β-Myosin Heavy Chain Variant Val606Met Causes Very Mild Hypertrophic Cardiomyopathy in Mice, but Exacerbates HCM Phenotypes in Mice Carrying Other HCM Mutations

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Rationale: Approximately 40% of hypertrophic cardiomyopathy (HCM) is caused by heterozygous missense mutations in β-cardiac myosin heavy chain (β-MHC). Associating disease phenotype with mutation is confounded by extensive background genetic and lifestyle/environmental differences between subjects even from the same family.

Objective: To characterize disease caused by β-cardiac myosin heavy chain Val606Met substitution (VM) that has been identified in several HCM families with wide variation of clinical outcomes, in mice.

Methods and Results: Unlike 2 mouse lines bearing the malignant myosin mutations Arg453Cys (RC+) or Arg719Trp (RW+), VM/+ mice with an identical inbred genetic background lacked hallmarks of HCM such as left ventricular hypertrophy, disarray of myofibers, and interstitial fibrosis. Even homozygous VM/VM mice were indistinguishable from wild-type animals, whereas RC/RC- and RW/RW-mutant mice died within 9 days after birth. However, hypertrophic effects of the VM mutation were observed both in mice treated with cyclosporine, a known stimulator of the HCM response, and compound VM/RC heterozygous mice, which developed a severe HCM phenotype. In contrast to all heterozygous mutants, both systolic and diastolic function of VM/RC hearts was severely impaired already before the onset of cardiac remodeling.

Conclusions: The VM mutation per se causes mild HCM-related phenotypes; however, in combination with other HCM activators it exacerbates the HCM phenotype. Double-mutant mice are suitable for assessing the severity of benign mutations. (Circ Res. 2014;115:227-237.)

Key Words: cardiomyopathies ■ genetics ■ hypertrophy ■ myocardial contraction ■ myosins

Hypertrophic cardiomyopathy (HCM) is the most frequent inherited disease of the heart. It is caused by missense mutations located in 1 of 28 different sarcomere genes.1 In ≈40% of cases, the defect is attributable to 1 of >300 known single amino acid substitutions in the β-cardiac myosin heavy chain (MHC; Figure 1) head domain.2,3 Hearts bearing β-MHC mutations usually start to develop significant thickening of ventricular walls and progressive myocardial fibrosis during the second decade of life.4 Because cardiac function is not compromised at early stages of HCM, affected individuals are often unaware of the disease until complications arise such as life-threatening arrhythmia and heart failure.

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Certain β-MHC mutations are associated with a more severe clinical course than others.5 Accordingly, they can be graded in benign and malignant mutations to predict disease progression and to stratify the clinical follow-up and medical treatment including implantable cardioverter-defibrillators for primary prevention of sudden death. For example, individuals affected with an Arg453Cys (RC) or an Arg719Trp (RW) mutation have severe myocardial remodeling and die at an average age of only 40 years.6,7 On the contrary, normal life expectancy was reported for 3 families carrying Val606Met (VM) mutation.6 However, many subsequent studies have questioned the benign nature of the VM mutation because they collectively identified 28 cases of cardiac death before the age of 30 years in 4 families that comprised 29 carriers of the mutation.8-12 Whether the VM mutation per
se is associated with a good or a poor prognosis of affected people remains uncertain.

Several factors have been associated with mutations that cause poor prognosis in patients with HCM. First, mutations that alter the charge of the encoded amino acid generally have a worse prognosis than mutations that encode amino acids of the same charge as the normal residue. Second, the location of the affected amino acids in specific functional myosin head domains such as the actin and ATP-binding sites or the converter domain at the head–rod junction has been associated with bad outcome (Figure 1). The VM mutation fulfills neither of these criteria raising the question, how a conservative amino acid substitution in the backbone of the cardiac myosin head may cause severe cardiac remodeling and premature death. The diversity of phenotypes between different kindreds and among affected family members suggests that the genotype–phenotype correlation of the VM mutation is highly influenced either by modifying genes or by nongenetic factors or both.

Dissecting the mechanisms that modify the response to a β-MHC mutation has been hindered by the limited number of affected individuals. Therefore, we have begun to evaluate the consequences of expressing the human β-MHC mutation VM in mice and compared the fate of the VM mutation with 2 previously described mouse models that carry human β-MHC mutations, RC and RW, in the mouse α-MHC gene.

All mutation-carrying mice were bred on the same genetic background and animals were housed under identical conditions from birth to death to reduce possible differences in background genetic modifiers and to minimize environmental influences. Furthermore, homozygous and compound heterozygous mice were cross-bred to determine the phenotypic consequences of a second mild or severe mutation in the myosin head. While heterozygous 26-week-old RC and RW mutants gradually developed hallmarks of HCM, no phenotype was detected in age-matched VM mice confirming the benign nature of this mutation. By contrast, mice carrying VM with either RC or RW mutations were much more severely affected. That is, even mild mutations substantially aggravated the morphological and functional heart phenotype and significantly reduce survival when placed in trans to more severe mutations. Extending these findings to humans, the huge impact of additional amino acid substitutions within the myosin head would suggest genetic testing of every patient harboring an HCM causing β-MHC mutation for additional genetic variants within this gene to better evaluate the clinical prognosis.

Methods

Detailed Methods are available in the Online Data Supplement, which includes the generation of gene-targeted animal models, cyclosporine A treatment, histological analyses using hematoxylin and eosin, Masson trichrome, Sirius Red, von Kossa, wheat germ agglutinin, and Hoechst 33258 staining as well as terminal deoxyuridine-5'-triphosphate (dUTP) nick-end labeling assays, assessment of myocyte size, mouse echocardiography, quantitative real-time polymerase chain reaction, left ventricular catheterization for assessment of hemodynamics, tissue bath measurements of force generation, transcriptional profiling using Affymetrix microarray, and statistical analysis.

Results

Mice Bearing Human β-MHC Mutations in α-MHC Develop Hallmarks of HCM

The HCM causing amino acid substitutions MHC arginine 453 cysteine substitution (RC), MHC arginine 719 tryptophane substitution (RW), and MHC valine 606 methionine substitution (VM) were introduced into the α-MHC gene of mice. Heterozygous animals bearing the RC/+ or RW/+ mutation showed progressive concentric hypertrophy (Figure 2). Although indistinguishable at young age, left ventricular wall thickness of 26-week-old hearts exceeded that of wild-type (wt) littermates by >20% (P<0.05). At this age, hallmarks of HCM such as myofiber disarray and interstitial fibrosis were also detected (Figure 2A). Regardless of morphological alterations, contractile function was good, at 26 weeks of age fractional shortening was 38±3% in wt mice, 43±3% in RW/+ mice, and 49±5% in RC/+ (P<0.05; Figure 2B and Table). At age 78 weeks, concentric hypertrophy of RC/+ and RW/+ hearts had further progressed, albeit at slower pace (Figure 2B). Fractional shortening was unchanged. The slow progression of disease during adolescence, the development of significant cardiac hypertrophy, fibrosis, and myofiber disarray in hearts...
with intact contractile function closely mimic the pathology observed in humans affected with the RC/+ or RW/+ mutation in the β-MHC gene.6,7

α-MHC\textsuperscript{VM} Mutation Produces Mild HCM That Can Be Exacerbated With Cyclosporine

To assess the severity of the VM/+ mutation, a novel knock-in mouse model was generated (Online Figure I). Unlike RC/+ and RW/+, mice carrying a VM/+ mutation in the α-MHC gene were indistinguishable from wt littermates throughout life (Figure 2, Table, Online Figure II). At age 26 weeks, end-diastolic left ventricular anterior wall thickness (0.77±0.04 mm [VM/+]) versus 0.75±0.02 mm [wt]; \(P=\text{NS}\) and dimensions (left ventricular diameter, 3.42±0.14 mm [VM/+]) versus 3.70±0.09 mm [wt]; \(P=\text{NS}\) as well as fractional shortening (44±5% [VM/+]) versus 38±3% [wt]; \(P=\text{NS}\) were normal. At very old age (78 weeks), VM/+ and wt mouse hearts still showed no detectable morphological differences (Figure 2B, Online Figure II). Invasive hemodynamic measurements in 8-week-old animals also yielded comparable values for left ventricular pressures, contraction and relaxation in VM/+ and wt mice (Figure 3C).

Figure 2. Morphological comparison of 3 different mouse lines (VM/+, RC/+, RW+) bearing human hypertrophic cardiomyopathy (HCM) causing mutations in the myosin heavy chain gene. A, Sections through wild-type (wt) hearts and heterozygous HCM mutants at the age of 26 weeks show concentric hypertrophy, myofiber disarray, and interstitial fibrosis only in RC/+ and RW/+ mice. \textit{Top}, Hematoxylin and eosin staining (bar, 100 μm). \textit{Bottom}, Transverse sections, collagen is stained in red by Sirius Red. B, Echocardiographic measurements at the age of 8, 26, and 78 weeks. FS indicates fractional shortening; LVAW, left ventricular anterior wall thickness; LVEDD, left ventricular end-diastolic diameter; and LVPW, left ventricular posterior wall thickness. \(n\geq3\) animals per genotype; \(*P<0.05, \text{RW/+ vs wt}; \#P<0.05, \text{RC/+ vs wt.} \)
Previous studies have demonstrated that cyclosporine exacerbates the hypertrophic response to MHC missense mutations. The response of VM/+ mice to cyclosporine was assessed (Figure 3A and 3B). After 3 weeks of cyclosporine treatment, VM/+ mice developed symmetrical left ventricular wall thickening (P<0.05) compared with wt mice. The benign nature of the VM substitution was further evaluated in homozygous animals (VM/VM) generated by cross-breeding of VM/+ mice (Figure 4A). The phenotype of mouse hearts carrying the VM substitution was further evaluated in homozygous animals (VM/VM) generated by cross-breeding of VM/+ mice (Figure 4A). Cardiac ventricles harvested shortly before death revealed significant upregulation of genetic markers of hypertrophy in 7-day-old hearts. Homozygous mutant hearts displayed patches of dying myocardial cells as suggested by increased numbers of terminal dUTP nick-end labeling–positive myocytes (data not shown) and areas in the myocardium with calcifications (Figure 4D).

### Compound Heterozygous Myosin Mutants (VM/RC) Develop Severe HCM

Next, we investigated the phenotype of mouse hearts carrying 1 benign (VM) and 1 malignant (RC) HCM causing myosin mutation. Cross-breeding of VM/VM and RC/+ mutants produced compound heterozygous VM/RC and VM/+ mice at 1:1 ratios. VM/RC developed normally and body weight did not differ from wt and heterozygous littermates (data not shown). Unlike RC/RC and RW/RW mice, VM/RC mutants lived to adulthood, but died prematurely at a mean age of 62±8 weeks, whereas wt and all heterozygous mouse lines lived for >100 weeks on average (Figure 5).

Histology of VM/RC myocardium was characterized by massive fibrosis, which was already detectable at the age of 10 weeks and progressed further over time (Figure 6A). Both at 10 and 26 weeks, collagen depositions in VM/RC hearts were 10-fold higher than in the hearts of VM/+ littermates (P<0.01). Hypertrophy of VM/RC hearts rapidly progressed and exceeded the wall thickness of VM/+ hearts by >50% at the age of 26 weeks (Figure 6A and 6B, Table). Compared with VM/+ siblings and wt hearts, left ventricular wall thickness of VM/RC heart was almost doubled (P<0.001). Heart-to-body weight ratios (6.7±0.5 mg/g) were higher than in wt (4.7±0.2 mg/g) and single heterozygous animals (VM/+, 4.5±0.1 mg/g; RC/+, 5.2±0.3 mg/g; P<0.05). Individual myocytes of VM/RC hearts, as determined in serial transverse sections through left ventricles after staining of cell membranes, were also enlarged at

#### Unlike VM/VM, Homozygous RC/RC and RW/RW Die Early

Inbreeding of RC/+ mice or RW/+ produced wt, heterozygous, and homozygous offspring at expected ratios (1:2:1). RC/+ and RW/+ animals lived for ≈2 years. In contrast, 100% of mice homozygous for either of the 2 HCM causing mutations (RC/RC and RW/RW) died within 9 days after birth (Figure 4A). Cardiac ventricles harvested shortly before death revealed significant upregulation of genetic markers of hypertrophy in 7-day-old hearts. Homozygous mutant hearts displayed patches of dying myocardial cells as suggested by increased numbers of terminal dUTP nick-end labeling–positive myocytes (data not shown) and areas in the myocardium with calcifications (Figure 4D).

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this age (myocyte area, 448±53 μm² [VM/RC] versus 273±13 μm² [VM/+]; P<0.05). Genetic markers of hypertrophy were clearly elevated already in 6- to 8-week-old VM/RC hearts, for example, atrial natriuretic peptide was 44±10-fold and brain natriuretic peptide 4.4±0.6-fold over wt (P<0.001; Figure 6C). Taken together, cardiac characterization of VM/RC mice indicated a synergistic (rather than an additive) behavior of the second mutation in amplifying the effects of 2 single mutations toward the development of myocardial hypertrophy and fibrosis. A similar heart phenotype as in VM/RC mice was observed in mice that are compound heterozygous for the benign VM and the malignant RW substitution (VM/RW) implying that the VM mutation also exacerbates HCM phenotypes of myosin mutations other than RC (Online Figure III).

Impaired Systolic and Diastolic Function of Compound Heterozygous VM/RC Hearts

VM/RC mice allowed the investigation of cardiac contractile function in hearts with severe HCM (Table, Figure 7). Although fractional shortening of 26-week-old animals was conserved (47.8±1.7% versus 44.0±4.6% in VM/+ and end-systolic volumes were low (5.5±0.9 μL [VM/RC] versus 10.8±1.6 μL [VM/+]; P<0.01), ventricular stroke volume was depressed (18.1±2.2 μL [VM/RC] versus 28.0±3.2 μL [VM/+]; P<0.05), most likely caused by the reduced end-diastolic volume of VM/RC hearts. Invasive pressure measurements in 6- to 8-week-old left ventricles further demonstrated depressed velocities of pressure rise (dp/dtmax) and maximal speed of pressure decay (dp/dtmin) were not different among groups; n≥4 animals per genotype. D, Gross morphology and histological sections of 1-year-old wild-type (wt) mice compared with age-, strain-, and sex-matched homozygous mutants (VM/VM). Heart sections were stained with hematoxylin and eosin (top) and with Sirius Red (bottom); bar, 100 μm. LVAd indicates left ventricular anterior wall thickness; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; and LVPW, left ventricular posterior wall thickness.

Depressed Force Generation of VM/RC Myocardium

Heterozygous HCM causing mutations are known to enhance systolic heart function, and hypercontractility was also
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found in RC/+ and RW/+ hearts. Therefore, we further dissected the cause of impaired systolic function in double heterozygous VM/RC mice by assessment of force production in isolated heart tissue. Left atria of 6- to 8-week-old VM/+ and VM/RC littermates were quickly excised, attached to a force transducer and kept in 37°C warm Tyrode's solution. After adjusting the pretension to 1 mN, tissue was electrically stimulated at a constant pace of 9 Hz to imitate physiological heart rate. Under these conditions, VM/RC tissue generated only 46% of the force produced by VM/+ tissue (0.18±0.04 versus 0.39±0.06 mN; P=0.01; Figure 7B). Also the speed of force generation (9.2±1.7 versus 19.2±2.8 mN/s; P=0.01) and the speed of force decay were clearly reduced (−6.5±1.0 versus −15.2±2.3 mN/s; P<0.01). These findings suggest that impaired force production of double-mutant sarcomeres may underlie depressed pressure buildup of VM/RC hearts.

Transcriptional Profiling of VM/RC Hearts

For characterization of the molecular defects imposed by compound VM/RC heterozygous mutations, expression profiles of 3 VM/RC and 4 wt mouse hearts were assessed by DNA microarray analysis (Affymetrix Mouse Gene 2.0 ST). We identified 754 genes that were upregulated (534 genes) or downregulated (220 genes) in VM/RC hearts by >50% at a statistical significance of P<0.05. All differentially expressed genes (P<0.05) were assigned to respective gene ontology functional categories (gene ontology terms) and enrichment P values were calculated (Online Table I). Among cellular components, differential gene expression was by far most significantly related to the gene ontology terms extracellular matrix (enrichment P value, P=1.99×10−36) and collagen (P=7.2×10−15). Gene ontology terms describing biological processes that were enriched for upregulated genes included the regulation of fibroblast proliferation (P=0.0001), cell death (P=0.00005), programmed cell death (P=0.00002), inflammatory (P=0.001), and immune responses (P=0.002) as well as the regulation of mitogen-activated protein kinase cascade (P=0.00003) and heart contraction (P=0.0002; Online Table I). Furthermore, microarray analysis and quantitative polymerase chain reaction identified differential regulation of genes encoding proteins involved in calcium binding and calcium transport suggesting that disturbed calcium homeostasis may play a pathogenetic role in the development of the HCM phenotype in VM/RC hearts (Online Figures IV and V).

Discussion

Genetically engineered mouse models carrying HCM causing β-MHC mutations in the mouse α-MHC gene closely resemble the cardiac pathology of the human disease.14,15,18,19 Here, we used such mice in an inbred genetic background to compare the phenotypic consequences of 3 different mutations. Similar to affected humans, RC/+ and RW/+ mice demonstrated slow progression of left ventricular hypertrophy during adolescence, interstitial fibrosis, and myofiber disarray at intact contractile function (Figure 2). Animals died early on when homozygous (Figure 4). Although heterozygous or homozygous VM hearts were indistinguishable from wt hearts (Figures 2 and 3C and 3D, Online Figure II, Table), the hypertrophic response could be exacerbated in VM/+ hearts either by cyclosporine treatment or by the combination of VM and RC mutations in a compound heterozygous model (VM/RC). VM/RC mice developed cardiac hypertrophy and interstitial fibrosis to a degree similar to animals after transverse
aortic banding (Figure 6). Cardiac contractile function was impaired early on and animals died prematurely at an average age of 62±8 weeks (Figures 5 and 7). The data indicate that the HCM stimulus caused by the VM mutation is much milder than that of the mutations RC and RW. Even homozygosity, which causes early lethality of RC/RC and RW/RW mice, did not induce signs of HCM in VM/VM hearts. Adverse effects attributable to the VM mutation only occurred in combination with other myosin mutations. However, in compound heterozygous mutants, the VM mutation severely boosted the morphological and functional HCM phenotype. These results indicate exceeding sensibility of the HCM causing RC mutation to additional variants within the cardiac myosin head. The individual genetic constitution of affected family members may bear great responsibility for the heterogeneity of resulting phenotypes as repeatedly described for the VM mutation.5-12 Presuming that our findings in mouse models can be extrapolated to humans, the data would suggest careful genetic testing of all individuals bearing a β-MHC mutation for additional genetic variants within the myosin head and possibly other sarcomere genes to stratify the individual risk and clinical management. Such analyses may be most important in patients carrying a benign β-MHC variant because the heart phenotype of mice carrying the VM mutation varied from no detectable changes to severe HCM and premature death contingent on the absence or presence of the RC mutation.

Unlike studies in humans, we observed little variation of measurements when characterizing the cardiac morphology and function of mouse models. Prediction of heart phenotypes based on age and genotype was always reliable recommending inbred mutant mouse lines for precise assessment of genotype-phenotype correlations in HCM. Here, we engineered human β-MHC mutations into the mouse α-MHC gene because mouse hearts predominantly express the faster α-isoform. Although

Figure 5. Survival of mice heterozygous (RC/+, VM/+) and compound heterozygous (VM/RC) for hypertrophic cardiomyopathy causing mutations in cardiac myosin compared with wild-type (wt) mice. Log-rank test, P<0.0001.

Figure 6. Characterization of compound heterozygous mouse hearts (VM/RC) and VM/+ littermates obtained from cross-breeding of RC/+ and VM/VM mice. A. Transverse sections and histology of 26-week-old hearts; upper, hematoxylin and eosin staining; lower, Sirius Red staining of left ventricular sections and quantitative analysis of red color indicating fibrosis of wild-type (wt), heterozygous (VM/+, RC/+), and compound heterozygous (VM/RC) hearts at age 10 weeks and 26 weeks, respectively; bar, 100 μm; serial sections of ≥3 hearts per genotype were analyzed; *P<0.05 vs wt of same age group. B, VM/RC hearts develop extensive hypertrophy as assessed by echocardiography (representative M-mode images from left ventricles are displayed, measurements of heart weight and myocyte cross-sectional area of 26-week-old hearts. *P<0.05; histological sections were stained with wheat germ agglutinin for detection of cell membranes (green) to determine myocyte size (right); nuclei (blue) stained with Hoechst 33258; bar, 25 μm; n≥4 animals per genotype. C, Expression of genetic markers of hypertrophy in 6- to 8-week-old mouse hearts. ANP indicates atrial natriuretic peptide; ASA, α-skeletal actin, BNP, brain natriuretic peptide; HW/BW, heart-to-body weight ratio; LVAW, left ventricular anterior wall thickness; LVPW, left ventricular posterior wall thickness; and MybpC, myosin-binding protein C. n=4 animals per genotype.
both isoforms share 93% identity in amino acid sequence, this is a drawback for the use of mice as genetic models of human HCM caused by β-MHC mutations. Nevertheless, knock-in mice heterozygous for the malignant RC and RW mutations in the α-MHC gene recapitulated morphological hallmarks of human HCM, such as gradual development of left ventricular hypertrophy, myofiber disarray, and fibrosis. Furthermore, functional analyses by echocardiography and left ventricular catheterization revealed hypercontractility of RC/+ and RW/+ mouse hearts (Figures 2B and 7, Table 1), and previous studies in these and other α-MHC knock-in mouse models demonstrated enhanced actin filament sliding, increased ATPase activity and force production of the myosin head on the molecular level.14,21–23 These findings in mouse α-MHC closely match biophysical analyses using patient biopsies with HCM causing mutations in the β-isofrom of MHC that described increased force, enhanced actin-myosin sliding velocities and actin-activated ATPase activity for a series of different mutations.24–27 In addition, Sommese et al28 recently reported a 50% increase of intrinsic motor force by the RC mutation in an elegant in vitro study using recombinant human β-MHC produced in adenovirus-infected myoblasts. Given such parallels for homologous HCM causing mutations in human β-MHC and mouse α-MHC it seems likely that the morphological and functional phenotype of VM mutants observed in this study may reflect characteristics of human hearts bearing the VM mutation in β-MHC.

In combination with the malignant RC substitution the cardiac phenotype of VM mice changed from a normal healthy heart to severe hypertrophy, interstitial fibrosis, systolic and diastolic impairment of contractile function, and premature death. Mice heterozygous for 2 malignant HCM causing myosin mutations (RC/RW) died shortly after birth (data not shown). Human carriers of >1 HCM causing mutation have been reported to develop the disease earlier, with a higher degree of left ventricular hypertrophy and a higher incidence of sudden death events caused by a double dose effect.29–31 Compound heterozygosity (different mutations

Figure 7. Functional characterization of VM/RC hearts. A, Left ventricular hemodynamics of 6- to 8-week-old wild-type (wt), heterozygous (RC/+ and VM/+), and compound heterozygous (VM/RC) myosin mutants. A, Maximal left ventricular pressure (LVP max), speed of pressure rise (dp/dt max), and pressure decay (dp/dt min) were measured at baseline (left) and during cardiac stimulation with dobutamine at increasing concentrations (right), n≥5 animals per genotype. B, Measurement of force generation in 6- to 8-week-old isolated left atria from heterozygous (VM/+) and compound heterozygous (VM/RC) mice. Force was measured at 1 mN pretension and electric stimulation at 9 Hz, dF/dt max indicates maximal speed of force generation; dF/dt min, maximal speed of force decay; and F max, maximal generated force.
in homologous alleles) or double heterozygosity (2 mutations in different genes) is thought to occur in ≤5% of families with HCM.\textsuperscript{29,30,32} The severe phenotype seen in VM/RC mouse hearts as well as in RC/RW and homozygous RC/RC and RW/RW mice suggests that in humans that carry 2 mutations ≥1 of the mutations must be mild. In fact, to date, no case has been reported with 2 β-MHC mutations that both are known to also cause HCM if expressed alone. A potential explanation for the synergistic effects of mutations in the myosin head may be the strong impact of the second myosin mutation on cardiac function (Figure 7). While contractility is normal or even enhanced in VM/+ and RC/+ hearts, it is severely depressed in VM/RC hearts. Given the extensive development of interstitial fibrosis, it would be conceivable that compound VM and RC mutations induce a fundamental defect in the transmission of force from the sarcomeres to the extracellular matrix. Analyses of isolated tissue under defined workload suggested markedly reduced force production of double-mutant sarcomeres to underlie impaired hemodynamics (Figure 7B). Possibly, in a heterozygous situation the roughly 50% of wt myosin heads are essential to compensate functional changes inflicted by the mutant myosin heads. By implication, even a minor change of the wt myosin by a benign substitution would lead to severe consequences on contractile function.

Transcriptional profiling revealed that genes important for extracellular matrix and fibroblast proliferation are most frequently differentially expressed in VM/RC hearts (Online Table I). These changes are consistent with our observation of severe myocardial fibrosis in these hearts at older age (Figure 6). A possible explanation for the massive induction of collagen is provided by the increased number of dying VM/RC myocytes and subsequent replacement fibrosis (Online Figure VII). The even distribution of singular terminal dUTP nick-end labeling–positive cells within the myocardium suggests that these cells may die by apoptosis. This assertion is substantiated by the over-representation of upregulated genes in gene ontology categories relevant for the regulation of cell death ($P=0.00005$) and apoptotic processes ($P=0.00003$; Online Table I, Online Figure VI).

Gene array analyses further provided evidence for involvement of pathogenic factors other than cell death in increased collagen production of VM/RC hearts. (1) We previously found transforming growth factor-β, which induces the synthesis of matrix molecules, to be required for the development of cardiac fibrosis in RW/+ mouse hearts by mediating nonmyocyte proliferation.\textsuperscript{15} In this study, microarray and quantitative polymerase chain reaction analyses detected upregulation of transforming growth factor-β and transforming growth factor-β receptors in VM/RC hearts suggesting that increased transforming growth factor-β signaling may also trigger fibrosis in VM/RC myocardium (Online Table II). (2) Along these lines, transcriptional profiling of VM/RC mouse hearts further hints at a possible involvement of inflammatory responses and immunologic processes in fibrotic myocardial remodeling (Online Table I). (3) Heart sections of an independent mouse model that develops severe dilated cardiomyopathy attributable to an Arg9Cys mutation in phospholamban revealed clearly more terminal dUTP nick-end labeling–positive cells than VM/RC hearts suggesting additional mechanisms to contribute to the high level of myocardial fibrosis observed in the latter (Online Figure VII). (4) Measurements of cardiac function indicated severe systolic and diastolic impairment of VM/RC left ventricles (Figure 7). Depressed hemodynamics would induce adverse myocardial remodeling attributable to both increased wall stress of ventricles and neurohumoral activation, that is, stimulation of the sympathetic system and the renin–angiotensin–aldosterone system.

In mice, there are 3 pertinent studies with multiple HCM causing sarcomere mutations though this is the first report on compound heterozygous myosin mutants. Tsoutsman et al\textsuperscript{33} characterized mice bearing a combination of the α-MHCArg403Gln mutation plus a transgene expressing the HCM causing Gly203Ser troponin I mutation in the mouse heart. These animals developed dilated cardiomyopathy, heart failure, and death within 3 weeks after birth. Also mice homozygous for an HCM causing loss of function mutation in the gene for cardiac myosin-binding protein C showed neonatal onset of progressive dilated cardiomyopathy.\textsuperscript{34} These animals lived for more than a year and heterozygotes developed just mild symptoms of HCM. Based on these observations, it seemed that an increased gene dosage or the combination of 2 cues that cause HCM would foster a dilated phenotype of the heart. Thus, it seemed likely that the combination of 2 HCM causing myosin mutations may also lead to cardiac dilatation, particularly in the light of β-MHC mutations that have been identified to induce dilated cardiomyopathy, such as the substitutions Ser532Pro and Phe764Leu.\textsuperscript{35} Nevertheless, VM/RC mice developed pure hypertrophy and typical features of HCM indicating that the combination of 2 heterozygous HCM causing mutations do not necessarily foster dilatation of the heart.

The comparison of 3 different knock-in mouse lines showed that the VM mutation seems to be benign. However, the VM mutation severely remodels the heart in a more than additive manner when combined with other heterozygous HCM causing myosin mutations. Along these lines, the combination of 2 malignant myosin mutations in homozygous RC/RC or RW/RW hearts is lethal. Genetically engineered mice seem to be suitable to assess the severity of human β-MHC mutations. The bold phenotypic boost in double-mutant mice further suggests compound mutants for assessment of severity. This strategy seems particularly useful for evaluating the consequences of benign mutations that do not induce significant phenotypes if expressed alone.

If every patient with HCM would carry 1 single sarcomere mutation and the prevalence of HCM is 1:500,\textsuperscript{36} the statistical risk to inherit an HCM causing mutation from either parent would be in the range of 1:1 million. However, compound heterozygosity or double heterozygosity have been described in up to 5% of families with HCM,\textsuperscript{29,31–33,37} and the 1000 Genomes Project database revealed multiple pathogenic mutations of sarcomeric genes in 5 of 1092 individuals suggesting that the majority of these gene variants would not be disease causing if expressed alone.\textsuperscript{38} Nevertheless, they may severely impact the severity of HCM as this study has shown in mice. A synthetic gain of phenotype would uncover relatively mild mutations and
also explain the occasionally striking phenotypic heterogeneity among individuals carrying the same HCM causing mutation. Finally, it would strongly suggest genetic testing of every HCM patient for additional genetic variants within sarcomere genes, to stratify the individual risk and clinical management.

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Disclosures

None.

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**Novelty and Significance**

**What Is Known?**

- Hypertrophic cardiomyopathy (HCM) is a leading cause of cardiac death in people aged <35 years.
- About 40% of HCM cases are caused by mutations in the β-cardiac myosin heavy chain (MHC) gene.
- Genotype–phenotype correlations can vary greatly, for example, in individuals with a heterozygous MHCVal606Met mutation (VM/+).

**What New Information Does This Article Contribute?**

- The VM mutation causes a mild phenotype in mice.
- Together with other HCM activators the VM mutation severely exacerbates the murine HCM phenotype.
- Double-mutant mice are suitable for assessing the severity of benign mutations.

The identification of the underlying genetic defect enables early diagnosis of HCM, but a clear prediction of clinical outcome requires careful assessment of confounding genetic and nongenetic factors. This work identified the human VM mutation as a benign mutation per se when modeled in mice. Nevertheless, in combination with either HCM causing MHC mutations or cyclosporine treatment VM knock-in mouse models showed a dramatic increase of cardiac hypertrophy and interstitial fibrosis leading to premature death of compound mutant animals. Therefore, even benign genetic defects that are not disease causing if expressed alone may act synergistically on the HCM phenotype suggesting careful genetic screening of all individuals bearing a β-MHC mutation for additional genetic variants.
β-Myosin Heavy Chain Variant Val606Met Causes Very Mild Hypertrophic Cardiomyopathy in Mice, but Exacerbates HCM Phenotypes in Mice Carrying Other HCM Mutations
Robert Blankenburg, Katarzyna Hackert, Sebastian Wurster, René Deenen, J.G. Seidman, Christine E. Seidman, Martin J. Lohse and Joachim P. Schmitt

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SUPPLEMENTAL MATERIAL

Detailed Methods

Generation of mutant mice. The VM substitution was introduced into the cardiac α-MHC gene using standard gene targeting techniques (Fig. S1).1,2 In brief, mutagenesis PCR was used to introduce the mutation into the targeting vector containing positive (neomycin resistance) and negative (thymidine kinase) selection markers. Neomycin-resistant embryonic stem cell clones (129/SvEv background) were screened for homologous recombination by Southern blot analyses and picked-out mutant cells injected into mouse blastocysts. Chimeric mice were mated with EIIa-Cre transgenic mice (129/SvEv background) for deletion of the neomycin resistance gene. RC and RW mutant mice have been described previously.3,4 Homozygous cardiac α-MHC mutants (VM/VM, RC/RC, RW/RW) were generated by crossbreeding of heterozygous mice. Double heterozygous mice (VM/RC) originated from crossbreeding of VM/VM and RC/+ animals. All animals were maintained and studied using protocols approved by the responsible ethics committees and government agencies (Regional Government of Lower Franconia, Germany). Inbred male mice in an 129/SvEv background were used in all studies. For cyclosporine treatment of mice 15 µg/g body weight cyclosporine A (CsA) in PBS were injected subcutaneously twice daily for up to 4 weeks.

Mouse heart weight, histopathology, cell size, fibrosis assessment and TUNEL staining. Mouse hearts were perfused with 4% paraformaldehyde at physiological pressures, blotted dry on tissue paper and weighed. Next, they were embedded in paraffin and sections were stained with hematoxylin and eosin (H & E), Masson’s trichrome, Sirius Red, vonKossa or wheat germ agglutinin (WGA, Alexa Fluor 488, Molecular probes Inc., Eugene, OR) according to the manufacturer’s protocol and as described previously.5 For fibrosis assessment, the relative proportion of red staining was determined in at least four whole transverse sections. Sections were derived from different regions of left ventricles.
>100µm apart from each other and stained with Sirius Red. Perivascular collagen was
excluded from the analyses. Cell size was determined from >100 WGA stained myocytes per
study group. Only round cells with a nucleus (visualized with Hoechst 33258 stain) present in
the center of the myocyte were chosen. Measurements were performed using ImageJ
software. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining
was performed on mouse heart sections using the In Situ Cell Death Detection Kit,
Fluorescein (Roche Applied Science, Penzberg, Germany) according to the manufacturer’s
protocol.

**qPCR.** After extraction of total RNA from mouse ventricular tissue according to the
manufacturer’s protocol (Qiagen, Hilden, Germany), cDNA was synthesized from 1µg total
RNA by *in vitro* transcription using Superscript II (Invitrogen) and oligo dt primers. Relative
quantification of mRNA expression levels was performed by qPCR (Thermal Cycler CFX96,
BioRad, Hercules, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as
endogenous control.

**Echocardiography.** Mice were anesthetized with pentobarbital (30µg/g body weight), placed
on a warm plate and attached to an ECG monitor. Using a Vevo 2100 High-Resolution In
Vivo Micro-Imaging System and RMV 707B Scanhead (VisualSonics Inc., Toronto, Canada),
M-mode traces of left parasternal long and short axes were obtained at a minimal heart rate
450 bpm, as described previously.⁶ Left ventricular volumes, left ventricular mass, stroke
volume fractional shortening and ejection fraction were calculated from measurements of left
ventricular dimensions and wall thicknesses. E/A ratios were determined by Doppler
echocardiography of left ventricular inflow velocities. A single individual performed all
echocardiographic studies and cardiac measurements, without knowledge of the genotype.

**Hemodynamic measurements.** Hemodynamic measurements were performed as
described previously.⁶,⁷ Briefly, the right carotid artery of anesthetized mice (300 µg/g body
weight tribromoethanol) was exposed and ligated. Proximal of the ligature a 1.4 F pressure catheter (Millar Instruments) was inserted and advanced to the left ventricle. Dobutamine was applied continuously via a polyethylene tubing placed in the left jugular vein and connected to a microinfusion pump (Braun, Melsungen, Germany). Pressure loops were recorded and analyzed for heart rate, maximal pressure, and the derivatives of pressure loops using a PowerLab system and Chart software (Chart 5.4, AD Instruments, Colorado Springs, CO).

**Tissue bath experiments.** For measurements of force generation in isolated left atria, 6 to 8 weeks-old mice were sacrificed, the hearts rapidly excised and placed in carbogenated modified Tyrode’s solution (119 mM NaCl, 5.4 mM KCl, 1.2 mM CaCl₂, 1 mM MgCl₂, 22.6 mM NaHCO₃, 0.42 mM NaH₂PO₄, 0.025 mM EDTA, 10 mM glucose, 0.2 mM ascorbic acid, pH 7.4). Left atria were isolated, tied with two 6-0 silk sutures and the ends attached to a force transducer. Atria were kept in a tissue bath (IOA-5301, Föhr Medical Instruments, Seeheim, Germany) with carbogenated 37°C modified Tyrode’s solution and electrically paced with 10 mV at 10 ms cycle length (Stimulator Type 215/l, HSE, March-Hugstetten, Germany). Signals from isometric force transducers were fed into a PowerLab system and analyzed using Chart software (Version 5.4, AD Instruments Colorado Springs, CO).

**Transcriptional profiling.** RNA was extracted from ventricles of 3 VM/RC and 4 wild-type male 129SvEv mice at the age of 7 weeks (RNeasy Midi kit, Qiagen, Hilden, Germany). RNA integrity was confirmed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and quantified by photometric Nanodrop measurement. Synthesis of cDNA and subsequent biotin labeling of cRNA was performed according to the manufacturer’s protocol (WT Plus Kit; Affymetrix, Inc., Santa Clara, CA). After fragmentation, labeled cRNA was hybridized to Affymetrix Mouse Gene 2.0 ST Gene Expression Microarrays, stained by
streptavidin/phycoerythrin conjugate and scanned as described in the manufacturer's protocol.

**Statistical analysis.** Normality testing using Prism software (Version 6.0) provided no indication of nonparametric data distribution. Thus, unpaired Student's t tests were used for comparisons of two groups and ANOVA for multiple comparisons. Survival curves were compared using log rank tests. P<0.05 was considered significant. Data are presented as mean ± SEM.

Affymetrix CEL files were analysed using Partek Genomics Suite software (v6.6; Partek Inc., St. Louis, MO). Raw expression values were background corrected by Robust Multi-array Average (RMA) and quantile normalized. Partek standard One-way ANOVA on log2 transformed expression data was used to determine differential gene expression with significance levels set at P<0.05 and P<0.01, respectively. Differentially expressed genes passing a fold-change cut-off >1.5 were further classified using Partek’s Gene Ontology (GO) enrichment tool in order to cluster differentially expressed genes into functional GO classes. A modified Fisher’s exact test was used to assess the probability that differentially expressed genes are statistically overrepresented within a GO class, expressed by a corresponding enrichment p-value. An enrichment P-value <0.05 was used to focus on highly enriched GO classes. Hierarchical clustering of differentially expressed genes enriched in distinct GO classes was performed using Euclidean similarity measures with Ward’s linkage.
Online Figure I

A wild type allele

HindIII SpeI

EcoRI HindIII

targeting construct

SpeI PGK neo

EcoRI

TK

V606M

homologous recombination

(EcoRI) HindIII

3.9kb fragment

floxed allele

HindIII SpeI

(EcoRI) HindIII

EcoRI

EcoRI HindIII

Cre recombination

PGK neo

V606M

mutant allele

HindIII SpeI

EcoRI

EcoRI HindIII

1 kb

V606M

B (+/+) VM/+ 20kb

3.9kb

C (+/) VM/+ 135bp

77bp

58bp

Online Figure I
Online Figure I. Generation of knockin mice carrying the human Val 606 Met (V606M) substitution in the cardiac α-myosin heavy chain gene. A, targeting strategy; the targeting construct carries a neomycin resistance gene (PGK neo) flanked by loxP sites (black triangles), the V606M mutation engineered into exon 16, and a thymidine kinase cassette (TK) as negative selection marker; below, after homologous recombination (large crosses) properly targeted sequence (floxed allele) before and after loxP/Cre-mediated deletion of the neomycin resistance cassette (PGK neo). B, floxed alleles were identified by Southern blot analysis; Southern blotting of Hind III-digested DNA from targeted embryonic stem cells, probed with the external EcoR I / Hind III fragment; blot showing two examples of properly targeted clones containing both the 20kb wild-type and the engineered 3.9kb fragments (right two lanes). C, gel electrophoresis of RT-PCR fragments after mutation-specific restriction digest with Rsa I confirming the missense mutation (V606M) in the targeted allele. The mutation abolishes an Rsa I site.
Online Figure II. Morphology of middle-aged (0.5 years) and old mouse hearts (1.5 years) carrying the human V606M substitution in the cardiac α-myosin heavy chain gene. A, heart-to-body weight ratios, left ventricular (LV) transsectional areas and myocyte size of 0.5 and 1.5 years-old wild-type, VM/+ and VM/VM mice. LV transsectional areas were determined as the area of the LV wall on a transverse heart sections just below the atrioventricular valves. BW, body weight. B, Hematoxylin and Eosin (H&E) and Sirius Red staining of wild-type and VM/+ mouse hearts aged 1.5 years.
Online Figure III

A

![Survival curve over age](image)

B

VM/RC

LA

LVW

C

VM/RC

Image of tissue sample
Online Figure III. Characterization of compound heterozygous VM/RW mouse hearts. A, survival curves of VM/RW mice compared to compound heterozygous VM/RC and wild type mice (wt); log rank-test of VM/RW vs. wt, P < 0.0001. B, Longitudinal section through a 25 weeks-old VM/RW mouse heart and H & E staining. Note the thick left ventricular free wall (LVW) and the big left atrium (LA). C, Sirius Red staining of the same heart as in B demonstrates marked collagen depositions in the myocardium. scale bar, 100 µm;
Online Figure IV. Gene expression profile of calcium-related genes in VM/RC compared to wild type mouse hearts. Heatmap and hierarchical clustering of genes involved in cellular calcium homeostasis. 39 genes were upregulated and 4 genes were downregulated in microarray analyses of 3 VM/RC compared to 4 age-, strain- and gender-matched wild-type mouse hearts (p<0.05). Color range displays the fold-change for each mRNA sample. Gene symbols and average fold change of each gene are displayed. Hierarchical clustering groups most similarly expressed genes and gene clusters.
Online Figure V

![Bar charts showing mRNA expression levels of different proteins relative to wild type. The proteins include Calsequestrin, Triadin, Junctin, Sorcin, and Calreticulin. Each bar chart compares wild type (wt) to different conditions labeled as RCo+, VMi+, and VMRC.](image-url)
Online Figure V. mRNA expression of calcium-binding genes of the sarcoplasmic reticulum (SR) in 7 weeks-old wild type, (wt), RC/+; VM/+ and VM/RC mouse hearts as assessed by qPCR. SR calcium-binding genes have been proposed to play an important role in the pathogenesis of HCM. Significant upregulation of calsequestrin and sorcin was found only for compound heterozygous VM/RC hearts. * P<0.05 vs. wt; n = 4 animals per genotype.
**Online Figure VI.** Gene expression profile of death-related genes. Heatmap and hierarchical clustering of death-related genes that were differentially regulated in microarray analyses of 3 VM/RC and 4 age-, strain- and gender-matched wild-type mouse hearts (p<0.05). 38 genes were up-regulated and 3 genes were down-regulated. Color range displays the fold-change for each mRNA sample. Gene symbols and average fold change of each gene are displayed. Hierarchical clustering groups most similarly expressed genes and gene clusters.
Online Figure VII. TUNEL staining of mouse hearts carrying the VM/+, VM/RC or a DCM causing human Arg9Cys mutation in the gene phospholamban (PLN<sup>R9C</sup>).<sup>10</sup> Left, heart section showing an example of a TUNEL positive myocyte; WGA, fluorescently tagged wheat germ agglutinin for staining of cellular membranes; Hoechst, staining of DNA with Hoechst 33258 stain; the arrow points at the TUNEL-positive nucleus of a myocyte. Right, counting results of TUNEL-positive myocytes.
Online Table I: Gene Ontology (GO) terms with significant enrichment of differentially expressed genes comparing 7 weeks-old compound heterozygous VM/RC and VM/+ mouse hearts.

<table>
<thead>
<tr>
<th>Enriched GO terms</th>
<th>Enrichment ( p )-value</th>
<th>number of genes</th>
<th>total genes in group</th>
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<tbody>
<tr>
<td><strong>cellular component</strong></td>
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<tr>
<td>extracellular matrix</td>
<td>1.99E-36</td>
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<td>298</td>
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<td>collagen</td>
<td>7.17E-15</td>
<td>18</td>
<td>89</td>
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<td><strong>biological process</strong></td>
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<td></td>
<td></td>
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<tr>
<td>positive regulation of fibroblast proliferation</td>
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<td>5</td>
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<td>regulation of fibroblast proliferation</td>
<td>1.16E-04</td>
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<td>66</td>
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<tr>
<td>regulation of cell-matrix adhesion</td>
<td>3.98E-05</td>
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<td>56</td>
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<td>cell proliferation</td>
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<td>regulation of cardiac muscle hypertrophy</td>
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<td>regulation of cell death</td>
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<td>regulation of apoptotic process</td>
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<td>response to cytokine stimulus</td>
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<td>regulation of cytokine production</td>
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<td>regulation of heart contraction</td>
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<tr>
<td>calcium ion binding</td>
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<tr>
<td>calcium ion transport</td>
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Online Table II: Increased expression of TGF-beta in VM/RC

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<th>gene</th>
<th>fold increase</th>
<th>p-value</th>
<th>method</th>
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</thead>
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<td>TGFB1</td>
<td>1.36</td>
<td>0.045</td>
<td>gene array</td>
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<tr>
<td>TGFB2</td>
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<td>TGFBR1</td>
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<td>0.033</td>
<td>gene array</td>
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<tr>
<td>TGFBR2</td>
<td>1.40</td>
<td>0.038</td>
<td>qPCR</td>
</tr>
</tbody>
</table>

TGFB, transforming growth factor beta (TGF); TGFBR, TGFbeta receptor; fold increase vs. VM/+ (gene array) or vs. wild-type (qPCR)


**Supplemental References**


