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Genetics and Disease of Ventricular Muscle

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Cardiomyopathies are a heterogeneous group of heart muscle diseases associated with heart failure, arrhythmias, and death. Genetic variation has a critical role in the pathogenesis of cardiomyopathies, and numerous single-gene mutations have been associated with distinctive cardiomyopathy phenotypes. Contemporaneously with these discoveries, there has been enormous growth of genome-wide sequencing studies in large populations, data that show extensive genomic variation within every individual. The considerable allelic diversity in cardiomyopathy genes and in genes predicted to impact clinical expression of disease mutations indicates the need for a more nuanced interpretation of single-gene mutation in cardiomyopathies. These findings highlight the need to find new ways to interpret the functional significance of suites of genetic variants, as well as the need for new disease models that take global genetic variant burdens, epigenetic factors, and cardiac environmental factors into account.

Cardiomyopathies are broadly defined as diseases of the heart muscle, and the ventricles are involved in most cases. These disorders are relatively common, occurring in one in 500 individuals, and are associated with an increased risk of heart failure, heart transplantation, malignant cardiac arrhythmias, stroke, and sudden death. Because of this high prevalence and substantial morbidity and mortality, cardiomyopathies represent a major health-care burden and cost to the community. Since the discovery of the first genetic cause of an inherited cardiomyopathy more than two decades ago (Geisterfer-Lowrance et al. 1990), linkage analysis and candidate gene screening in families and sporadic cases have identified hundreds of mutations in genes encoding diverse subcellular components of the cardiomyocyte (Seidman and Seidman 2011; Watkins et al. 2011). Despite these efforts, the genetic basis of disease in many families is unknown, and the promise of gene-based treatments has been largely unmet. Recently, next-generation sequencing technologies have ushered in a new era of discovery for understanding

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the genetic underpinnings of human disease, and nowhere is this more apparent than in the field of cardiomyopathies. Sequencing a person's entire genome is both feasible and affordable, and initial studies have revealed an extraordinary extent of genetic variation in every person. A significant and surprising finding has been that affected and unaffected individuals carry numerous novel and potentially disease-causing variants (including nonsense, frameshift insertion/deletions, and splice-site-altering variants) in cardiomyopathy genes. These observations raise important questions about how to interpret the clinical significance of any single variant and the potential need to consider the role of combinations of variants in disease pathogenesis. The genetics revolution will undoubtedly challenge current mechanistic paradigms for inherited cardiomyopathies and may profoundly impact patient management. As genome sequencing has begun to enter "the clinic," it is timely to review what is known about the genetics of cardiomyopathies, insights gained to date from personal genome sequencing, and future directions.

DEFINITIONS

Classification methods for stratifying subtypes of cardiomyopathies have evolved over time. In 1980, the World Health Organization (WHO) defined cardiomyopathies as "heart muscle diseases of unknown cause" (WHO/ISFC Task Force 1980). This was updated in 1995 to "diseases of myocardium associated with cardiac dysfunction" and included hypertrophic cardiomyopathy (HCM), dilated cardiomyopathy (DCM), arrhythmogenic right ventricular cardiomyopathy (ARVC), and restrictive cardiomyopathy (RCM) (Richardson et al. 1995). The most current consensus definition was formulated in 2006 by an expert panel composed of members of four working groups of the American Heart Association: "Cardiomyopathies are a heterogeneous group of diseases of the myocardium associated with mechanical, and/or electrical dysfunction that usually (but not invariably) show inappropriate ventricular hypertrophy or dilatation and are a result of a variety of causes that frequently are genetic." This definition encompassed genetic and nongenetic causes of HCM, DCM, ARVC, and RCM and, for the first time, included a range of cardiac rhythm abnormalities under the umbrella of "electrical" muscle disorders (Maron et al. 2006). Although each of the cardiomyopathies can be defined by characteristic features, making a diagnosis in an individual case is not always straightforward owing to the frequent occurrence of overlapping phenotypic manifestations. For example, left ventricular systolic dysfunction can develop in patients with end-stage HCM, ARVC, LVNC, and RCM; left ventricular diastolic dysfunction can be seen in patients with HCM or DCM; and ventricular arrhythmias can occur with any of the cardiomyopathies. The imprecision of defining cardiomyopathies based on clinical features creates a need for improved classification systems. However, as outlined in subsequent sections of this review, gene-based classifications improve specificity better than labels such as "diseases of the sarcomere" and "diseases of the cytoskeleton" and when combined with conventional morphological descriptors (HCM/DCM) can indicate clinically meaningful mechanisms. For example, recognition that DCM is caused by either an *MYH7* or *LMNA* mutation implies that either diminished motor function (*MYH7*) or increased sensitivity to biophysical stress (*LMNA*) is involved, information that could indicate distinct therapeutic strategies. With the emergence of mechanism-based therapies for cardiomyopathies, incorporation of genetic etiology into classification systems will be increasingly meaningful to clinicians.

FROM PHENOTYPE TO GENOTYPE

Genetics studies of cardiomyopathies have typically started with cardiomyopathy phenotypes and sought to identify associated genotypes. These studies have been undertaken using genome-wide linkage analysis in large kindreds followed by resequencing of promising candidate genes within the linkage interval, or direct candidate gene screening in small families or cohorts of unrelated sporadic cases. Several cri-

teria have been used to support specific variants as potentially being disease causing, including cosegregation with affection status in families, absence from a control population (typically more than 100 healthy subjects), location at a residue that is highly conserved across species, protein-altering variant type (nonsense, frameshift insertion–deletion, splice site change, nonsynonymous), and predicted or experimentally validated functional effects. Because criteria for candidate gene selection often include cardiac expression, known functions in the heart, and functional similarity to established disease genes, analyses are inherently biased by current concepts of disease pathogenesis and may result in overestimation of the relative importance of some gene groups. Variants in numerous genes have been identified with each of the cardiomyopathies and functionally linked to myocardial defects.

Hypertrophic Cardiomyopathy

HCM is characterized by the presence of left ventricular hypertrophy that occurs in the absence of associated cardiac or systemic disorders. The diagnosis is made on the basis of left ventricular wall thickness determined by echocardiography or cardiac magnetic resonance imaging. Disproportionate hypertrophy of the interventricular septum is frequently seen, although other patterns may occur, including diffuse thickening with noncontiguous areas of hypertrophy or focal thickening confined to specific regions such as the ventricular apex (Maron and Maron 2013). The severity of hypertrophy can vary from massive to mild, with some genotype-positive individuals having little or no hypertrophy (Klues et al. 1995; Watkins et al. 1995; Maron and Maron 2013). Left ventricular chamber size is normal or small, and systolic function is normal or hyperdynamic. Other echocardiographic features that may be seen include left ventricular outflow tract obstruction and mitral valve abnormalities. Although cardiac biopsy is not required for the diagnosis of HCM and is rarely performed, there are characteristic histopathological features of myocyte hypertrophy, myofiber disarray, and interstitial fibrosis. Complications of HCM include heart failure caused by left ventricular diastolic dysfunction in those with preserved left ventricular systolic function or left ventricular systolic dysfunction in a minority of cases with end-stage disease. Left ventricular diastolic abnormalities may also cause left atrial dilation, and atrial fibrillation is seen in 20% of patients (Olivotto et al. 2001). Sudden cardiac death caused by ventricular arrhythmias is a devastating but relatively infrequent complication and is mainly seen in younger patients (Maron and Maron 2013). The differential diagnosis of HCM includes physiological causes of left ventricular hypertrophy such as athlete's heart, cardiac metabolic storage disorders, and a range of secondary causes of hypertrophy (Maron et al. 2006).

Individuals with HCM may have a positive family history, with an autosomal-dominant inheritance pattern, or present as a sporadic case. More than 1400 mutations have been associated with HCM, the majority of these located in genes that encode proteins in the thick and thin filaments of the sarcomere, with a small number of mutations also seen in genes encoding Z-disc components and calcium-handling proteins (Table 1). The precise number of disease genes for HCM has been debated, ranging from eight to 11 or more depending on varying interpretation of the evidence for pathogenicity (Hershberger et al. 2009; Seidman and Seidman 2011; Watkins et al. 2011; Landstrom and Ackerman 2012; Maron et al. 2012; Maron and Maron 2013; Teekakirikul et al. 2013). Mutations in *MYH7*, which encodes the β-myosin heavy chain, and *MYBPC3*, which encodes cardiac myosin-binding protein C, each account for 30%–40% of genotyped cases. Genetic testing of known disease genes usually detects mutations in ~60% of familial cases and 40% of sporadic cases (Ho 2010). In testing panels, it is useful to include the *PRKAG2*, *LAMP2*, and *GLA* genes that encode the γ-regulatory subunit of the AMP-activated protein kinase, lysosome-associated membrane protein-2, and α-galactosidase, respectively, as these have been associated with phenotypes that can mimic HCM (Sachdev et al. 2002; Arad et al. 2005).

Table 1. Genes associated with childhood-onset and adult-onset cardiomyopathies and related phenotypes

Gene		HCM	DCM	LVNC	RCM	ARVC	Arrhythmic syndromes[a]	Other
Sarcomere								
ACTC1	Cardiac actin	X	X	X	X			
MYBPC3	Myosin-binding protein C	X	X	X				
MYH6	α-Myosin heavy chain	X	X					Congenital heart defects
MYH7	β-Myosin heavy chain	X	X	X	X			Congenital heart defects
MYL2	Myosin light chain (R)	X			X			
MYL3	Myosin light chain (E)	X			X			
MYLK2	Myosin light chain kinase 2	X						
TNNC1	Troponin C	X	X					
TNNI3	Troponin I	X	X	X	X			
TNNT2	Troponin T	X	X	X	X			
TPMI	α-Tropomyosin	X	X	X	X			
TTN	Titin	X	X			X		
Z-disc								
ACTN2	α-Actinin	X	X					
ANKRD1	Cardiac ankyrin repeat protein	X	X					Congenital heart defects
BAG3	Bcl-2-associated athanogene 3		X					Myofibrillar myopathy: LVH + RCM
CSRP3	Muscle LIM protein	X	X					
FHL2	Four and half LIM protein-2		X					
LDB3	Cypher/ZASP	X	X	X				
MURC	Muscle-restricted coiled-coil		X					
MYOZ2	Myozenin	X						
MYPN	Myopalladin		X					
NEBL	Nebulette		X					
NEXN	Nexilin	X	X					
TCAP	Telethonin	X	X					
VCL	Vinculin	X	X					
Cytoskeleton								
DES	Desmin		X		X			Myofibrillar myopathy: ARVC
DMD	Dystrophin		X					
Sarcolemma/extracellular matrix								
CAV3	Caveolin 3	X					X	DCM + skeletal myopathy
CHRM2	M2-muscarinic acetylcholine receptor		X					
DTNA	α-Dystrobrevin			X				
ILK	Integrin-linked kinase		X					
LAMA4	Laminin-α4		X					

Continued

Table 1. *Continued*

Gene		HCM	DCM	LVNC	RCM	ARVC	Arrhythmic syndromes[a]	Other
SGCB	β-Sarcoglycan		X					
SGCD	δ-Sarcoglycan		X					
Desmosome								
DSC2	Desmocollin-2		X			X		
DSG2	Desmoglein-2		X			X		
DSP	Desmoplakin		X	X		X		
JUP	Plakoglobin					X		
PKP2	Plakophilin-2		X			X		
Nucleus								
EMD	Emerin		X					EDMD
EYA4	Eyes absent homolog 4		X					
GATAD1	GATA zinc finger domain-containing protein 1		X					
LMNA	Lamin A/C		X	X				EDMD, extracardiac disorders
NKX2–5	NKX2–5		X	X				Congenital heart defects
PRDM16	PR domain-containing 16		X					
RBM20	RNA-binding protein 20		X					
TMPO	Thymopoietin		X					
Sarcoplasmic reticulum/Ca^{2+} channels[a]								
CASQ2	Calsequestrin 2	X		X			X	
JPH2	Junctophilin	X						
PLN	Phospholamban	X	X					
RYR2	Ryanodine receptor					X	X	
K^+, Na^+ ion channels[a]								
ABCC9	SUR2A subunit, K_{ATP} channel		X					
SCN5A	Cardiac sodium channel		X	X			X	
Mitochondria								
COX15	COX15 homolog, cytochrome *c* oxidase assembly protein							Infantile LVH
DNAJC19	TIM14							DCMA syndrome: DCM, LVNC
SDHA	Succinate dehydrogenase							Neonatal DCM
TAZ	Tafazzin							Barth syndrome: DCM, LVNC
Cytoplasm/transmembrane								
CRYAB	αβ-Crystallin		X					
FKTN	Fukutin		X					
GLA	α-Galactosidase							Fabry disease: LVH
LAMP2	Lysosomal membrane-associated 2							Danon disease: LVH, DCM

Continued

Table 1. *Continued*

Gene		HCM	DCM	LVNC	RCM	ARVC	Arrhythmic syndromes[a]	Other
MIB1	Mind bomb homologue 1			X				
PRKAG2	AMPK subunit γ2							Glycogen storage disease: LVH, ventricular pre-excitation
PSEN1	Presenilin-1		X					
PSEN2	Presenilin-2		X					
TMEM43	Transmembrane protein 43					X		
TTR	Transthyretin							Amyloidosis: LVH, RCM
YWHAE	14–3–3ε			X				

ARVC, Arrhythmogenic right ventricular cardiomyopathy; DCM, dilated cardiomyopathy; DCMA, DCM with ataxia; EDMD, Emery-Dreifuss muscular dystrophy; HCM, hypertrophic cardiomyopathy; LVH, left ventricular hypertrophy; LVNC, left ventricular noncompaction; RCM, restrictive cardiomyopathy.

[a]Mutations in the following genes encoding ion channels and related proteins have been associated with ventricular arrhythmic syndromes but not with other types of ventricular cardiomyopathy: *KCNQ1*, *KCNH2*, *KCNE1*, *KCNE2*, *KCNE3*, *KCNJ2*, *KCNJ5*, *SCN1B*, *SCN3B*, *SCN4B*, *CACNA1C*, *CACNA2D1*, *CACNB2*, *GDP1L*, *HCN4*, *SNTA1*, *AKAP9*.

Dilated Cardiomyopathy

DCM is characterized by increased chamber size and impaired systolic contraction of the left and/or right ventricles. In addition to these defining features, there may be a range of additional echocardiographic and ECG abnormalities. Although ventricular wall thickness may be normal or reduced on transthoracic echocardiography, overall left ventricular mass is usually increased. Dilatation of the atria is frequently seen and may result from left ventricular diastolic filling defects, mitral valve regurgitation, and/or atrial myopathy. DCM as a result of any cause may be complicated by progressive heart failure, supraventricular and ventricular arrhythmias, thromboembolic stroke, and sudden death. In genetic forms of DCM, there may be distinctive cardiac or extracardiac features, including conduction-system abnormalities, congenital heart defects, valvular defects, left ventricular noncompaction (LVNC), skeletal myopathy, lipodystrophy, and sensorineural deafness. The differential diagnosis of DCM encompasses a broad range of genetic and acquired factors (Maron et al. 2006). Defining the etiology of DCM has clinical utility because some etiologies—including viral or bacterial infections, drugs, toxins, autoimmune, metabolic, endocrine, or nutritional disorders—are potentially treatable. However, among the 50% of cases without an identified cause, one in four individuals has a family history of DCM, suggesting an underlying genetic basis (Petretta et al. 2011).

Genetic variants have been associated with sporadic cases of DCM or identified in families that usually show an autosomal-dominant inheritance pattern, with autosomal-recessive or X-linked inheritance in a minority of cases. More than 40 genes have been associated with a predominant clinical phenotype of adult-onset DCM, and these encode diverse components of the sarcomere, Z-disc, cytoskeleton, sarcolemma, and nucleus (Table 1). Relatively few mutations have been identified in most of these genes, and the yield of genetic testing has been only 20%–30% (Hershberger and Siegfried 2011; Millat et al. 2011; Teekakirikul et al. 2013; van Spaendonck-Zwarts et al. 2013). One notable exception is the *LMNA* gene, which causes DCM and conduction-system disease (Fatkin et al. 1999, 2010). Because of its distinctive phenotype, the *LMNA* gene has been frequently screened, and this has yielded high num-

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bers of mutations. Recently, truncating mutations in the *TTN* gene that encodes the giant protein titin were identified in 25% of cases of familial DCM and 18% of sporadic DCM cases (Herman et al. 2012). Although these findings have yet to be confirmed in independent patient cohorts, these results point to the *TTN* gene being the most common cause of familial DCM, and inclusion of this gene in genetic testing panels should substantially increase the yield of positive results.

Arrhythmogenic Right Ventricular Cardiomyopathy

ARVC is characterized histologically by progressive myocyte loss and fibrofatty replacement of the right ventricle, with left ventricular involvement in up to 75% of cases. There can be a wide range of phenotypical features, including ventricular tachyarrhythmias, syncope, or sudden death, and segmental or global chamber dilatation and contractile defects. The clinical diagnosis of ARVC can be challenging, and sets of major and minor criteria have been devised by task forces of international experts that take into account structural and functional abnormalities, tissue characterizations, ECG abnormalities, arrhythmias, and family history (McKenna et al. 1994; Marcus et al. 2010). Because of the high frequency of biventricular abnormalities, clinical differentiation of individuals with ARVC from those with DCM is difficult.

Families with ARVC have generally shown autosomal-dominant inheritance patterns. ARVC has also been reported as an autosomal-recessive disorder in two syndromes: Naxos syndrome, which is associated with woolly hair and palmarplantar keratoderma, and Carvajal syndrome, associated with prominent left ventricular as well as skin involvement. Mutations in nine genes have been associated with ARVC with varying levels of evidence; five of these encode the desmosomal proteins, plakophilin-2, plakoglobin, desmoplakin, desmocollin, and desmoglein-2 (Teekakirikul et al. 2013). Approximately 50% of ARVC cases have a desmosomal gene mutation, most commonly (~40%) in the plakophilin-2 gene (van Tintelen et al. 2006; den Haan et al. 2009). Desmosomal gene mutations have also been identified in ~5% of subjects with a clinical diagnosis of DCM (Elliott et al. 2010). A small number of mutations in genes encoding the nondesmosomal proteins, transmembrane protein 43, transforming growth factor β3, cardiac ryanodine receptor, and titin have been associated with ARVC.

Left Ventricular Noncompaction

During normal heart development, thick trabeculations form in the early embryonic ventricle, and these subsequently compress to become the endocardium. LVNC is considered to be a developmental defect in which trabecular compaction fails to occur. The resulting noncompacted endocardium adjacent to compacted epicardium gives rise to a two-layered appearance of the myocardial wall that is detectable on imaging modalities such as transthoracic echocardiography or magnetic resonance imaging. These changes also result in deep intertrabecular sinusoidal recesses that have a predilection for blood clot formation. LVNC is usually detected as an asymptomatic finding during cardiac imaging and may occur in isolation or in association with other congenital heart abnormalities. The trabecular thickening in LVNC may be difficult to distinguish from left ventricular hypertrophy that results from other causes, and the differential diagnosis includes HCM, hypertensive cardiomyopathy, endocardial fibroelastosis, apical thrombus, and tumors (Oechslin and Jenni 2011). Left ventricular systolic function can deteriorate over time, and patients with LVNC often experience heart failure, ventricular arrhythmias, or thromboembolic events. LVNC may be sporadic or familial, with autosomal-dominant, autosomal-recessive, and X-linked inheritance reported. Mutations in sarcomere protein genes have been found in up to 50% of LVNC cases, with the majority of variants in the *MYH7* gene (Hoedemaekers et al. 2007; Klaassen et al. 2008; Pantazis and Elliott 2009; Probst et al. 2011). Mutations in Z-disc, cytoskeletal, and mitochondrial genes have also been found (Table 1). Deletion and loss-of-function dominant mutations in *PRDM16*, a

gene of unknown function(s), have also been identified to cause LVNC that occurs in isolation or in the context of the chromosome 1p36 deletion syndrome (Arndt et al. 2013).

Restrictive Cardiomyopathy

RCM is a rare cardiomyopathy associated with impaired ventricular diastolic filling and increased end-diastolic pressure, which results in normal or reduced ventricular dimensions and biatrial dilatation. Ventricular wall thickness and systolic function are initially normal, although systolic dysfunction and heart failure may develop with increasing age. The overall prognosis in RCM is poor, especially in pediatric cases, and heart transplantation is often required. RCM can occur as a primary cardiomyopathy with a genetic etiology or occur secondary to infiltrative or systemic disorders such as amyloidosis and sarcoidosis. Mutations in seven sarcomere protein genes (Table 1), as well as in *DES*, encoding the cytoskeletal protein desmin, have been found in sporadic cases and families with RCM (Parvatiyar et al. 2010a; Sen-Chowdry et al. 2010; Caleshu et al. 2011). The differential diagnosis of RCM includes HCM, which can also manifest with restrictive diastolic filling defects and atrial dilatation. The high prevalence of sarcomere protein gene mutations does raise the question as to whether RCM and HCM are truly distinctive disorders or represent different points along a spectrum of left ventricular diastolic dysfunction. Mixed RCM and HCM phenotypes frequently coexist in the same families, and the relatively more severe and early onset of disease in many RCM cases may be attributed to a higher "dose" of mutant protein associated with homozygous mutations or compound mutations, some of which may occur de novo (Sen-Chowdry et al. 2010; Caleshu et al. 2011; Pinto et al. 2011). For example, Caleshu et al. (2011) identified one index case that presented with RCM and severe heart failure requiring cardiac transplantation. She was found to be homozygous for an *MYL3* variant and heterozygous for an *MYL2* variant. Her mother was heterozygous for both mutations and clinically unaffected. A second index case with RCM was homozygous for a *TPMI* variant. Both parents were heterozygous carriers of this variant, with the father having a diagnosis of HCM. On a mechanistic level, both RCM and HCM have been associated with increased myofilament calcium sensitivity with varying effects on ATPase activity (Parvatiyar et al. 2010b; Willat et al. 2010; Pinto et al. 2011)

Arrhythmic Syndromes

A number of ventricular arrhythmic syndromes, collectively referred to as "ion channelopathies," have been associated with mutations in genes encoding cardiac sodium, potassium, and calcium channels. These disorders include long QT syndrome, short QT syndrome, Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia, and sudden unexplained nocturnal death syndrome. The characteristics of each of these arrhythmias have been reviewed elsewhere and include ventricular tachycardia, ventricular fibrillation, and death (Cerrone et al. 2012; Webster and Berul 2013). Variants in some of the same ion channel genes have been identified in patients with the "structural" cardiomyopathies, DCM, ARVC, and LVNC, in which ventricular arrhythmias often feature prominently (Tiso et al. 2001; Hershberger et al. 2008; Shan et al. 2008; McNair et al. 2011; Mann et al. 2012).

Overlapping Phenotypes, Overlapping Genes

The increased knowledge base of genetic causes of cardiomyopathies and longitudinal follow-up of affected individuals have led to the recognition that there is substantial overlap not only in phenotypical features but also in molecular etiology. Some phenotypical manifestations (increased cardiac mass, contractile dysfunction, arrhythmias, and sudden death) are common to all cardiomyopathy subtypes. Moreover, mutations in the same gene can cause different cardiomyopathies. For example, mutations in the *MYH7* gene have been associated with HCM (Geisterfer-Lowrance et al. 1990), DCM (Kamisago et al. 2000), LVNC (Hoedemaekers et al. 2007; Vermeer et al. 2013), and RCM

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(Karam et al. 2008). These observations raise the question of how distinctive the cardiomyopathies really are, and how the phenotypical differences and commonalities can be reconciled at a mechanistic level.

Detailed functional characterizations that have been performed on a few pathogenic mutations provide plausible links to myocardial dysfunction. For example, *MYH7* mutations that cause HCM (R403Q) and DCM (F764L or S532P) have reciprocal (increased or decreased, respectively) effects on force production by the sarcomere (Schmitt et al. 2006; Debold et al. 2007; Chuan et al. 2012). Secondary responses triggered by these mutations may be similar (e.g., increased energy utilization, re-expression of fetal genes), whereas other responses, presumably the unidentified signals that drive cardiac remodeling along dilated or hypertrophic pathways, are different. Delineation of these signals will be critical to fully understand genotype–phenotype relationships. The considerable genetic heterogeneity of cardiomyopathies poses additional questions. How do mutations in *LMNA* (Fatkin et al. 1999) or *PLN* (Schmitt et al. 2003), which encode, respectively, a nuclear membrane protein and the physiological inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase, or in sarcomere protein genes, for example, *TTN*, *MYH7*, and *ACTC1* (McNally et al. 2013), produce the overlapping phenotype of DCM? Are there multiple mechanisms by which DCM can emerge, or do mutations in molecules with disparate functions converge onto a final common pathway?

FROM GENOME TO PHENOTYPE

Sequencing of the entire human genome is now available and affordable, and next-generation sequencing technologies have already facilitated the discovery of genetic defects responsible for several rare disorders (Choi et al. 2009; Ng et al. 2010; Worthey et al. 2011). Because disease-causative variants are enriched in the 1% of the human genome that contains protein-coding sequences, there has been substantial interest in evaluating these regions using whole-exome sequencing (WES). Databases of whole-genome and WES sequences obtained from many thousands of individuals in large-scale initiatives, such as the 1000 Genomes Project and the NHLBI-funded Exome Sequencing Project (ESP), are documenting the extent and range of genetic variation within populations of varying racial background and have yielded some surprising results. In particular, WES studies have revealed a staggering extent of personal genetic variation with about 20,000 single nucleotide polymorphisms (SNPs) identified in individual European samples and about 24,000 in African American samples (Bamshad et al. 2011).

An Emerging Conundrum: Novel Functionally Deleterious Variants Are Present in the General Population

Several recent studies that have evaluated variation in cardiomyopathy genes in sequence databases have made an important observation. A substantial number of individuals in the general population carry variants that meet many of the conventional criteria used to define pathogenic variants in disease cohorts, including novelty, high-impact variant type (nonsense, frameshift insertion/deletion, loss or gain of splice donor or acceptor sites, etc.), and predicted or validated protein-altering effects. These observations prompt reevaluation of the criteria for pathogenicity and indicate that single novel missense or loss-of-function variants identified in a few subjects with cardiomyopathy can no longer be assumed to be disease causing.

Variant novelty has been thought to be an important factor, and many of the gene variants that have been considered responsible for cardiomyopathies have been "private" mutations seen only in a single family. With insights gained from sequence data in many thousands of individuals in the general population, it has now become apparent that, in fact, a substantial proportion of human coding sequence variants are rare. A good example of this was provided in the data reported by Pan et al. (2012), who looked at SNPs in coding regions of 46 cardiomyopathy genes in more than 5000 individuals

in the ESP database and found that 9103 (91%) of the 9974 total variants were rare (minor allele frequency <1%) and that 5448 (60%) of these were novel and seen only in one individual. In traditional Sanger-sequencing studies of selected candidate genes, any single high-impact variants identified have been assumed to be pathogenic. However, bioinformatics analyses of next-generation sequencing outputs are revealing that the average human genome contains more than 100 of these types of variants, with at least 20 encoded proteins being completely inactivated (Bamshad et al. 2011; MacArthur et al. 2012). Furthermore, the distribution of rare deleterious variants varies significantly among different racial and ethnic ancestries (Fu et al. 2013).

There are now increasing numbers of examples where specific variants reported in the literature to be causative for cardiomyopathy have subsequently been identified in population databases. Norton et al. (2012) looked at 197 rare variants associated with DCM and found that 33 (16.8%) were present in the ESP database. Similarly, Pan et al. (2012) found that four of 46 (8.7%) reported pathogenic variants in the *MYH7*, *MYBPC3*, and *TNNT2* genes were present in the ESP database. Golbus et al. (2012) evaluated known and predicted pathogenic variants in the *MYH7*, *MYBPC3*, and *TTN* genes in 1092 individuals in the 1000 Genomes Project database. Twenty-one rare or low-frequency protein-altering variants (missense, nonsense, splice site) were present in *MYH7* and 22 in *MYBPC3*, with 700 protein-altering variants in *TTN*. Twenty-two SNPs in these three genes were present in the Human Genome Mutation database, a comprehensive catalog of disease-associated mutations and in the 1000 Genomes Project database. Several variants in desmosomal genes that were initially reported as disease causing in ARVC cohorts have similarly been identified in general population control subjects (Milting and Klauke 2008; Christensen et al. 2010).

Do these reports highlight a disturbing prevalence of false-positive disease mutations, or are there other explanations? A limitation of both the 1000 Genomes Project and ESP databases is that there is a lack of phenotype information, raising the possible explanation that individuals with asymptomatic cardiomyopathy (or other diseases) and/or clinically undiagnosed disease are included in these cohorts. However, Bick et al. (2012) sequenced eight sarcomere protein genes in 3600 individuals from the Framingham Heart Study (FHS, European American) and the Jackson Heart Study (JHS, African American) cohorts, for which there are detailed clinical cardiovascular phenotype data, which for many FHS participants spans many decades. Among these cohorts, 11.2% individuals had rare, protein-altering variants: 14 in 1637 FHS participants and eight in 1963 JHS participants had been reported in the literature as likely pathogenic. The overall prevalence of likely pathogenic variants was 0.6%, which is approximately twice the estimated prevalence of HCM. Review of echocardiographic results indicated that only four of the 22 variant carriers had undetected HCM. In addition to identifying a subset of individuals with undiagnosed HCM, the results of Bick et al. (2012) show that an overall phenotypical impact of rare sarcomere protein variants and individuals who carried these variants had an increased long-term risk of left ventricular dilatation and adverse cardiovascular events.

New Genetic Models

Genetic studies in cohorts of patients with cardiomyopathy have an inherent ascertainment bias that may lead to potentially erroneous conclusions about the disease causality of some variants. Next-generation sequencing studies provide an expanded perspective and the opportunity to generate new genetic models that take into account each individual's total burden of genetic variants. Although Mendelian inheritance still indicates the predominant effect of a single mutation, inclusion of genomic information from affected patients and the healthy population can improve this model by excluding false positives and accounting for phenotypic variation among patients with a shared pathogenic mutation. In families with a Mendelian disorder, there is likely to be only one rare var-

iant with a large functional effect that is primarily responsible for disease. However, the demonstration that a rare variant is absent from small cohorts of healthy controls, alters a conserved amino acid residue, and has predicted or demonstrable protein-altering effects is no longer sufficient evidence for disease causation, in particular, when genome databases show a population prevalence of comparable deleterious variants that exceed the disease prevalence. In contrast, the addition of genetic data such as statistically significant cosegregation within a large family, identification of additional deleterious variants in the same gene from multiple affected families, and functional recapitulation of the phenotype in an animal model should reduce false-positive conclusions about causality. In such families, gene-based diagnosis should be quite accurate. Incorporating genomic data, particularly on functionally related molecules and within protein networks, should improve insights into modifiers that influence phenotype expression. The presence of double and multiple pathogenic cardiomyopathy mutations is known to account for intrafamilial differences in disease onset and severity (Richard et al. 2003; Girolami et al. 2010; Saltzman et al. 2010), but the substantial numbers of unique or rare variants with predicted functional consequences in every person provides the opportunity to fully explore genetic modifiers of disease. Specific and cumulative genomic variation in molecules that may enhance or attenuate the consequences of an inherited pathogenic mutation warrants consideration. An extension of these considerations is that some cardiomyopathies might reflect the collective effects of multiple genetic variants rather than any single dominant mutation. This model would be reflected by non-Mendelian inheritance patterns in families. Researchers and clinicians alike are now confronted by the need to take global burdens of genetic variation into consideration, but these new genetic paradigms will be difficult to test because functional characterization of every variant will be impractical and different variant combinations may have additive, synergistic, or epistatic effects. New parameters such as composite genetic risk scores will need to be devised, and these will need to be related to sensitive, readily measurable functional end points.

From Simplicity to Complexity: New Disease Paradigms

The cardiomyopathy disease genes are often grouped to define potentially unifying phenotype-specific mechanistic pathways. Hence, HCM has been termed a "disease of the sarcomere," DCM a "disease of the cytoskeleton," and ARVC a "disease of the desmosome." These simplistic concepts of disease pathogenesis have their limitations, particularly because a single disease gene may have several biological functions, subcellular locations, and phenotypes, and new disease genes that do not conform to these groupings continue to be discovered. In the light of personal genomic data, new models that incorporate multiple genetic variants potentially affecting diverse aspects of cardiomyocyte structure and function need to be considered.

Genetic variation is only part of the story in cardiomyopathies, however; and over recent years, it has been become apparent that there are numerous factors affecting gene expression and protein function that could determine how each gene mutation is manifested. These contextual factors may be indirect effects of the gene mutations or attributable to the development of cardiomyopathy or comorbidities (Fig. 1). These changes could have an impact on the cardiomyopathy disease genes and encoded proteins, interacting partner proteins, or the myocardial contractile milieu, with net functional sequelae.

Many factors intrinsic and extrinsic to the cardiomyocyte are now known to influence gene expression profiles. Some of the most exciting recent discoveries relate to epigenetic mechanisms that regulate gene expression, including DNA methylation, histone modifications, ATP-dependent chromatin remodeling, and noncoding RNAs, including lincRNAs and microRNAs (Leach et al. 2010; Movassagh et al. 2011; Chang and Brunear 2012; Schonrock et al. 2012; Papait et al. 2013; Udali et al. 2013). MicroRNAs

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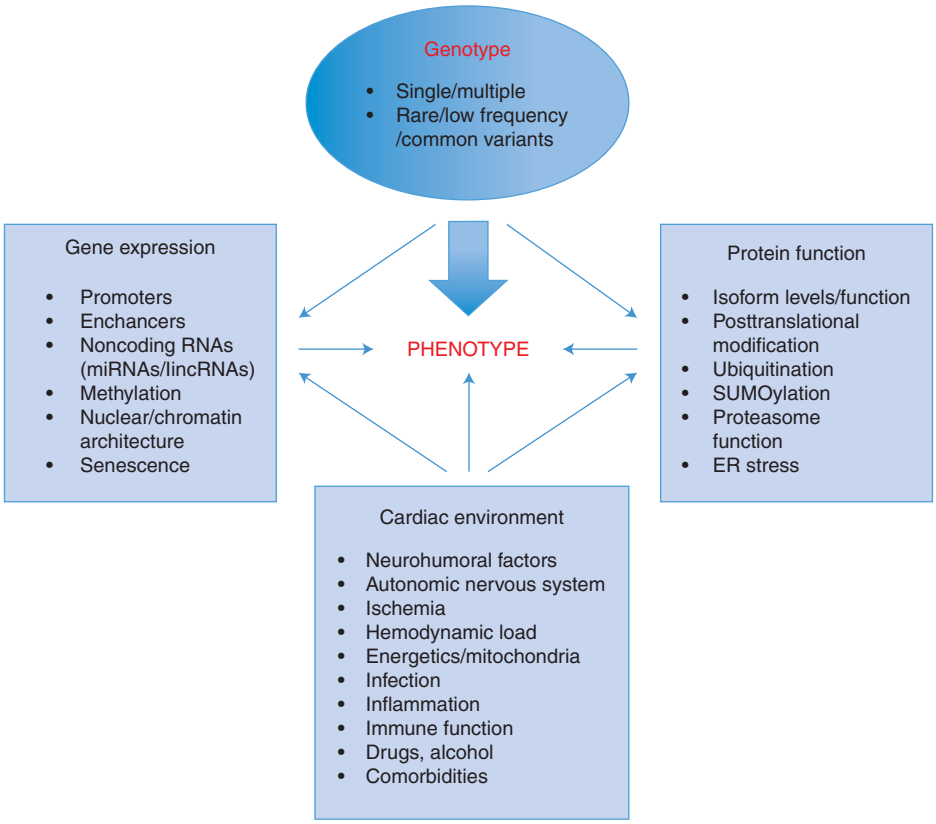

Figure 1. Schematic showing potential roles of genetics and other factors that are intrinsic and extrinsic to the cardiomyocyte as determinants of cardiomyopathy phenotype.

are small (~22 nucleotide) noncoding RNAs that bind to the 3′-untranslated regions of target mRNAs and typically reduce mRNA levels by inhibiting translation or stimulating mRNA decay. Each microRNA has multiple mRNA targets, and there are estimated to be more than 2000 microRNAs that collectively regulate most protein-coding genes. Since the first report by van Rooij et al. (2006), there has been an explosion of interest in cardiac microRNAs, with several hundred papers describing their roles in cardiac development, adult heart function, and heart diseases (Thum et al. 2007; Divakaran and Mann 2008; Rao et al. 2009; Mendell and Olson 2012). Relatively few genetic variants that alter microRNA function in the heart have been reported. In one study, a human miR-499 variant identified in an unselected population of 2600 individuals was modeled in transgenic mice. Whereas wild-type miR-499 transgenic mice developed progressive DCM, the mutant mice had more favorable mRNA expression profiles and less severe cardiac dysfunction (Dorn et al. 2012). In another study in an arrhythmia cohort, a variant in the *MIR133A2* gene that encodes the cardiac microRNA miR-133a was shown to alter duplex processing and the mRNA target spectrum (Ohanian et al. 2013). The role of epigenetic factors, including microRNAs, has not yet been extensively evaluated in the inherited cardiomyopathies, and this promises to be a fruitful area for further research.

Protein activation and turnover are determinants of normal cardiomyocyte function and can be altered by specific gene mutations or generalized disorders. For example, a mutation in the cardiac transcription factor *NKX2–5* identified in a family with congenital heart dis-

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ease and DCM was shown to have reduced DNA-binding affinity, and there was also increased expression of mutant protein because of reduced degradation by the ubiquitin–proteasome system (Costa et al. 2013). Dysfunction of the endoplasmic reticulum can occur in a variety of pathological conditions and has diverse effects on protein synthesis, degradation, trafficking, and posttranslational modification (Groenendyk et al. 2010). Posttranslational modifications are important for the activity of many proteins, including the giant sarcomere protein titin. The mechanical properties of titin's cardiac-specific N2B spring and the PEVK region are altered by phosphorylation, which is, in turn, modulated by β-adrenergic stimulation and heart failure (Hidalgo and Granzier 2013). The relative levels of expression of titin isoforms containing different N2A, N2B, and PEVK sequences determine passive stiffness of cardiomyocytes, and these ratios can be altered in heart failure and by mutations in *RBM20*, a recently described DCM disease gene (Guo et al. 2012; LeWinter and Granzier 2013).

Taken together, these observations indicate a need to look beyond the single variant as an explanation for pathogenesis of cardiomyopathies and shift toward more complex models that take into consideration the actions and interactions of multiple genetic, epigenetic, and environmental factors (Fig. 1). The collective burden of all these influences on myocardial function can be expected to determine the cardiac phenotype.

FUNCTIONAL EVALUATION OF GENETIC VARIANTS

Induced Pluripotent Stem (iPS) Cells

The functional consequences of single genetic variants are often evaluated in transfected cells. Although useful, these types of in vitro experiments are unable to take into account the effects of background genetic variation or the plethora of factors that affect protein function in the beating heart. The landmark discovery that somatic cells derived from patient tissue samples can be reprogrammed to become induced pluripotent stem (iPS) cells that can be subsequently differentiated into mature cells of interest, such as cardiomyocytes, generated enormous interest worldwide and for the first time permitted patient-specific functional evaluation of genetic variants and responses to therapy (Takahashi and Yamanaka 2006). Several human cardiomyopathy and arrhythmia mutations have already been modeled in iPS cells; however, there remain substantial technical challenges to fully differentiate iPS cells into adult cardiomyocytes. The immaturity of iPS-derived cardiomyocytes can have significant functional implications. For example, in murine and human iPS cells, t-tubules and associated proteins are typically absent, and this results in non-uniform propagation of calcium transients and incomplete electrical coupling of cells (Lieu et al. 2009). iPS cells are also not subjected to the hemodynamic load that critically impacts the function of in vivo myocytes. Although many features of the human cardiomyopathy phenotypes have been recapitulated, relatively few novel mechanistic insights have as yet been gained into how different mutations impact phenotype (Moretti et al. 2010; Itzhaki et al. 2011; Sun et al. 2012; Kim et al. 2013; Knollman 2013; Priori et al. 2013). Nevertheless, with further refinements of culturing techniques and emerging tools for genome editing, analyses of isogenic iPS cells may well become a valuable asset for gene variant evaluation.

Zebrafish as a Model for Cardiovascular Disease

Genetically modified mice have been the animal model of choice to evaluate in vivo effects of human gene mutations. Murine models have several limitations, however, including the time and expense of generating, breeding, and aging adequate numbers of experimental mice. Zebrafish are rapidly gaining popularity as a model organism because they are easy to breed, have rapid development and numerous progeny, and genetic manipulation can be readily achieved (Lieschke and Currie 2007; Dahme et al. 2009; Santoriello and Zon 2012; Verkerk and Remme

2012). Two distinctive properties of zebrafish during the first weeks of life are their transparency, which enables heart development and function to be directly observed in real time, and their independence from a functioning circulatory system for oxygen supply to the tissues. Although the zebrafish heart has only two chambers, many aspects of cardiac physiology are similar to that of humans (Milan et al. 2006; Milan and MacRae 2008; Verkerk and Remme 2012). There are some notable anatomical differences, however, such as the lack of coronary arteries and pulmonary vasculature. Although a majority of human genes have zebrafish orthologs, a genomic duplication event that occurred during teleost evolution has resulted in duplicate pairs of many zebrafish genes, which is an important consideration for gene knockdown experiments. Naturally occurring or chemically induced zebrafish mutations that result in cardiac phenotypes can be studied to identify new candidate genes for human cardiomyopathies and arrhythmias (Fig. 2A) (Bendig et al. 2006; Dahme et al. 2009). Conversely, transient or sustained knockdown of zebrafish genes using morpholino-modified antisense oligonucleotides or new gene-editing techniques such as transcription activator-like effector nucleases (TALENs), respectively, can be used to study the effects of genes with no known cardiac functions in which variants may be identified by WES or other sequencing methods (Sander et al. 2011; Santoriello and Zon 2012; Arndt et al. 2013). Finally, human gene variants can be modeled using transgenic zebrafish (Fig. 2B) (Huttner et al. 2013). These models will be invaluable for elucidating molecular mechanisms of disease and for evaluating therapies.

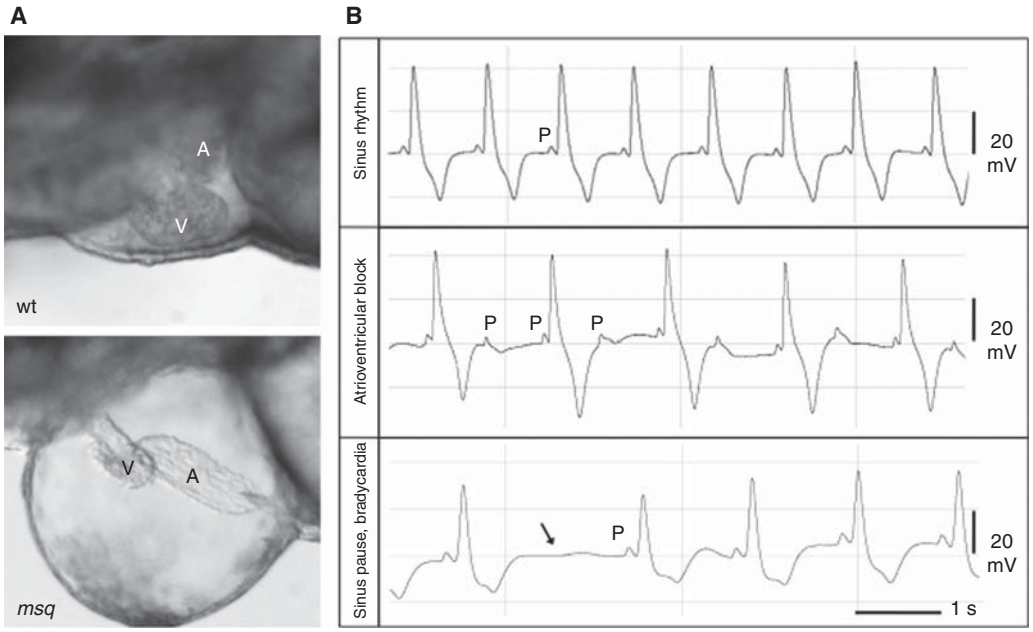

Figure 2. Effects of cardiac gene mutations in zebrafish. (*A*) Lateral view of embryonic zebrafish hearts at 3 d postfertilization. (*Top* panel) Wild-type (wt) heart; (*lower* panel) heart of a *mainsqueeze* (*msq*) mutant showing pericardial edema because of loss of ventricular contractility. A, atrium; V, ventricle. Genetic studies of the ENU-generated *msq* mutant identified an L308P variant in the *integrin-linked kinase* (*ilk*) gene (Bendig et al. 2006). *ILK* mutations have subsequently been associated with human DCM. (*B*) ECG tracings from anesthetized adult zebrafish. (*Top* panel) Normal sinus rhythm in a wild-type zebrafish. (*Middle* and *lower* panels) 2:1 Atrioventricular block and sinus bradycardia with pauses, respectively, in transgenic zebrafish expressing the human *SCN5A* D1275N mutation (Huttner et al. 2013).

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CLINICAL IMPLICATIONS

Genetic Testing

Current expert consensus guidelines for genetic testing in cardiomyopathies have been formulated with the objectives of establishing the etiology of disease in index cases and genotyping asymptomatic relatives to ascertain those at risk of developing disease (Hershberger et al. 2009). Targeted sequencing tests of the most common disease genes for each of the cardiomyopathies have been available for some time in diagnostic laboratories worldwide, and the relative cost, yield, and reliability of these tests in comparison to WES for clinical use is a subject of current debate (Sikkema-Raddatz et al. 2013; Teekakirikul et al. 2013). A major challenge for both genetic testing techniques is data analysis and interpretation of the significance of variants. Given the increasing appreciation of the frequency of novel and functionally deleterious variants in the general population, increasingly robust evidence will be required to be confident that any particular variant is truly disease causing, and there will undoubtedly be an exponential rise in the numbers of "variants of unknown significance." Caution is also required in giving negative results to asymptomatic family members and releasing them from follow-up, because the absence of a one-family-gene variant does not necessarily preclude the presence of additional potentially deleterious variants in other genes.

Genetics as a Guide to Therapy?

Treatment guidelines for heart failure and arrhythmias in cardiomyopathies are similar to those for nongenetic causes of these disorders. There are no specific therapies in current clinical use that change gene mutations. However, the R222Q *SCN5A* mutation has recently provided a proof-of-principle example in which the effect of a mutant protein can be directly targeted. This variant has been reported in several families with DCM and complex ventricular arrhythmias and has been shown to have an activating effect on cardiac sodium channels (Hershberger et al. 2008; McNair et al. 2011; Mann et al. 2012). Treatment of affected family members with drugs that have sodium-channel-blocking properties markedly reduced the numbers of ventricular ectopic beats and improved ventricular contractile function (Mann et al. 2012). Various therapies directed toward reversing biological processes downstream from the initiating genetic trigger have been shown to be effective in murine cardiomyopathy models, and some of these have been evaluated subsequently in human clinical trials. Examples of this include treatment with L-type calcium channel inhibitors, HMG CoA reductase inhibitors, antioxidants in HCM models, and β-blockers and extracellular signal-regulated kinase (ERK) inhibitors in DCM models (Patel et al. 2001; Semsarian et al. 2002; Lombardi et al. 2009; Muchir et al. 2009; Chandar et al. 2010; Yeoh et al. 2011). Other new approaches to therapy that are currently under investigation include reversal of microRNA effects with antagomirs, and stem-cell therapies. It is envisaged that the wealth of genetic data provided by whole genome and WES will reveal tractable targets for novel drug therapies. The ultimate goal of genetics studies is to enable personalized approaches to diagnosis, treatment, and prevention. This will require high-throughput bioinformatics analysis, identification and validation of actionable variants, and prospective clinical assessment.

CONCLUDING REMARKS

These are exciting times in cardiovascular genetics when genomic sequencing on an unprecedented scale promises to revolutionize understanding of cardiomyopathy pathogenesis as well as approaches to investigation and treatment. New ways to rapidly assess the functional significance of variants singly, and in combination, are required. Several fundamental questions need to be addressed, including why patients with the same phenotype have different genotypes, and why those with the same genotype develop different phenotypes. The answers to this question will no doubt need to take into account each person's entire profile of rare and common genetic variants as well as epigenetic

and environmental factors, and future strategies for diagnosis, early detection, and management of cardiomyopathy may need to focus on endpoint myocardial functional defects.

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