals and their relatives was based on epidemiological data (5,6). As the genetic bases for many types of CHMs are elucidated, genetic counseling for such families is increasingly refined and effective. The ability to offer genetic testing will aid in identifying those at-risk individuals who may present with normal cardiac evaluations but who may be susceptible to complications later in life, or for having children with more severe presentations of disease. A detailed three-generation family history is useful in the evaluation of the individual with a CHM. Additionally, a peripheral blood karyotype is warranted in those individuals with multiple congenital anomalies.

CHMs can be detected and diagnosed prenatally, and, in some instances, may be amenable to fetal intervention (7). Furthermore, vast improvements in the surgical and medical management of individuals with CHMs during the last several decades has dramatically increased the number of adults living with CHMs (4). ASDs, which occur more commonly in women than men, are among the most common CHMs to be identified in the adult CHM patient (4). Because these individuals have their own families, clinicians are likely to see an increase in clusters of familial CHMs.

At present, genetic testing for the majority of the genes implicated in CHMs is available only on a research basis (8). Continued research to better understand the incidence, penetrance, and expressivity of mutations in these and other genes involved in CHMs is critical before such tests warrant widespread clinical application. In this chapter, we discuss syndromes that are characterized by the presence of ASDs and/or VSDs and their causative genes. We provide an overview of some of the better understood genes investigated to date, but this overview is not a comprehensive list. Mendelian syndromes and chromosomal etiologies with associated CHMs that can include septation defects are listed in **Table 1 (9–11)**. The number of genes associated with ASDs and VSDs in patients is rapidly increasing.

Although the technology best used to screen for mutations (e.g., denaturing high-performance liquid chromatography, denaturing gel electrophoresis, single-strand conformational polymorphism analysis, and automated sequencing) can be legitimately debated, and largely reflects institutional and laboratory biases, we do not focus on this technical aspect. Instead, we focus on the biological processes underlying different genetically determined septation defects so that such understanding may appropriately inform the choice of genes to be screened for mutations in any given patient. From the perspective of routine clinical testing, it is already impractical to screen all septation defect-associated genes in any given patient, and the selection of genes to be screened by the clinician evaluating the patient to undergo testing will be an increasingly challenging problem. Thus, the methods used to make this selection should rely on a thorough understanding of the phenotypes, expression patterns, and functional developmental defects associated with mutations in each gene.

### **1.1. Holt–Oram Syndrome: TBX5**

Holt–Oram syndrome (HOS) is an autosomal-dominant disorder characterized by structural and/or conductive heart deformities in the setting of upper-limb radial ray anomalies (12). HOS is estimated to occur in approx 1 in 100,000 live births, and most individuals represent sporadic cases, i.e., new mutations. There is significant phenotypic variability observed between affected individuals even in a single family, suggesting a role for modifying genetic factors. The limb findings associated with HOS are fully penetrant and may be unilateral, bilateral/symmetric, or bilateral/asymmetric. A range of upper-limb deformities (ULDs) with varying severity may be observed, such as triphalangeal or absent thumb(s), severe limb hypoplasia, phocomelia, abnormal forearm pronation and supination, possible sloping shoulders, and restriction of shoulder joint movement. Polydactyly is not a feature of HOS. Left-sided ULDs are often more severe than right-sided ULDs. In some affected

**Table 1**  
**Mendelian and Chromosomal Forms of ASD, VSD, and AVSD**

Disease	Gene (chromosomal location)	OMIM or reference no.	Clinical features, including CHMs
Autosomal-dominant conditions			
Alagille syndrome	<i>JAG1</i> (20p12)	118450	Peripheral PS, ASD, TOF, deafness, decreased number of intrahepatic bile ducts, posterior embryotoxon, triangular facies
ASD with AV conduction defects	<i>NKX2.5</i> (5q34)	108900	ASD progressive AV block, TOF, VSD, tricuspid valve anomalies
ASD2	<i>GATA4</i> (8p23.1-p22)	607941	ASD, VSD, AVSD, PS, mitral and aortic valve regurgitation
Autosomal-dominant ASD	(5p)	<b>10</b>	Incomplete penetrance, aortic stenosis, atrial septal aneurysm, persistent left superior vena cava
ASD1	(6p21.3)	108800	Autosomal-dominant ASD
AVSD	(1p31-p21)	600309	Autosomal-dominant AVSD, incomplete penetrance, and variable expressivity
AVSD2	<i>CRELD1/CIRRN</i>	607170	Partial AVSD with heterotaxy
DiGeorge syndrome	<i>TBX1</i> (del22q11)	188400	VSD, right sided aortic arch, TOF, learning disabilities, secondary palate clefts
Holt–Oram syndrome	<i>TBX5</i> (12q24.1)	142900	Ostium secundum ASD, VSD, progressive heart block, upper-limb deformity
Duane–Radial Ray syndrome (Okilhiro syndrome)	<i>SALL4</i> (20q13.13-q132)	607323	Radial ray abnormalities, Duane anomaly, sensorineural and/or conductive deafness, ASD (infrequent), Hirschsprung disease, anal stenosis, imperforate anus, renal abnormalities, fused cervical vertebrae, hypoplasia of pectoral and upper-limb musculature

Noonan syndrome	<i>PTPN11</i> (12q24.1)	163950	PS, left ventricular hypertrophy, cardiac septal defects, webbed neck, postnatal short stature, characteristic facial features, cryptorchidism, bleeding diathesis, vertebral anomalies
Autosomal-recessive conditions			
Ellis-van Creveld syndrome	<i>EVC</i> (4p16), <i>EVC2</i> (4p16)	225500	Common atrium, ASD, AVSD, short limbs, short ribs, postaxial polydactyly, dysplastic teeth and nails
Ivemark syndrome	Not identified	208530	Asplenia or polysplenia, abnormal lobation of lungs, malposition and maldevelopment of abdominal organs, ASD, VSD, AVSD, PS
McKusick-Kaufmann syndrome	<i>MKKS</i> (20p12)	236700	Hydrometrocolpos, postaxial polydactyly, ASD, VSD, AVSD, common atrium
Smith-Lemli-Opitz	<i>DHCR7</i> (11q12-q13)	270400	ASD, VSD, AVSD, PDA, aortic coarctation, short stature, failure to thrive, low cholesterol levels, facial dysmorphism, abnormal male external genitalia
TARP syndrome	Xp11.23-q13.3	300442	Club foot, ASD, Robin sequence, persistent left superior vena cava, lethal in infancy
Chromosomal etiologies			
Chromosomal anomaly	Clinical features		
Down syndrome/trisomy 21	AVSD, common neonatal findings include hypotonia, flat facial profile, poor motor reflex, slanted palpebral fissures, joint hyperflexibility, excess skin on posterior neck, transpalmar crease		
Trisomy 13	AVSD, holoprosencephaly-type defects, severe mental defect, minor motor seizures, polydactyly, rocker-bottom feet, mild microcephaly, single umbilical artery		

(continued)

**Table 1 (continued)**

Disease	Clinical features
Trisomy 18	AVSD, clenched hand and overlapping fingers, polyhydramnios, single umbilical artery, hypertonic, mental deficiency, short sternum, small nipples, inguinal or umbilical hernia, hypoplastic nails
3pdel	AVSD, mental and growth retardation (prenatal onset), hypotonia, microcephaly, bilateral ptosis, upper limb postaxial polydactyly
Turner syndrome (45,X)	Bicuspid aortic valve, aortic coarctation, MVP, AVSD, short stature (female), congenital lymphedema, broad chest with widely spaced nipples, ovarian dysgenesis, narrow convex nails
Interstitial dupl 19p	VSD, urinary tract anomalies, renal anomalies, rocker-bottom feet, small thorax, mild hydrops fetalis, nuchal edema
ASD, atrial septal defect; AV, atrioventricular; AVSD, AV septal defect; MVP, mitral valve prolapse; PDA, patent ductus arteriosus; PS, pulmonic stenosis; TOF, Tetralogy of Fallot; VSD, ventricular septal defect.	

individuals, the ULD may involve only the carpal bones and be visible only on X-ray. Initial clinical evaluation should, therefore, include posterior–anterior hand X-rays of the affected individual's parents to rule out any carpal bone anomalies. This information, in the absence of mutational analysis, is critical for offering accurate recurrence risk estimates for the family.

Heart defects in HOS are incompletely penetrant, occurring in approx 75% of affected individuals (13). When they do occur, expressivity is highly variable. The most typical CHMs associated with HOS are ostium secundum ASDs, which may be associated with isomerism and can present as a common atrium. Similarly, VSDs, particularly of the muscular trabeculated septum are frequent manifestations (13,14). Such defects are often associated with abnormal ventricular trabeculation. Numerous other complex CHMs have been associated with HOS, but conotruncal anomalies and ostium primum ASDs are not cardinal features of this syndrome. All individuals with HOS, regardless of the presence of a CHM, are at risk for cardiac conduction disease. Such conduction disease can be progressive, resulting in complete heart block with or without atrial fibrillation, and mandates routine electrocardiographic evaluation for all individuals with HOS.

Mutations in *TBX5*, a member of the T-box transcription factor gene family, cause HOS. This gene is located at chromosome 12q24.1 (14,15). The T-box gene family is characterized by a highly conserved T-box DNA-binding domain. Of the many *TBX5* mutations identified to date, most are nonsense or frameshift mutations, splice mutations, or chromosomal rearrangements that are predicted to produce HOS through *TBX5* haploinsufficiency. *TBX5* missense mutations do occur, and, unlike *TBX5* haploinsufficiency mutations, they have been associated in large family studies with more significant organ bias in their manifestations (13). Because such genotype–phenotype correlations are only evident as statistically significant findings in population studies, they are not useful in predicting the individual patient's phenotype (13,16). Only a single family potentially affected by HOS (17) has been shown not to be linked to chromosome 12q24.1, and, thus, if any intergenic genetic heterogeneity exists in HOS, it is very small.

The specific role of *TBX5* in heart development is slowly being elucidated. *TBX5* has been shown to play a role in chamber specification (18) as well as in inhibition of cardiomyocyte proliferation (19). Therefore, *TBX5* may participate in cardiogenesis by regulating cell proliferation in specific cardiac domains, resulting in the regional morphological features of the heart. Creation of a *Tbx5* knockout mouse (20) has provided an *in vivo* method for identifying downstream targets of *Tbx5*. Although these embryos died early in development, before cardiac looping, several observations were made before their death. Failed development of posterior sinoatrial structures resulted in a

common atrium and a distorted right ventricle, thus, adding credence to the hypothesis that *Tbx5* plays a role in cardiac septation. In addition, expression of several cardiac-specific genes, such as *Mlc2v*, *Irx4*, *Hey2*, *ANF*, *Nkx2.5*, and *Gata-4* were noted to be either decreased or absent in the *Tbx5*-null mice. However, only expressions of *ANF* and *Cx40* were altered in heterozygous *Tbx5* mice, which were also observed to have enlarged hearts with ASDs. Bruneau et al. (20) further demonstrated that *Tbx5* acts synergistically with *Nkx2.5* to transactivate expression of *Cx40*. Other biochemical studies have confirmed this finding and have demonstrated a synergy between *Tbx5* and *Nkx2.5* to transactivate expression of *ANF* (21,22). Recent data from Garg et al. (23) suggests that *Tbx5* also interacts with *Gata-4*. Identification of the downstream targets and cofactors of *TBX5* is helpful in understanding the role of this transcription factor in cardiogenesis.

### **1.2. Familial ASDs With Progressive AV Block: NKX2.5**

Schott et al. (24) identified mutations in *NKX2.5* (a member of the highly conserved NK homeobox transcription factor gene family) as the underlying cause for an autosomal-dominant cardiac disorder characterized by ostium secundum ASDs and postnatal progressive AV conduction defects. Unlike individuals with HOS, these patients did not exhibit limb deformity (25). The families in this cohort had members with various other CHMs, including VSD, tetralogy of Fallot (TOF), subvalvular aortic stenosis, ventricular hypertrophy, pulmonary atresia, and mitral valve malformations. In another case, Pauli et al. (26) reported a patient with a distal 5q deletion, including the *NKX2.5* locus and who presented with an ASD, AV block, and ventricular myocardial noncompaction.

Benson and colleagues (27) provided further evidence for a diverse role for *NKX2.5* mutations in a variety of forms of congenital heart disease, including ASD, muscular VSD, VSD associated with TOF or double-outlet right ventricle (DORV), tricuspid valve abnormalities, and familial cardiomyopathy. Goldmuntz et al. (28) identified *NKX2.5* mutations in nonsyndromic TOF and estimated that *NKX2.5* mutations account for at least 4% of such cases. In a series of 114 prospectively enrolled individuals with TOF (in whom 22q11 deletions were excluded), they identified six individuals with *NKX2.5* mutations. The combination of right-sided aortic arch and pulmonary atresia were more common in the mutation-positive group. The authors demonstrated that reduced penetrance and variable expressivity were associated with these mutations, and that AV conduction disease was not associated with mutations located outside of the gene's DNA-binding homeodomain.

*NKX2.5* mutations are proposed to contribute to a small but significant proportion of sporadic cases of ASD and hypoplastic left-heart syndrome (HLHS)



(29). One hundred forty-six individuals with a diagnosis of secundum ASD ( $n = 102$ ), patent foramen ovale complicated by paradoxical embolism ( $n = 25$ ), or HLHS ( $n = 19$ ) were analyzed. Ten percent of the patients with ASD or patent foramen ovale reported a family history of heart defects, and 4% of the patients had AV block. A missense mutation, T178M, was identified in an ASD family without AV block, and in one child in the family with HLHS. A second previously reported disease-causing mutation, E21Q, was also identified, but did not segregate with disease in a family with ASD, leading one to question the significance of this variant. These findings also highlight the difficulty in establishing whether unique sequence variants found in isolated individuals with CHMs are truly responsible for disease pathogenesis.

McElhinney et al. (30) reported *NKX2.5* mutations in 3% of a population of prospectively enrolled patients afflicted with the following CHMs: TOF, secundum ASD (with and without AV block), truncus arteriosus, DORV, left transposition of the great arteries, interrupted aortic arch, HLHS, and aortic coarctation. Almost 90% of patients with *NKX2.5* mutations in this group reportedly had no family history of CHMs. Heterozygote status was confirmed in some available parents, suggesting decreased penetrance of these mutations. Interestingly, none of the 18 mutations, which consisted of missense mutations, an in-frame deletion, and an insertion resulting in premature translation termination, were localized to the homeodomain region. Thus, these studies highlighted the importance of mutational analysis, suggesting *NKX2.5* in nonhomeodomain *NKX2.5* regions.

Given the various cardiac phenotypes observed in the previously mentioned families, the role of *NKX2.5* in other forms of CHMs as well as cardiac morphogenesis seems diverse. Although genotype–phenotype correlations have not borne out, the variable expressivity among affected individuals may reflect various interactions between *NKX2.5* and other modifier genes. Located on chromosome 5q35 (31), the *NKX2.5* gene, the human homolog of *Drosophila* gene, *tinman*, is a marker of the cardiac lineage and is expressed in atrial and ventricular myocardium during development (32). Mutations in the *Drosophila* gene, *tinman*, result in absence of the dorsal vessel, a relative of the vertebrate heart (33). In vertebrates, *NKX2.5* is an important regulator of cardiac-restricted gene activity required during cardiogenesis; as demonstrated in mice deficient in *Nkx2.5*, which died before looping of the linear heart tube (32). Biochemical analysis of mutant proteins demonstrated *NKX2.5* haploinsufficiency as a cause of these cardiac defects (34). The wide range of *NKX2.5* mutational cardiac phenotypes suggests a primary involvement of this transcription factor with other modifier genes at various stages of cardiogenesis. Ongoing efforts to uncover the downstream targets and synergistic partners of *NKX2.5* have

revealed a number of myocardial genes, including *ANF*, *MLC-2v*, *N-Myc*, *Msx2*, *eHAND*, *MEF2C* (32), *TBX5* (21), and *GATA-4* (35).

### 1.3. Familial ASDs and VSDs: GATA4

A subset of familial, nonsyndromic cardiac septal defects has recently been linked to mutations in *GATA-4* (23), a member of the conserved GATA zinc-finger transcription factor family. Before this, Pehlivan demonstrated that individuals with a chromosomal deletion of 8p23.1, where *GATA-4* localizes, characteristically had a variety of cardiac septal defects (36). Taken together, these data suggest that *GATA-4* and, potentially, other 8p genes, regulate septogenesis during cardiac development.

Initially, linkage of these CHMs to a single locus on chromosome 8p22–23 in a large, multigenerational family with an autosomal-dominant inheritance of ASDs revealed the presence of *GATA-4* within this region. Through sequence analysis of *GATA-4* in two large kindreds, Garg et al. (23) were able to associate two *GATA-4* mutations with these congenital heart defects. Besides the characteristic ASDs found in all affected members, individuals also presented with a variety of other congenital heart defects, including VSDs, AV septal defects (AVSDs), and pulmonic valvular stenosis. The association with AVSD and conotruncal anomalies distinguishes this syndrome from HOS. Nevertheless, missense mutation of *GATA-4* diminished DNA-binding activity and transcriptional activity of the transcription factor, and it abrogated a physical interaction between *GATA-4* and *TBX5*.

*GATA-4* mutations were not demonstrated in some ASD and VSD kindreds that were analyzed (23). This observation, in addition to the finding that *GATA-4* is not consistently deleted in all individuals with CHMs who have 8p mutations (36), suggests a role for other genes in close proximity to *GATA-4* as causative for the familial clusters. It is also conceivable that such genes may mediate the effects of *GATA-4* on cardiogenesis.

During mouse cardiogenesis, *Gata-4* is expressed in the atrial and ventricular myocardium, endocardium, endocardial cushions, and outflow tract (37). Disruption of *Gata-4* in the mouse has revealed a phenotype that is consistent with its expression pattern. Deletion of *Gata-4* in mice causes arrested development between embryonic days 7.0 and 9.5, caused by defects in cardiac morphogenesis, specifically cardia bifida (38). However, Pu et al. (39) showed that a conditional deletion of mouse *Gata-4* leads to DORV, common AV canal, and marked hypoplasia of both the trabecular and compact myocardium. In vitro experiments using embryonic stem cells showed that *GATA-4* is essential for survival of cardioblasts and for terminal cardiomyocyte differentiation (40,41). Several genes, such as  $\alpha$ - and  $\beta$ -*MHC*, *ANF*, and cardiac *TnC*, are transcriptionally regulated by *Gata-4* (42) when studied by in vitro assays.

Data also show that *Gata-4* acts synergistically with *Nkx2.5* to activate *ANF* (43) as well as cardiac  $\alpha$ -actin via the serum response factors (44), and is influenced by interactions with FOG-2 to modulate GATA-dependent transcriptional activation, thus, acting as either an activator or repressor (45,46). A potentially cooperative relationship between TBX5 and GATA-4 (23) is appealing, given the overlap in cardiac phenotypes associated with the mutations in these genes.

#### 1.4. Ellis–van Creveld Syndrome: EVC and EVC2

Unlike other Mendelian forms of CHMs, Ellis van–Creveld syndrome (EvC) is inherited in an autosomal-recessive manner (47). The hallmark features of EvC are congenital heart defects, which occur in at least 50% of affected individuals, as well as chondroectodermal dysplasia and bilateral postaxial polydactyly (48,49). Such findings have not been reported in syndromes with septation defects caused by mutations in *TBX5*, *NKX2.5*, or *GATA-4*. Cardinal cardiac features include ostium secundum or ostium primum ASDs, as well as a common atrium. Complex CHMs have also been observed (48–52). Occasional findings include polydactyly of the feet, and genitourinary anomalies (49,51). EvC has been reported in a number of ethnic groups, but is particularly common in the Lancaster County, PA Amish population, where more than 12% of individuals are carriers for the disease, and approx 1 in 5000 people are affected (50).

By studying affected families of Amish, Mexican, Ecuadorian, and Brazilian descent, Polymeropoulos et al. (52) initially mapped the genetic locus for EvC to chromosome 4p16.1. Subsequent positional cloning studies of affected Amish individuals enabled investigators to identify the novel *EVC* gene at this locus as a disease gene. The EVC protein is predicted to contain a leucine zipper, as well as putative nuclear localization signals and a transmembrane domain (53), but its function remains unknown. Galdzicka et al. (54) also identified the *EVC2* gene. This gene is mutated in individuals affected by EvC, and also localizes to chromosome 4p16. Ruiz-Perez et al. (55) reported mutations in *EVC2* in a number of other affected individuals of varying ethnicities, but not in Amish families. *EVC* and *EVC2* are arranged in the genome in a “head-to-head” configuration, with transcription start sites separated by 2624 bp in humans. Expression of *EVC* and *EVC2* could be coordinated by the same promoter or by shared elements of overlapping promoters (55). The *EVC2* protein is predicted to encode a transmembrane domain, three coil-coiled regions, and a RhoGEF domain, and shares sequence homology to class IX nonmuscle myosin tail domains, but is not homologous to *EVC*. Northern blot analysis of *EVC2* revealed expression in the heart, placenta, lung, liver, skeletal muscle, kidney, and pancreas (54). *In situ* hybridization of human embryonic tissue for *EVC*

revealed low levels of mRNA in the developing bone, heart, kidney, and lung. Specifically, *EVC* expression was observed in the atrial and ventricular myocardium, as well as the atrial and interventricular septa (53).

The availability of genetic testing, particularly in the setting of ultrasound diagnosis (56), is an important advance in the management of at-risk pregnancies and newborns with EvC. By identifying new mutations in the *EVC* and *EVC2* genes, more information can be gained to understand how these two genes participate in cardiac and skeletal development. Further studies to elucidate the downstream targets may explain not only the mechanism by which mutations in *EVC* and *EVC2* lead to the manifestations of disease observed in affected individuals, but also may explain the mechanism by which these mutations produce either a skeletal or cardiac phenotype, or both.

### **1.5. VSD: 22q11.2 Genes and Other Candidates**

VSDs are the most common form of CHM observed. They may occur as isolated defects, or in conjunction with other CHMs, such as those discussed in **Subheading 1.3**. VSDs are a common occurrence in individuals with 22q11-deletion syndrome, also referred to as DiGeorge syndrome and velocardiofacial syndrome (57,58). Individuals with 22q11 chromosomal deletions often have characteristic facial features, palatal anomalies, mental/learning deficiencies, and endocrine and immunological findings, in addition to the cardiovascular defects. These characteristics aid in the diagnosis. McElhinney et al. (59) sought to determine the contribution of 22q11 deletion to VSDs in a group of 125 prospectively enrolled individuals with conoventricular, posterior malalignment, or conoseptal hypoplasia VSD. Although the authors identified 22q11 deletion in 10% of those individuals enrolled, the strongest predictors for chromosomal deletion were anomalies of aortic arch branching or sidedness, anomalies of the cervical aortic arch, or discontinuous pulmonary arteries in conjunction with VSD. In general, the contribution of 22q11 deletion to certain isolated VSDs is predicted to be minimal.

Recent evidence suggests that many features associated with DiGeorge syndrome are caused by haploinsufficiency of the *TBX1* gene, a member of the T-box gene family (60–62). Mice with a hemizygous deletion of the 1.5-Mb region corresponding to human chromosome 22q11 exhibited conotruncal and parathyroid defects. After insertion of a human bacterial artificial chromosome containing the *TBX1* gene, however, only the conotruncal defects could be partially rescued (62). Individuals who have the phenotypical features of 22q11.2-deletion syndrome, but have intact 22q11.2 chromosomes, have been shown to have mutations in *TBX1*, which localizes to this chromosomal region. Thus, *TBX1* may be a major determinant of the 22q11.2-deletion syndrome (63).

Membranous VSD, with variable muscular septal involvement, has been observed in association with biventricular cardiomyopathy in transgenic mice lacking *Hey-2/CHF1* (64). Based on its expression pattern, *Hey-2/CHF1* has been implicated in the development of the ventricle, vasculature, somites, and retina (65,66). The *Drosophila* gene, *gridlock*, a member of the hairy/enhancer of split-related family of genes, is expressed in a pattern similar to the mouse *Hey* genes, and functions downstream of Notch (67). Although these genes have yet to be implicated in human CHMs, other Notch ligands have been implicated. Mutations in the *JAG1* gene, a Notch-receptor ligand, result in the autosomal dominantly inherited Alagille syndrome (68,69), a multisystemic disorder with frequent cardiac involvement. Primarily, peripheral pulmonic stenosis occurs, although ASD, VSD, and TOF can also occur.

### 1.6. AV Septal Defects

AVSDs, also referred to as endocardial cushion defects or AV canal defects, result from developmental anomalies of the AV canal. The resulting AVSDs can range in severity and complexity, and may be partial (atrial or ventricular form) or complete (involve atrial and ventricular septa), which could reflect a polygenic basis for the various clinical presentations.

Epidemiological data on AVSDs from the Baltimore–Washington infant study have supported the idea of genetic heterogeneity and have provided other useful information regarding the various subtypes of AVSD (70). Family history of CHMs is more commonly observed in individuals with the complete form of AVSD and with the partial atrial form. Also of note, complete AVSD and the partial ventricular form are more likely to occur in association with extracardiac anomalies, whereas nearly 55% of the partial atrial form occur as an isolated finding (70). Preconceptual maternal diabetes is a risk factor for the complete form of AVSD, as well as other cardiac and noncardiac complications in newborns, thus, highlighting the importance of eliciting a detailed pregnancy history in the evaluation of such cases (71).

In humans, AVSDs are most commonly associated with Down syndrome (trisomy 21). Down syndrome cell adhesion molecule has been proposed as a candidate gene for CHMs associated with Down syndrome (70). AVSDs are also commonly associated with other chromosomal anomalies, including 3p25 deletion, 8p2 deletion, trisomy 13, and trisomy 18, often presenting as ventricular AVSDs (70,72). Such chromosomal etiologies are generally characterized by additional clinical manifestations, including mental and growth retardation, as well other multiple congenital anomalies. AVSD is occasionally detected in Turner syndrome (chromosomal complement 45,X) and 22q11.2-deletion syndrome (72). Autosomal-dominant AVSD, exhibiting

incomplete penetrance and variable expressivity, has also been reported, with linkage to chromosomes 1p31–p21 and 3p25 (73,74).

The *CRELD1*, or *cirrin*, gene was proposed as an AVSD candidate gene on 3p25. *CRELD1* is a cell adhesion molecule that is expressed during heart development (75). Robinson and colleagues (76) analyzed the *CRELD1* gene in individuals with both the complete and partial forms of AVSD. They reported distinct missense mutations in three patients, two patients with isolated partial AVSD (ostium primum ASD) and one patient with AVSD and heterotaxy. There were no mutations identified in 13 individuals with complete AVSD, suggesting genetic heterogeneity among the various subtypes. Each of the detected mutations occurred at highly conserved amino acid residues. Paternal inheritance was confirmed in one of the individuals with isolated partial AVSD, and the mutation was also detected in two of the subject's siblings. Neither the father nor the siblings had any identifiable CHM on echocardiogram, suggesting incomplete penetrance. These findings prompted Robinson et al. (76) to conclude that *CRELD1* is an AVSD susceptibility gene and that mutations in *CRELD1* are associated with an increased risk of developing partial AVSDs.

In some cases, Noonan syndrome, an autosomal-dominant, genetically heterogeneous syndrome that occurs in approximately every 1 in 1000 to 2500 live births, has been shown to result from mutations in the *PTPN11*, or *SHP-2*, gene on chromosome 12q24.1 (77). This gene has been demonstrated to have a role in cardiac semilunar valvulogenesis, among other developmental activities. The CHMs typically associated with Noonan syndrome are pulmonic stenosis and hypertrophic cardiomyopathy, although AVSD has been reported in approx 15% of cases (78). Individuals with Noonan syndrome also commonly have other diagnostic features, including webbed neck, dysmorphic facial features, proportionate short stature, chest deformity, cryptorchidism, mental retardation, and bleeding diatheses. Mutations in *PTPN11* have also been identified in individuals with multiple lentigines, electrocardiographic-conduction abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retardation of growth, and sensorineural deafness syndrome (79).

AVSD has been reported, although very rarely, in individuals suggested to have other Mendelian syndromes (e.g., EvC and HOS), but genetic analyses of genes associated with these syndromes have not been reported to confirm these clinical diagnoses. Similar to EvC, many autosomal-recessive syndromes in which AVSD and other CHMs have been observed involve skeletal abnormalities. McKusick–Kaufman syndrome, similar to EvC, is common in the Amish population and is caused by mutations in the *MKKS* gene on chromosome 20p12 (80). This gene encodes a protein similar to the chaperonin proteins.



Clinical features include hydrometrocolpos in females, postaxial polydactyly, and CHMs. The autosomal-recessive oral–facial–digital syndromes, including short-rib polydactyly, have also been reported to have a risk for AVSD.

In addition to the syndromes discussed in **Subheading 1.6.**, AVSD is also commonly seen in association with developmental defects involving left–right axis formation. One example is Ivemark syndrome, characterized by asplenia, CHMs (mostly ASDs, AVSDs, and conotruncal malformations) (**81**), malposition and maldevelopment of the abdominal organs, and abnormal lobation of the lungs. Ivemark syndrome is a recessive condition, but is most often sporadic.

Individuals with Smith–Lemli–Opitz syndrome, a recessive condition caused by mutations in the sterol  $\delta$ -7-reductase on chromosome 11q12–q13 (**82**), are also afflicted with AVSDs. In one report, approx 21% affected individuals had a diagnosis of AVSD (**83**). Other common features of Smith–Lemli–Opitz include failure to thrive, low birth weight, dysmorphic facies, renal anomalies, genital anomalies in males, postaxial polydactyly, mental retardation, and low cholesterol levels.

### **1.7. Pre-Implantation Genetic Diagnosis for Congenital Heart Defects**

The ability to offer genetic testing for CHM has been limited until recent years. Now that several causative gene defects, as discussed in **Subheadings 1.1.–1.7.**, have been identified for some cases of familial CHMs, increasing numbers of options are available. Before identification of these genes, couples interested in prenatal diagnosis for CHMs were limited to genetic counseling and general recurrence risk assessments, and to imaging modalities (such as ultrasound) or testing for chromosomal causes of CHMs in conjunction with amniocentesis or chorionic villus sampling. With the identification of single genes responsible for Mendelian forms of CHMs, the ability to offer pre-implantation genetic diagnosis (PGD) in combination with in vitro fertilization (IVF) is now a potential option for some couples who prefer earlier diagnosis than afforded by amniocentesis or chorionic villus sampling.

Successful PGD for CHM has been achieved for a pregnancy at 50% risk for HOS (**84**) in a couple pursuing IVF for premature ovarian failure. The proband had a previously reported mutation in exon 3 of *TBX5*, predicting a truncated protein(Glu69ter) (**14**). The proband and his spouse wished to achieve a pregnancy unaffected by HOS, if possible.

## **2. Materials**

1. *TBX5* oligonucleotide primers.
2. Polymerase chain reaction (PCR) reagents and equipment.

3. DNA-sequencing equipment.
4. DNA from single blastomeres and genomic DNA from parents and controls.
5. DNA restriction enzymes.
6. Lymphocyte separation gradient.
7. Single-cell separation methods.

### 3. Methods

#### 3.1. IVF and Blastomere Biopsy

IVF procedures, intracytoplasmic sperm injection (ICSI), and blastomere biopsies were performed by the Cornell Center for Reproductive Medicine and Infertility using standard techniques (85,86). This particular case required synchronization of an oocyte donor and the proband's spouse. Sperm from the proband, who has a personal history of HOS, was used for ICSI. Informed consent was obtained for all clinical procedures, blastomere biopsies, and genetic testing, in accordance with Weill Medical College of Cornell University Committee on Human Rights in Research.

#### 3.2. Sequence Analysis for *TBX5* Glu69ter Mutation

DNA was isolated from single blastomere biopsies. Biopsies were performed at 3 d after fertilization on five blastocysts obtained from fertilization (by ICSI) of five donor oocytes, using methods previously described (85). DNA was isolated from peripheral blood samples from the proband (positive control heterozygous for Glu69ter), as well as the oocyte donor (negative control), who was previously determined not to carry a *TBX5* mutation by bidirectional sequence analysis of protein-coding exons 2 to 9.

Exon 3 of *TBX5*, as well as its flanking introns, was amplified from the blastomeres using a nested PCR approach. This approach was used because of the limited template DNA able to be isolated from these single cells. The validity of this method was confirmed by analyses of single lymphocytes isolated from whole-blood samples from the proband, the oocyte donor, and controls, in anticipation of testing the blastomeres.

Lymphocytes were separated from whole-blood samples by Ficoll–Paque density gradient, using previously described techniques (14). Single lymphocytes were selected by direct micropipetting. Single lymphocytes and blastomeres were prepared using techniques described by Xu et al. (86). Single cells and blastomeres were placed into 500- $\mu$ L microcentrifuge tubes, stored at  $-30^{\circ}\text{C}$  with 25  $\mu$ L of lysis buffer. These samples were heated at  $65^{\circ}\text{C}$  for 10 min and placed immediately on ice, and 5  $\mu$ L of neutralization buffer was added.

PCR was performed using standard techniques previously described for amplification of exon 2 and exons 4 to 9 of *TBX5*. A nested PCR approach was used to amplify exon 3. First-round PCR for exon 3 and its flanking introns



was performed using primers 3GRN2 (5'-GGAAGGAGGAGCAGTCTC TGTGTT-3') and 3GFN (5'-GTGTCTTTTCTCCTCGTCCCTCTCTCTAC ACA-3') (84). Standard PCR reagents and AmpliTaqGold polymerase (Applied Biosystems [ABI], Foster City, CA) were used with PCR conditions of 40 cycles of (95°C for 20 s; 67°C for 30 s; and 72°C for 45 s). The resultant PCR product was 242 bp.

Second-round PCR (final volume, 50  $\mu$ L) was performed using 6  $\mu$ L of first-round PCR product (1:100 dilution) as a template. Nested primers for the second-round PCR amplification of exon 3 were: 3GRN (5'-AGTTTGGGG AAGGAATGCCCCACTAC-3') and 3GF1 (5'-GTGTCTTCTACACAACAA ACCATCTCACCTT-3') (84). PCR was again performed with standard reagents and AmpliTaq Gold polymerase with PCR conditions of 35 cycles of (95°C for 20 s; 65°C for 30 s; and 72°C for 45 s). The product of the second-round amplification was 182 bp. Negative controls were included in both rounds of PCR. Two second-round PCR reactions were performed in parallel. One reaction used a NED fluorescent dye (ABI)-labeled 3GRN (84) primer to allow for visualization of restriction fragment length polymorphism (RFLP) products. After PCR amplification, sequence and RFLP analysis of the PCR products were performed.

Bidirectional sequence analysis was performed on an ABI 377 automated sequencer, using BigDye Terminator sequencing (ABI) reagents, according to the manufacturer's recommended techniques.

### 3.3. RFLP Analysis

Twenty-five microliters of amplified PCR product were digested with *Dde*I [4U] (Promega, Madison, WI) for 90 min at 37°C and analyzed by gel electrophoresis, as previously described (14). The presence of the Glu69ter mutation resulted in a 123-bp product that could be visualized because of the use of the fluorescent 3GRN primer (see Notes 1 and 2).

Four of the five blastocysts were determined to be homozygous normal for *TBX5*. One blastocyst was determined to carry the Glu69ter mutation. The four normal blastocysts continued to grow well in culture after the blastomere biopsies. Two of the four blastocysts were transferred to the recipient. The two remaining blastocysts that were *TBX5* homozygous normal were cryopreserved through the clinical IVF program at Cornell for future transfer (see Note 3).

### 3.4. Clinical Follow-Up

Confirmation of pregnancy was confirmed by serum  $\beta$ -human chorionic gonadotropin. High-resolution ultrasound and amniocentesis were performed in the second trimester. There were no ultrasound anomalies suggestive of HOS. Sequence and RFLP analysis of DNA extracted from waste amniocytes

confirmed the homozygous normal *TBX5* genotype. Clinical karyotype was normal. At delivery, cord blood was obtained for *TBX5* genotyping, which again was normal.

### 3.5. Conclusion

We have discussed several human genetic disorders that have primary malformations of the atrial and/or ventricular septa. Although great strides have been made in our understanding of the molecular basis of cardiac development, specifically septation of the heart, many modulatory genes and signaling pathways remain unknown or poorly characterized. Gene mutations may cause a spectrum of cardiac defects. Not yet available on a routine clinical basis, molecular genetic testing is performed via scientific research protocols. Mutational screening of genomic DNA derived from patient blood samples is routinely performed using DNA confirmation-sensitive techniques and automated sequencing. Although these standard genetic testing methods have allowed basic scientists to rapidly screen numerous kilobases of genomic DNA, they remain costly and are often not reimbursable by the patient's health insurance coverage. In the future, molecular genetic testing will become more readily available as a standard clinical diagnostic test, allowing clarification of risks for an individual's family members, for reproductive options, and for possible progression of their own cardiac disease. Choosing which specific genes to be analyzed based on specific clinical presentation of probands will remain a critical challenge.

## 4. Notes

1. RFLP analysis has been successfully used for identification of gene mutations in individuals (**14**). The presence of an RFLP is detected in DNA products obtained from PCR amplification. Although RFLP analysis could be performed on primary PCR products, the yield of DNA obtained after PCR amplification from single-cell biopsies is rather low in many cases. Therefore, it may be necessary to perform a nested PCR reaction by using the product from the primary PCR as a template and to perform the RFLP analysis on the DNA generated from this secondary PCR reaction (**84,86**).
2. The presence of a mutation should be confirmed by both sequence and RFLP analyses of two distinct nested PCR reactions. It is necessary to examine two distinct nested PCR reactions to exclude the likelihood that PCR-induced errors have been introduced in the DNA (**84,86**).
3. After we successfully performed PGD for HOS using the assay design presented in **Subheading 3.2.**, the usefulness of coamplifying flanking single tandem repeats to address preferential allelic amplification has been suggested, and such a strategy should be taken into consideration when designing PGD assays (**87**).

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