nature medicine

A GRK5 polymorphism that inhibits β-adrenergic receptor signaling is protective in heart failure

Stephen B Liggett[1,6,7], Sharon Cresci[2,7], Reagan J Kelly[3,7], Faisal M Syed[1], Scot J Matkovich[2], Harvey S Hahn[1], Abhinav Diwan[1], Jeffrey S Martini[4], Li Sparks[1], Rohan R Parekh[1], John A Spertus[5], Walter J Koch[4], Sharon L R Kardia[3] & Gerald W Dorn II[1,2]

β-adrenergic receptor (βAR) blockade is a standard therapy for cardiac failure and ischemia. G protein–coupled receptor kinases (GRKs) desensitize βARs, suggesting that genetic GRK variants might modify outcomes in these syndromes. Re-sequencing of GRK2 and GRK5 revealed a nonsynonymous polymorphism of GRK5, common in African Americans, in which leucine is substituted for glutamine at position 41. GRK5-Leu41 uncoupled isoproterenol-stimulated responses more effectively than did GRK5-Gln41 in transfected cells and transgenic mice, and, like pharmacological βAR blockade, GRK5-Leu41 protected against experimental catecholamine-induced cardiomyopathy. Human association studies showed a pharmacogenomic interaction between GRK5-Leu41 and β-blocker treatment, in which the presence of the GRK5-Leu41 polymorphism was associated with decreased mortality in African Americans with heart failure or cardiac ischemia. In 375 prospectively followed African-American subjects with heart failure, GRK5-Leu41 protected against death or cardiac transplantation. Enhanced βAR desensitization of excessive catecholamine signaling by GRK5-Leu41 provides a 'genetic β-blockade' that improves survival in African Americans with heart failure, suggesting a reason for conflicting results of β-blocker clinical trials in this population.

Heart failure is an incurable syndrome arising from multiple causes that will affect one in five adults, conferring mortality rates of ~25% within a year of diagnosis and ~50% at 5 years after diagnosis[1,2]. The management of heart failure is complicated by disease heterogeneity in both inherited genetic cardiomyopathies[3] and the more common non-familial dilated and ischemic cardiomyopathies[4–7]. We and others have proposed that interindividual differences in genetic polymorphisms involving catecholamine signaling pathways can modify heart failure risk, prognosis or response to treatment. Especially relevant would be pharmacogenomic interactions between genetic variants of catecholamine receptors or their effectors and βAR antagonism (β-blockade), which is a standard therapy for heart failure and myocardial ischemia[8]. This therapy prolongs life and ameliorates symptoms but concomitantly impairs a key mechanism for acutely increasing cardiac output in response to physiological stress. Genetic polymorphisms that influence the balance between beneficial and toxic effects of βAR signaling may therefore be crucial to outcomes of cardiac disease.

An important mechanism for downregulating βAR signaling in heart failure is increased expression of myocardial GRK2, which phosphorylates cardiac βAR, leading to recruitment of β-arrestin and receptor uncoupling from G proteins and downstream signaling effectors[9]. Although several studies have shown that expression of a GRK2-inhibiting peptide can improve cardiac function in experimental models of heart failure[10,11], cardiac-specific ablation of *GRK2* in mice actually accelerates catecholamine-induced heart failure[12]. Thus, the effects of GRK2 on heart function seem to depend both on its expression level and on pathophysiological context. The function of the other GRK with high levels of cardiac expression, GRK5 (ref. 13), has not been as well defined. Genetic ablation of *GRK5* is not associated with a cardiac phenotype in mice[14], but massive cardiac overexpression of bovine GRK5 depresses cardiac βAR responsiveness[15,16]. Differences between GRK5 and GRK2 in subcellular localization, mechanism of activation and receptor specificity suggest that they may have nonredundant modulatory roles in the heart. Of particular interest is rapid up- and downregulation of GRK2 that correlates with ventricular function[17,18], implying that GRK2 may function predominantly in the acute regulation of βAR signaling. GRK5 expression seems to be less dynamic, suggesting that this GRK may be more important for chronic regulation[19,20]. As such, GRK5-mediated βAR desensitization might provide adaptive, beneficial effects during early ventricular decompensation, before frank cardiac failure.

To examine this notion, we searched for human genetic variants of GRK2 and GRK5 that might modify the risk or outcome of heart failure, or alter the response to heart failure therapy. We identified a

[1]Department of Internal Medicine, University of Cincinnati, 231 Albert Sabin Way, Cincinnati, Ohio 45267, USA. [2]Center for Pharmacogenomics, Washington University, 660 S. Euclid Avenue, St. Louis, Missouri 63110, USA. [3]Department of Epidemiology, School of Public Health, University of Michigan, 109 Observatory Road, Ann Arbor, Michigan 48109, USA. [4]Center for Translational Medicine, Thomas Jefferson University, 1025 Walnut Street, Philadelphia, Pennsylvania 19107, USA. [5]University of Missouri, 4401 Wornall Road, Kansas City, Missouri 64111, USA. [6]Current address: Department of Medicine, Cardiopulmonary Genomics Program, University of Maryland, 20 Penn Street, Baltimore, Maryland 21201, USA. [7]These authors contributed equally to this work. Correspondence should be addressed to G.W.D. II (gdorn@im.wustl.edu).

Received 28 December 2007; accepted 6 March 2008; published online 20 April 2008; doi:10.1038/nm1750

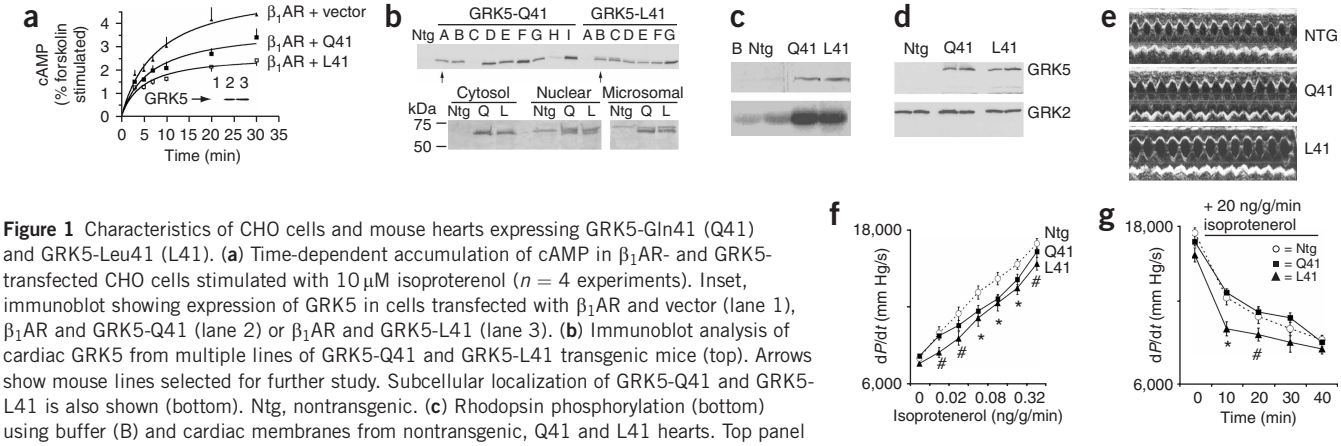

Figure 1 Characteristics of CHO cells and mouse hearts expressing GRK5-Gln41 (Q41) and GRK5-Leu41 (L41). (**a**) Time-dependent accumulation of cAMP in β_1AR- and GRK5-transfected CHO cells stimulated with 10 μM isoproterenol ($n = 4$ experiments). Inset, immunoblot showing expression of GRK5 in cells transfected with β_1AR and vector (lane 1), β_1AR and GRK5-Q41 (lane 2) or β_1AR and GRK5-L41 (lane 3). (**b**) Immunoblot analysis of cardiac GRK5 from multiple lines of GRK5-Q41 and GRK5-L41 transgenic mice (top). Arrows show mouse lines selected for further study. Subcellular localization of GRK5-Q41 and GRK5-L41 is also shown (bottom). Ntg, nontransgenic. (**c**) Rhodopsin phosphorylation (bottom) using buffer (B) and cardiac membranes from nontransgenic, Q41 and L41 hearts. Top panel shows an immunoblot for GRK5 from the same fractions. The blots shown are representative of three experiments. (**d**) Immunoblot analysis of GRK5 and GRK2 in pairs of mouse hearts. (**e**) Representative (of ten mice per group) M-mode echocardiograms from GRK5-Q41, GRK5-L41 and Ntg mice. (**f**) *In vivo* contractile dose response (d*P*/d*t*) to isoproterenol in GRK5-Q41, GRK5-L41 and Ntg mice ($n = 12$ mice per group). $^*P < 0.05$ for Q41 versus Ntg and L41 versus Ntg; $\#P < 0.05$ for Leu41 versus Ntg. (**g**) Mean data for time-dependent desensitization of d*P*/d*t* response to infused isoproterenol in closed-chest *in vivo* catheterization studies ($n = 12$ mice per group). $^*P < 0.05$ for L41 versus Ntg or Q41; $\#P = 0.081$ for L41 versus Ntg or Q41.

polymorphism in GRK5 that changes amino acid 41 in the noncatalytic regulatory domain from glutamine (the most common variant) to leucine. We characterized the effects of this polymorphism in transfected cells, transgenic mice and two independent cohorts of cardiac disease among African Americans, in whom the polymorphism is common. We show that the GRK5-Leu41 variant augments βAR desensitization and represents a form of genetic β-blockade that diminishes βAR signaling, confers resistance to experimental catecholamine-induced cardiomyopathy and protects against early death in African Americans with heart failure.

RESULTS

Genetic variability in *GRK5* but not *GRK2*

Of the seven GRK isoforms present in humans, GRK5 and GRK2 predominate in myocardium[10,12,13]. We searched for polymorphisms in the 16 exons coding for GRK5 and the 21 exons coding for GRK2 by re-sequencing 96 DNA samples (Human Variation Collection of the Coriell Institute, http://ccr.coriell.org/nigms/cells/humdiv.html) from individuals of diverse ethnicity (40 European Americans, 40 African Americans and 16 people of Chinese descent), providing a 98% probability of detecting a polymorphism with an allele frequency as low as 0.02. Four nonsynonymous polymorphisms were detected for *GRK5*, at cDNA nucleic acid positions 122 (A/T), 840 (G/A), 1274 (C/T) and 1624 (C/G), resulting in amino acid changes at residues 41 (glutamine to leucine; US National Center for Biotechnology Information (NCBI) Single Nucleotide Polymorphism (SNP) number rs17098707), 304 (arginine to histidine; rs12718341), 425 (threonine to methionine) and 542 (proline to alanine). The GRK5-Q41L variant was the only one with an allele frequency greater than 2% in any ethnic group and was therefore chosen for further study. In contrast to *GRK5*, we did not find any nonsynonymous polymorphisms in *GRK2*; in particular, we did not identify the D457V and K465M variants reported in dbSNP (NCBI SNP Cluster numbers rs1977983 and rs1977982). Furthermore, when we sequenced the DNA samples from the Whitehead Institute in which these two polymorphisms had been identified, we were unable to confirm their presence.

GRK5-Gln41 and GRK5-Leu41 differentially affect βAR

Amino acid 41 of GRK5 is adjacent to a lipid- and calmodulin-binding domain. To determine the effect of the glutamine to leucine substitution at this position on the ability of GRK5 to desensitize β_1AR, the effects of GRK5-Gln41 and GRK5-Leu41 on β_1AR desensitization were examined during continuous exposure to the βAR agonist isoproterenol. Chinese hamster ovary (CHO) cells were transfected with human β_1AR (the Arg389 variant, which is the most common) and either GRK5-Gln41 or GRK5-Leu41. Receptor expression (determined by radioligand binding) and GRK expression (determined by western blotting) were equivalent in experiments using GRK5-Gln41 and those using GRK5-Leu41 (**Fig. 1a** and data not shown). The rate and maximal level of cAMP accumulation over time in response to 10 μM isoproterenol in these cells is a measure of receptor coupling to $G\alpha_s$, the cAMP stimulatory G-protein, and to adenylyl cyclase, and is inversely related to receptor desensitization. GRK5-Leu41 cells had a different desensitization pattern from GRK-Gln41 cells ($P < 0.001$ by ANOVA): GRK5-Leu41 cells, as compared to GRK5-Gln41 cells, had a ∼25% lower rate of cAMP accumulation (time to half-maximal accumulation, 4.7 ± 1.2 min versus 6.4 ± 1.9 min, $P < 0.05$) and a ∼33% lower maximal response (2.6% ± 0.2% versus 3.8% ± 0.4% of the forskolin-stimulated response, $P < 0.05$) (**Fig. 1a**). Thus, the GRK5-Leu41 polymorphism decreases β_1AR signaling by enhancing agonist-promoted desensitization.

Pharmacogenomics of GRK5-Leu41 and β-blockade

Diminished isoproterenol-stimulated βAR signaling by GRK5-Leu41 resembles receptor antagonism by pharmacological β-blockers, suggesting that this polymorphism might interact with or mimic β-blockade in human cardiac syndromes for which β-blockers are standard therapy. Accordingly, we performed genotyping to determine whether individuals had the GRK5-Gln41 or GRK5-Leu41 variant in two independent case-control cohorts of cardiac disease subjects closely matched for ethnicity, sex and age. The first cohort consisted of 810 individuals (568 European Americans and 242 African Americans) from Cincinnati with New York Heart Association class II–IV heart failure, and the second cohort consisted of 822 individuals (580 European Americans and 242 African Americans) from Kansas City and Atlanta with acute cardiac ischemia. Clinical characteristics of the two study groups are presented in **Table 1**. An unaffected control group consisted of 513 subjects (406 European Americans and 107 African Americans) with negative histories and physical examinations for cardiac disease, normal electrocardiograms and normal

Table 1 Descriptive statistics of the case-control heart failure and acute coronary ischemia cohorts

	Heart failure		Acute coronary ischemia	
	European American $n = 568$	African American $n = 242$	European American $n = 580$	African American $n = 242$
Variable	Mean ± s.d.	Mean ± s.d.	Mean ± s.d.	Mean ± s.d.
Age at enrollment (years)	53 ± 13	51 ± 13	62 ± 12	55 ± 11
Males (%)	69.3	55.0	65.7	57.4
Follow up (years)	2.3 ± 2.2	2.3 ± 2.1	3.8 ± 1.1	3.3 ± 1.4
Height (cm)	172 ± 10	172 ± 10	171 ± 10	169 ± 10
Weight (kg)	86 ± 21	90 ± 26	86 ± 18	83 ± 21
Left ventricular EF (%)	28 ± 14	33 ± 15	48 ± 12	44 ± 16
Hypertension (%)	45.1	80.2	62.8	81.0
β-blocker use (%)	69.2	80.2	82.2	73.4

Diagnosis (%)

	Heart failure			Acute coronary ischemia	
	European American	African American		European American	African American
Nonischemic CHF	54.8	71.1	STEMI	30.9	15.7
Ischemic CHF	43.1	26.8	NSTEMI	33.1	54.1
Other	2.2	1.5	UA	36.0	30.2

EF, left ventricular ejection fraction; CHF, congestive heart failure; STEMI, ST segment elevation myocardial infarction; NSTEMI, non-ST segment elevation myocardial infarction; UA, unstable angina.

echocardiograms. Among European Americans, allele frequencies for the gene encoding GRK5-Leu41 were 0.013 (in unaffected individuals), 0.024 (heart failure) and 0.010 (acute ischemia), with no association between the presence of the GRK5-Leu41 variant and either type of cardiac disease. Among African Americans, allele frequencies for the gene encoding GRK5-Leu41 were approximately ten-fold higher than in European Americans, but again did not differ among unaffected individuals (0.23), individuals with heart failure (0.24) and individuals with acute ischemia (0.28). The heterozygous allele frequency for the gene encoding GRK5-Leu41 among African Americans was 0.35 and the homozygous frequency was 0.062, consistent with predictions from Hardy-Weinberg equilibrium ($P = 0.57$). Thus, the allele for the GRK5-Leu41 variant is rare in European Americans but common in African Americans and is not disproportionately represented in individuals with cardiac disease.

GRKs desensitize only ligand-occupied receptors, which is consistent with our ability to detect a functional difference between GRK5-Gln41 and GRK5-Leu41 only after catecholamine challenge (**Fig. 1**). Because catecholamine excess and chronic βAR stimulation occur after the onset of cardiac disease, it did not seem surprising that this GRK5 polymorphism does not alter the risk of developing either heart failure or ischemia. Like protection afforded by β-blockade, the clinical consequences of GRK5-mediated βAR desensitization may affect cardiac disease only after the accompanying catecholamine excess develops. We tested this notion by examining survival rates in human heart failure and acute coronary ischemia cohorts for an interaction between the presence of the GRK5-Leu41 variant and β-blocker usage.

Among African Americans with either heart failure or acute coronary ischemic syndromes, age- and gender-adjusted Cox proportional hazards modeling showed significant interactions between GRK5-Leu41 and β-blocker usage on the endpoint of death (likelihood ratio tests with $P = 0.036$ and $P = 0.023$ for individuals with heart failure and acute coronary ischemia, respectively). In European Americans, in whom the presence of even a single allele encoding GRK5-Leu41 is rare, there was no significant interaction in either the heart failure ($P = 0.46$) or the acute coronary ischemia ($P = 0.72$) cohorts. These results reveal a pharmacogenomic interaction between the GRK5-Leu41 variant and β-blocker therapy for heart failure and acute ischemic syndromes in African Americans only.

GRK5-Leu41 enhances *in vivo* cardiac β_1AR desensitization

As human gene association studies do not address molecular mechanisms and are subject to unknown epistatic effects from other genetic variation, we further characterized the effects of GRK5-Gln41 and GRK5-Leu41 in mice in which each variant was expressed only in cardiac myocytes. Because 30-fold overexpression of bovine GRK5 in transgenic mouse hearts substantially depresses basal and catecholamine-stimulated cardiac function[15,16], we created a large number of founder lines to identify mice expressing human GRK5-Gln41 or GRK5-Leu41 with low and comparable levels of expression and no differences in subcellular localization (**Fig. 1b**). The selected lines (Gln41A and Leu41B; **Fig. 1b**) each showed four- to six-fold increases in myocardial GRK activity as assessed by rhodopsin phosphorylation assay (**Fig. 1c**) and immunoblotting (**Fig. 1b–d**), with no compensatory regulation of GRK2 (**Fig. 1d**). Notably, no abnormalities of cardiac size, histological appearance, disease-related gene expression or basal contractile function (assessed by M-mode echocardiography) were detected in GRK5-Gln41 or GRK5-Leu41 mice up to 6 months of age (**Fig. 1e** and data not shown). Thus, the presence of transgenic GRK5 expression at these levels does not cause dysfunction, and there were no effects attributable to the Leu41 variant under baseline conditions.

To determine the effects of GRK5-Gln41 and GRK5-Leu41 expression on βAR stimulation of cardiac contractility, we catheterized mouse hearts to measure left ventricular peak positive dP/dt (the maximal rate of change of instantaneous pressure during systole) at baseline and in response to increasing doses of intravenous isoproterenol. Both GRK5-Gln41 and GRK5-Leu41 mice showed rightward shifts of the dose-response curves in comparison with nontransgenic

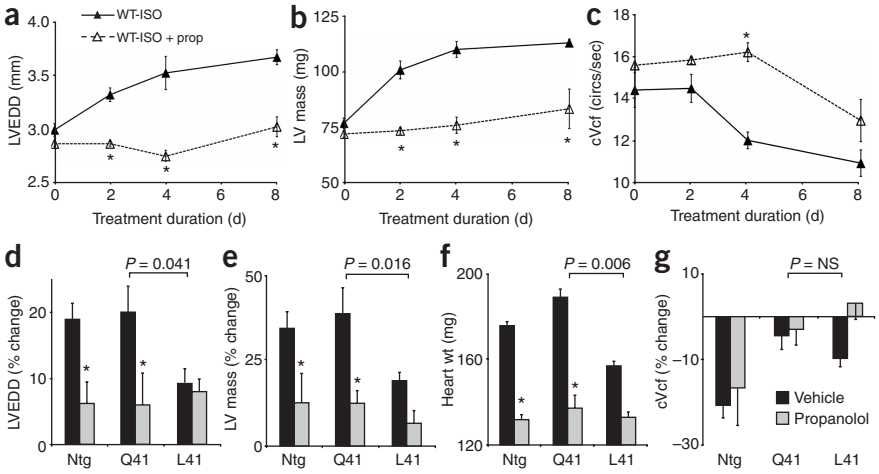

Figure 2 Cardiac expression of GRK5-L41, but not GRK5-Q41, confers resistance to catecholamine-induced cardiomyopathy. (**a**–**c**) Time course of the development of catecholamine cardiomyopathy in normal mice (WT-ISO) and the effect of β-blockade with propanolol (prop). LVEDD, left ventricular end diastolic dimension; LV mass, left ventricular mass; cVcf, velocity of circumferential shortening corrected for heart rate reported as circumferences/second (circs/sec) ($n = 6$ per group, $^*P < 0.05$ versus no propanolol). (**d**–**g**) Changes in cardiac parameters before and after 8 d of chronic isoproterenol treatment in vehicle-treated ($n = 12$ per group) and propanolol-treated ($n = 6$ per group) mice. Numerical P values compare GRK5-L41 and GRK5-Q41 responses. $^*P < 0.05$ for propranolol versus vehicle in each genotype. Responses in nontransgenic mice (Ntg) are shown for comparison. NS, not significant.

mice (**Fig. 1f**; half-maximal effective concentration (EC_{50}) was 0.135 ± 0.041 ng/g/min for GRK5-Gln41 transgenic mice and 0.086 ± 0.025 ng/g/min for GRK5-Leu41 transgenic mice, compared to 0.046 ± 0.011 ng/g/min for nontransgenic mice, $P = 0.02$, for either GRK5-Leu41 or GRK5-Gln41 transgenic mice versus nontransgenic mice), reproducing previous findings with transgenic expression of GRK2 or GRK5[10,15,16]. In contrast, desensitization of βAR-stimulated contraction, measured after 10 min of continuous high-dose isoproterenol infusion, was greater (that is, peak positive dP/dt response was lower) in GRK5-Leu41 mice than in either GRK5-Gln41 mice or nontransgenic control mice ($P < 0.001$ $n = 12$ per group; **Fig. 1g**). This trend continued at 20 min ($P = 0.081$), but after 30 min of continuous isoproterenol infusion, desensitization in all groups had reached similar levels ($P = 0.133$). These data show that GRK5-Leu41 is more effective than GRK5-Gln41 in desensitizing cardiac βAR under conditions of acute catecholamine excess.

GRK5-Leu41 protects against experimental heart failure

In experimental mouse models, overexpression of βARs[21] or their $G\alpha_s$ G protein signaling transducer[22] causes cardiac dilation and failure. Conversely, genetic ablation of the βAR and $G\alpha_s$ downstream effector, adenylyl cyclase, preserves myocardial function after physiological stress[23]. The finding that GRK5-Leu41 accelerates isoproterenol-promoted βAR desensitization suggested that, like β-blockers[22], it might protect hearts from the effects of persistent βAR stimulation, that is, cardiac dilation, ventricular hypertrophy and heart failure (**Fig. 2a–c**)[12]. To directly examine this possibility, we chronically administered isoproterenol to GRK5-Gln41 and GRK5-Leu41 transgenic mice and to nontransgenic mice using an implanted osmotic minipump. Whereas GRK5-Gln41 expression did not protect against isoproterenol-mediated increases in left ventricular chamber size (LVEDD; **Fig. 2d**) and left ventricular mass (**Fig. 2e**,**f**), GRK5-Leu41 expression at the same level was protective (**Fig. 2d–f**). Furthermore, whereas β-blockade ameliorated the effects of catecholamine cardiomyopathy on LVEDD and LV mass in nontransgenic and GRK5-Gln41 transgenic mice, in GRK5-Leu41 transgenic hearts, β-blockade had no additional protective effect (**Fig. 2d–f**). Both GRK5-Gln41 and GRK5-Leu41 blunted isoproterenol-mediated deterioration in cardiac contractility, whereas β-blockade had relatively little effect on this parameter (**Fig. 2g**). These results indicate that enhanced βAR desensitization by GRK5-Leu41 can ameliorate catecholamine cardiotoxicity, protecting against left ventricular remodeling and cardiomyopathy development similarly to pharmacological β-blockade.

GRK5-Leu41 prolongs survival in human heart failure

To further define the magnitude of the clinical benefit of the *GRK5* polymorphism, we undertook a prospective analysis of 375 African-American subjects with heart failure, comparing time to death or cardiac transplantation as a function of *GRK5* genotype and β-blocker treatment status. Characteristics of the prospective study group, classified by *GRK5* genotype and β-blocker use, are shown in **Table 2**.

Table 2 Descriptive statistics of African-American heart failure subjects in the prospective study

	Two alleles encoding GRK5-Q41		One or two alleles encoding GRK5-L41	
Variable	No β-blocker use ($n = 34$)	β-blocker use ($n = 182$)	No β-blocker use ($n = 27$)	β-blocker use ($n = 132$)
Fractional shortening (%)	26 ± 12	22 ± 11	24 ± 10	23 ± 12
LV ejection fraction (%)	34 ± 25	34 ± 14	31 ± 14	34 ± 14
Nonischemic cardiomyopathy (%)	76	66	69	71
Ischemic cardiomyopathy (%)	21	31	27	29
LV mass indexed to body weight (g)	160 ± 51	178 ± 58	180 ± 72	186 ± 69
Percentage predicted LV mass (%)	175 ± 55	178 ± 56	179 ± 55	198 ± 69
Hypertension (%)	85	80	67	81
Female (%)	56	43	48	46

LV, left ventricular.

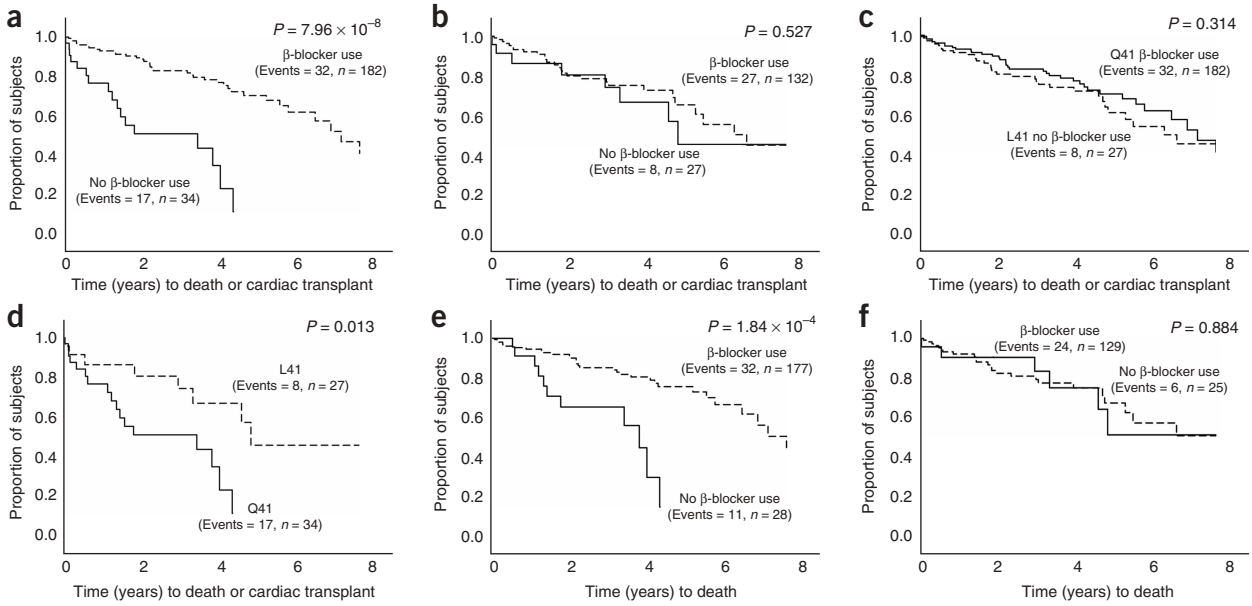

Figure 3 Prospective analysis of the interaction between *GRK5* polymorphism and β-blockade as a determinant of heart failure outcome in African Americans. Kaplan-Meier curves for time from diagnosis of heart failure to death or cardiac transplantation (**a**–**d**) or to death alone (**e**,**f**). (**a**) Comparison of GRK5-Q41 only subjects with and without β-blocker use. (**b**) Comparison of GRK5-L41 carrier subjects with and without β-blocker use. (**c**) Comparison of GRK5-Q41 only subjects treated with β-blockers to GRK5-L41 carrier subjects without β-blocker use. (**d**) Comparison of GRK5-Q41 only and GRK5-L41 carrier subjects, both without β-blocker use. (**e**) Comparison of GRK5-Q41 carrier subjects with and without β-blocker use for time to death only. (**f**) Comparison of GRK5-L41 carrier subjects with and without β-blocker use for time to death only.

Individuals homozygous for the more common GRK5-Gln41 who received β-blockers had longer transplantation-free survival times than people of the same genotype who were β-blocker naive (hazard ratio, 0.22; 95% confidence interval, 0.12–0.40; $P < 0.001$; **Fig. 3a**), results that are consistent with the well-established therapeutic benefits of β-blocker treatment in heart failure[4,24–26]. In contrast, there was no difference in outcome among GRK5-Leu41 carrier (homozygous or heterozygous) subjects with heart failure with or without β-blocker treatment (hazard ratio, 0.78, 95% confidence interval, 0.35–1.7; $P = 0.53$; **Fig. 3b**). To formally evaluate the effect of interaction between β-blocker use and GRK5-Leu41 on time to death or heart transplantation, we compared Cox proportional hazards models that included age, sex, β-blocker usage with and without an interaction term between β-blocker usage and *GRK5* carrier status. The model with the interaction term was significantly better than the reduced model (likelihood ratio test $P = 0.005$). The β-blocker–GRK5-Leu41 carrier status interaction term was significant at $P = 0.004$, indicating an interaction between *GRK5* genotype and β-blocker usage for the endpoint of death or transplantation. β-blocker treatment fully mimicked the survival advantage of GRK5-Leu41 (**Fig. 3c**). β-blocker–naive GRK5-Leu41 subjects had transplantation-free survival times significantly longer than β-blocker–naive Gln41 subjects ($P = 0.013$; **Fig. 3d**). Similar results were obtained when all-cause mortality alone was considered (**Fig. 3e**,**f**). When the β-blocker–naive homozygous GRK5-Gln41 group was set as the reference to derive age- and sex-adjusted hazard ratios for genotype and β-blocker treatment, β-blocker–naive GRK5-Leu41 carriers (individuals with either one or two *GRK5* alleles encoding GRK5-Leu41) were protected from the primary endpoint of combined occurrence of all-cause mortality and cardiac transplantation (**Table 3**) as well as from the secondary endpoint of all-cause mortality (data not shown). In β-blocker–naive subjects homozygous for the *GRK5* allele encoding GRK5-Leu41, the hazard ratio for the primary endpoint was 0.081 (95% confidence interval, 0.035–0.19), which, by comparison to the hazard ratio of 0.28 (95% confidence interval, 0.12–0.66) in heterozygous subjects, suggests a gene-dose response.

Although not a prospective study, a cohort analysis within the case-control study of individuals with acute coronary ischemia also showed an interaction between *GRK5* genotype and β-blocker use. The specific effect of genotype was most evident in the group receiving β-blockers, in which GRK5-Leu41 carriers showed an improvement in survival compared to GRK5-Gln41 only subjects (hazard ratio, 0.45; 95% confidence interval, 0.238–0.853; $P = 0.01$).

Given the reported associations between the β_1AR-Arg389 polymorphism and the response to β-blocker treatment in heart failure[27], and between β_1AR-Arg389 and a deletion polymorphism in the α_{2c}AR, $\alpha_{2c}AR_{\Delta 322-325}$, and the risk of having heart failure[28], we examined whether interactions among these functionally related polymorphisms and GRK5-Leu41 might provide a more predictive model. We observed no significant interaction affecting time to death or cardiac transplantation between GRK5-Leu41 and β_1AR-Arg389 ($P = 0.46$), between GRK5-Leu41 and $\alpha_{2c}AR_{\Delta 322-325}$ ($P = 0.21$) or among all three polymorphisms ($P = 0.87$).

Unrecognized differences in racial admixture within African-American study subjects could result in spurious genotype-phenotype associations. Therefore, we estimated the percentage of African ancestry of African-American individuals in the prospective study on the basis of genotyping 13 race-informative short tandem repeats[29,30]. No difference in racial admixture was found between the β-blocker–treated (African ancestry, 73.4%) and β-blocker–naive groups (African ancestry, 76.8%; $P = 0.22$). We then used the estimated African ancestry as an additional term to adjust the Cox proportional hazards model, which did not significantly change the hazard ratios and *P* values (**Table 3**).

The clinical applicability of an association between a genetic variant and outcome depends upon its reproducibility and predictive accuracy. Internal reproducibility of the β-blocker–GRK5 interaction

Table 3 Cox proportional hazards of GRK5 variation and β-blocker use for the combined occurrence of all-cause mortality or transplantation[a]

Group			Hazard ratio	95% CI	*P* value
GRK5 variant[b]	β-blocker use	*n* (number of events)			
Without adjustment for percentage of African ancestry[c]					
Gln41	–	34 (17)	1.0 (reference)	–	–
Gln41	+	182 (37)	0.19	0.10–0.34	<0.001
Leu41	–	27 (8)	0.28	0.12–0.66	0.004
Leu41	+	132 (27)	0.20	0.05–0.80	0.02
With adjustment for percentage of African ancestry[d]					
Gln41	–	34 (17)	1.0 (reference)	–	–
Gln41	+	182 (37)	0.19	0.10–0.37	<0.001
Leu41	–	27 (8)	0.31	0.13–0.73	0.007
Leu41	+	132 (27)	0.20	0.05–0.84	0.03

[a]Adjusted for age at heart failure diagnosis and sex ($n = 375$). [b]Leu41 represents heterozygotes; Results section contains data for the effects of Leu41 homozygotes. [c]Likelihood ratio test *P* value for the interaction of GRK5-Gln41 status and β-blocker use = 0.005. [d]Likelihood ratio test *P* value for the interaction of GRK5-Gln41 status and β-blocker use = 0.01. CI, confidence interval.

in our prospective African-American heart failure cohort was assessed by a sequential analysis procedure[31]. Fifty randomly chosen subjects were used as the training set, and the remaining test sample had a *P* value of 0.01 in 86 of 100 instances, which is considered a very high degree of internal reproducibility. The predictive utility of the β-blocker–GRK5 interaction, as assessed by leave-one-out cross-validation, showed that the relative risk ratios were significant predictors of transplantation-free survival ($P < 0.001$), with an R^2 value of 0.039 (**Supplementary Table 1** online). The GRK5-Leu41 variant compares favorably to standard clinical risk factors for heart failure outcome (combined age, sex and hypertension), which had an R^2 value of 0.023. The overall R^2 value increased to 0.05 when clinical predictors were included in the β-blocker–GRK5 variant interaction model.

DISCUSSION

Several genetic polymorphisms affecting proteins in the βAR signaling pathway have been proposed as modifiers of heart failure risk[7,8,27,32]. Because of the morbidity, mortality and healthcare costs of heart failure[33–36], efforts are underway to identify additional genetic markers that will indicate prognosis and guide management of patient care. Here we examined the genes encoding GRK5 and GRK2 because these proteins constitute a crucial regulatory node for the pathophysiologically important cardiac βAR signaling pathway that has not been previously explored for genotype-phenotype interactions in human heart disease. These candidate genes are of particular interest because the role of their protein products in signaling is to modify receptor coupling to G proteins and downstream adenylyl cyclase, key effectors of βAR function that are perturbed in heart failure. Furthermore, GRK5 and GRK2 have the potential to modify signaling through both the β_1- and β_2AR subtypes, as well as other important receptors involved in heart failure[16,35]. Compared to the highly polymorphic receptors they regulate, we found these two human GRKs to be relatively invariant, in that screening the complete coding sequences of both genes in 192 chromosomes identified only one common nonsynonymous polymorphism. There is also striking cross-species similarity in the amino-acid sequence of GRK5 among mammals, with 96% identity between human and mouse GRK5 and absolute conservation of glutamine at position 41 in organisms ranging from humans to zebrafish (**Supplementary Fig. 1** online). The rarity of polymorphic variations in human GRK2 and GRK5 and the high degree of sequence conservation among species for GRK5 suggest that minor sequence changes can have considerable functional and physiological consequences, as we found for GRK5-Leu41.

A major function of GRKs is the uncoupling of ligand-occupied receptors from signaling effectors, resulting in decreased cellular responsiveness (desensitization) and a time-dependent loss of agonist-promoted function at the organ level[9]. In human heart failure, increases in myocardial GRK2 activity[17,18] and marked desensitization of cardiac β_1- and β_2ARs[36] represent endogenous processes that may protect the heart from high circulating catecholamine levels[37,38]. Here we found a gain-of-function genetic polymorphism in *GRK5* that augments βAR desensitization. As expected for a kinase that specifically modulates the agonist-occupied form of G protein–coupled receptors, the GRK5-Gln41 and GRK5-Leu41 variants could not be functionally distinguished in the absence of agonist. Indeed, within the African-American population, in which the allele coding for the GRK5-Leu41 variant is fairly common (~40% of the African-American individuals we studied carry at least one allele), it did not alter the risk of developing heart failure. However, GRK5-Leu41 was markedly more effective than GRK5-Gln41 in promoting isoproterenol-mediated β-receptor desensitization in transfected cells and transgenic mice, showed a β-blocker–like protective effect in the context of chronic catecholamine excess in transgenic mice, and was associated with prolonged survival in clinical heart failure. In the transgenic mice, expression was targeted specifically to cardiac myocytes, indicating a major role for this polymorphism in direct cardioprotection. Taken together, these data indicate that GRK5-Leu41 acts to attenuate βAR signaling in a manner similar to partial βAR antagonism with β-blockers, favoring protection against remodeling and improving survival.

Mechanistic experiments in transfected cells and transgenic mice were an important feature of this work, as they revealed experimental phenotypes that aided in designing the human studies and showed that the *GRK5* polymorphism (rather than an unknown locus in linkage disequilibrium with *GRK5*) is the basis for the observed effects. Delineation of pharmacogenetic risk modifiers may help in the interpretation of clinical studies. Clinical trials of β-blockers in heart failure have generally shown disappointing results in African-American populations compared to European-American subjects[39] but have not considered the unique genetic characteristics of these different populations. Because ~40% of African Americans carry an allele encoding GRK5-Leu41, and these individuals are genetically

protected from adverse outcomes once they develop heart failure, African Americans analyzed as a group would therefore paradoxically seem to have less response to β-blocker therapy compared to European Americans in whom the polymorphism is rare. Our findings should encourage the use of genetic profiling in clinical trials to assure proper identification of subpopulations who can benefit from individualized therapy tailored to personal genetic makeup.

METHODS

Study subjects. Human study protocols were approved by the Institutional Review Boards of the University of Cincinnati and Washington University. Subjects provided written informed consent. Enrollment criteria for the heart failure case-control study were an age of 18–80 years, a left ventricular ejection fraction of less than 40% and class II–IV heart failure (New York Heart Association). Enrollment criteria for the acute coronary ischemia case-control study were an age of 18–80 years and hospital admission with the confirmed diagnosis of acute myocardial infarction or unstable coronary syndrome. Unaffected controls were recruited from the greater Cincinnati area. Classification as European American or African American was self-reported.

In the prospective GRK5–β-blocker interaction study, 402 African-American subjects with heart failure gave informed consent between May 1, 2000 and June 1, 2006, 242 of whom were also in the observational study. Of these, 383 subjects had their blood drawn to obtain DNA. *GRK5* genotypes were not obtained from five individuals (success rate of 98.7%), and three individuals (0.8%) were lost at follow-up. These three individuals were homozygous for the gene encoding GRK5-Gln41. The primary clinical endpoint was the combined occurrence of all-cause mortality and cardiac transplantation; secondary endpoints were the occurrence of all-cause mortality or the occurrence of cardiac transplantation. The average follow-up period was 30 months. β-blocker use was defined as continuous therapy for at least 6 months. 73% of the individuals treated with β-blockers received carvedilol, 22% received metoprolol and 5% received other β-blockers. Decisions regarding β-blocker treatment were made by the subjects' physicians.

Sequencing and genotyping. We used PCR to amplify the exons encoding human GRK2 (Entrez Gene number 156) and human GRK5 (Entrez Gene number 2869) from genomic DNA (PCR primers for GRK5-coding exons are in **Supplementary Table 2** online). Polymorphism discovery and *GRK5* genotyping were achieved by bidirectional automated sequencing and outputs were aligned with the reference sequence using SeqScape v2.5. Variants were individually verified by an investigator (R.R.P.). African-American subjects with heart failure were further genotyped at 13 race-informative short tandem-repeat loci[29].

***In vitro* βAR desensitization studies.** We transfected CHO cells with cDNAs encoding human β_1AR and either GRK5-Gln41, GRK5-Leu41 or empty vector. We treated cell monolayers with isoproterenol for the indicated times at 37 °C and quantified cAMP as previously described[40].

Experimental heart failure. We generated transgenic mice (FVB/N background) by using the α-myosin heavy chain promoter to express human GRK5-Gln41 and GRK5-Leu41 using methods similar to those previously described[41]. We identified multiple founders by genomic Southern analysis of tail clip DNA. We killed F_1 or F_2 mice and analyzed myocardial GRK content by immunoblotting with GRK2- or GRK5-specific antisera (Santa Cruz). Subcellular GRK5 localization was assessed in the 800*g* nuclear pellet, the 100,000*g* cytosolic supernatant and the 100,000*g* microsomal pellet after differential centrifugation. Rhodopsin phosphorylation was measured as described[42]. Mouse lines with equivalent cardiac GRK5-Gln41 and GRK5-Leu41 protein expression were propagated for study. We treated mice in accordance with approved University of Cincinnati Animal Care and Use Committee protocols.

To assess βAR responsiveness and desensitization in transgenic mice, we catheterized the left ventricle[41] during graded infusions (3 min/dose) of the nonselective β-agonist isoproterenol (0.01–0.32 ng/g/min) followed by a sustained 30-min infusion of isoproterenol of 20 ng/g/min to evoke desensitization[43]. We induced heart failure by chronic isoproterenol infusion by osmotic minipump[12]. We assessed cardiac remodeling by transthoracic echocardiography.

Statistical analysis. We used Student's *t*-tests and chi-square tests to assess significant differences in variables between ethnic groups and between genotype classes within ethnic groups. We assessed Hardy-Weinberg equilibrium in each ethnic group separately. The primary clinical endpoint for the prospective study was the combined occurrence of all-cause mortality and cardiac transplantation; secondary endpoints were the occurrence of all-cause mortality or the occurrence of cardiac transplantation. We assessed differences in time from diagnosis to endpoint with Kaplan-Meier curves and log-rank tests[44]. We obtained hazards ratios by Cox proportional hazards modeling[45] using an additive genetic model[46] after adjustment for age at diagnosis, β-blocker usage, hypertension status and sex. To assess internal reproducibility of the association between *GRK5* allele and survival, we used an analytical strategy that lowers the probability of type 1 errors through sequential hypothesis testing[31]. We used the smallest possible sample sizes to reject the null hypothesis of no GRK5–β-blocker interaction, and the remaining samples were used to confirm those findings[47]. The type 1 error (α), type 2 error (β) and effect size (D) were preset to $\alpha = 0.05$, $\beta = 0.8$ and $D = 0.5$ to create stopping rules for the procedure, where the sample size, n, is considered a random variable. Starting with $n = 50$, we tested the null hypothesis of no GRK5–β-blocker interaction with the Wald test and then sequentially added another individual and retested.

To assess the predictive value of Cox proportional hazards models, we used leave-one-out cross-validation. Each individual was sequentially left out and a Cox proportional hazards model for time to death or transplant was fitted. Using the coefficients estimated with the n–1 individuals, an overall relative risk was calculated for the individual left out. These relative risks were then used as the predictor in a new Cox proportional hazards model. Because each individual is omitted from the model used to calculate his or her relative risk, the performance of a model using these relative risks as predictors approximates the predictive ability of the association in an independent sample drawn from the same population. We assessed the performance of each model using a measure of its overall statistical significance given by Cox and Snell R^2, a measure of the model's predictive ability[48]. We used two-tailed tests and an α level of 0.05 to assess significance. We estimated percentage African ancestry with the program Structure[30].

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

Supported by US National Heart, Lung, and Blood Institute Special Clinical Centers of Research in Heart Failure grants P50 HL77101 and HL77113 and by R01 HL87871.

AUTHOR CONTRIBUTIONS

S.B.L., adenylyl cyclase studies and manuscript preparation; S.C., ischemic cohort genotyping; R.J.K., statistical analysis and manuscript preparation; F.M.S., clinical heart failure studies; S.J.M., transgenic mouse studies; H.S.H., clinical heart failure and transgenic mouse studies; A.D., clinical heart failure and transgenic mouse studies; J.S.M., rhodopsin kinase studies; L.S., heart failure cohort genotyping; R.R.P., polymorphism discovery and heart failure cohort genotyping; J.A.S., clinical ischemia studies; W.J.K., supervised studies; S.L.R.K., supervised statistical analyses; G.W.D. II, conceived and directed polymorphism discovery, heart failure genomics and transgenic mouse studies and prepared manuscript.

Published online at http://www.nature.com/naturemedicine
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Levy, D. *et al.* Long-term trends in the incidence of and survival with heart failure. *N. Engl. J. Med.* **347**, 1397–1402 (2002).
2. Roger, V.L. *et al.* Trends in heart failure incidence and survival in a community-based population. *J. Am. Med. Assoc.* **292**, 344–350 (2004).
3. Franz, W.M., Muller, O.J. & Katus, H.A. Cardiomyopathies: from genetics to the prospect of treatment. *Lancet* **358**, 1627–1637 (2001).
4. The Merit HF Investigators. Effect of metoprolol CR/XL in chronic heart failure: Metoprolol CR/XL Randomised Intervention Trial in Congestive Heart Failure (MERIT-HF). *Lancet* **353**, 2001–2007 (1999).
5. The β-Blocker Evaluation of Survival Trial Investigators. A trial of the β-blocker bucindolol in patients with advanced chronic heart failure. *N. Engl. J. Med.* **344**, 1659–1667 (2001).

6. van Campen, L.C., Visser, F.C. & Visser, C.A. Ejection fraction improvement by β-blocker treatment in patients with heart failure: an analysis of studies published in the literature. *J. Cardiovasc. Pharmacol.* **32** (suppl. 1), S31–S35 (1998).
7. Wagoner, L.E. *et al.* Polymorphisms of the β_2-adrenergic receptor determine exercise capacity in patients with heart failure. *Circ. Res.* **86**, 834–840 (2000).
8. Liggett, S.B. Pharmacogenetic applications of the Human Genome project. *Nat. Med.* **7**, 281–283 (2001).
9. Kohout, T.A. & Lefkowitz, R.J. Regulation of G protein–coupled receptor kinases and arrestins during receptor desensitization. *Mol. Pharmacol.* **63**, 9–18 (2003).
10. Koch, W.J. *et al.* Cardiac function in mice overexpressing the β-adrenergic receptor kinase or a β ARK inhibitor. *Science* **268**, 1350–1353 (1995).
11. Koch, W.J. Genetic and phenotypic targeting of β-adrenergic signaling in heart failure. *Mol. Cell. Biochem.* **263**, 5–9 (2004).
12. Matkovich, S.J. *et al.* Cardiac-specific ablation of G protein–receptor kinase 2 redefines its roles in heart development and β-adrenergic signaling. *Circ. Res.* **99**, 996–1003 (2006).
13. Premont, R.T., Koch, W.J., Inglese, J. & Lefkowitz, R.J. Identification, purification, and characterization of GRK5, a member of the family of G protein–coupled receptor kinases. *J. Biol. Chem.* **269**, 6832–6841 (1994).
14. Gainetdinov, R.R. *et al.* Muscarinic supersensitivity and impaired receptor desensitization in G protein–coupled receptor kinase 5–deficient mice. *Neuron* **24**, 1029–1036 (1999).
15. Chen, E.P., Bittner, H.B., Akhter, S.A., Koch, W.J. & Davis, R.D. Myocardial function in hearts with transgenic overexpression of the G protein–coupled receptor kinase 5. *Ann. Thorac. Surg.* **71**, 1320–1324 (2001).
16. Rockman, H.A. *et al.* Receptor-specific *in vivo* desensitization by the G protein–coupled receptor kinase-5 in transgenic mice. *Proc. Natl. Acad. Sci. USA* **93**, 9954–9959 (1996).
17. Ungerer, M., Bohm, M., Elce, J.S., Erdmann, E. & Lohse, M.J. Altered expression of β-adrenergic receptor kinase and β_1-adrenergic receptors in the failing human heart. *Circulation* **87**, 454–463 (1993).
18. Ungerer, M. *et al.* Expression of β-arrestins and β-adrenergic receptor kinases in the failing human heart. *Circ. Res.* **74**, 206–213 (1994).
19. Oyama, N. *et al.* Angiotensin converting enzyme inhibitors attenuated the expression of G protein–coupled receptor kinases in heart failure patients. *Circ. J.* **70**, 362–363 (2006).
20. Dzimiri, N., Basco, C., Moorji, A., Afrane, B. & Al Halees, Z. Characterization of lymphocyte β_2-adrenoceptor signalling in patients with left ventricular volume overload disease. *Clin. Exp. Pharmacol. Physiol.* **29**, 181–188 (2002).
21. Liggett, S.B. *et al.* Early and delayed consequences of β_2-adrenergic receptor overexpression in mouse hearts: critical role for expression level. *Circulation* **101**, 1707–1714 (2000).
22. Asai, K. *et al.* β-adrenergic receptor blockade arrests myocyte damage and preserves cardiac function in the transgenic $G_s\alpha$ mouse. *J. Clin. Invest.* **104**, 551–558 (1999).
23. Okumura, S. *et al.* Disruption of type 5 adenylyl cyclase gene preserves cardiac function against pressure overload. *Proc. Natl. Acad. Sci. USA* **100**, 9986–9990 (2003).
24. Bristow, M.R. β-adrenergic receptor blockade in chronic heart failure. *Circulation* **101**, 558–569 (2000).
25. Packer, M. Current role of β-adrenergic blockers in the management of chronic heart failure. *Am. J. Med.* **110** (suppl. 7A), 81S–94S (2001).
26. Waagstein, F. *et al.* Beneficial effects of metoprolol in idiopathic dilated cardiomyopathy. Metoprolol in Dilated Cardiomyopathy (MDC) Trial Study Group. *Lancet* **342**, 1441–1446 (1993).
27. Liggett, S.B. *et al.* A polymorphism within a conserved β_1-adrenergic receptor motif alters cardiac function and β-blocker response in human heart failure. *Proc. Natl. Acad. Sci. USA* **103**, 11288–11293 (2006).
28. Small, K.M., Wagoner, L.E., Levin, A.M., Kardia, S.L. & Liggett, S.B. Synergistic polymorphisms of β_1- and α_{2C}-adrenergic receptors and the risk of congestive heart failure. *N. Engl. J. Med.* **347**, 1135–1142 (2002).
29. Barnholtz-Sloan, J.S., Chakraborty, R., Sellers, T.A. & Schwartz, A.G. Examining population stratification via individual ancestry estimates versus self-reported race. *Cancer Epidemiol. Biomarkers Prev.* **14**, 1545–1551 (2005).
30. Pritchard, J.K., Stephens, M. & Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959 (2000).
31. Bechhoffer, R.E., Kiefer, J. & Sobel, M. *Sequential Identification and Ranking Procedures* (University of Chicago Press, Chicago, 1986).
32. Liggett, S.B. β-adrenergic receptors in the failing heart: the good, the bad, and the unknown. *J. Clin. Invest.* **107**, 947–948 (2001).
33. Lloyd-Jones, D.M. *et al.* Lifetime risk for developing congestive heart failure: the Framingham Heart Study. *Circulation* **106**, 3068–3072 (2002).
34. Thom, T. *et al.* Heart disease and stroke statistics—2006 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* **113**, e85–e151 (2006).
35. Kim, J. *et al.* Functional antagonism of different G protein–coupled receptor kinases for β-arrestin–mediated angiotensin II receptor signaling. *Proc. Natl. Acad. Sci. USA* **102**, 1442–1447 (2005).
36. Port, J.D. & Bristow, M.R. Altered β-adrenergic receptor gene regulation and signaling in chronic heart failure. *J. Mol. Cell. Cardiol.* **33**, 887–905 (2001).
37. Cohn, J.N. *et al.* Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. *N. Engl. J. Med.* **311**, 819–823 (1984).
38. Bristow, M.R. Why does the myocardium fail? Insights from basic science. *Lancet* **352** (suppl. 1), SI8–SI14 (1998).
39. Shekelle, P.G. *et al.* Efficacy of angiotensin-converting enzyme inhibitors and β-blockers in the management of left ventricular systolic dysfunction according to race, gender, and diabetic status: a meta-analysis of major clinical trials. *J. Am. Coll. Cardiol.* **41**, 1529–1538 (2003).
40. Liggett, S.B. *et al.* Altered patterns of agonist-stimulated cAMP accumulation in cells expressing mutant β_2-adrenergic receptors lacking phosphorylation sites. *Mol. Pharmacol.* **36**, 641–646 (1989).
41. D'Angelo, D.D. *et al.* Transgenic Galphaq overexpression induces cardiac contractile failure in mice. *Proc. Natl. Acad. Sci. USA* **94**, 8121–8126 (1997).
42. Koch, W.J., Inglese, J., Stone, W.C. & Lefkowitz, R.J. The binding site for the $\beta\gamma$ subunits of heterotrimeric G proteins on th eβ-adrenergic receptor kinase. *J. Biol. Chem.* **268**, 8256–60 (1993).
43. Odley, A. *et al.* Regulation of cardiac contractility by Rab4-modulated β_2-adrenergic receptor recycling. *Proc. Natl. Acad. Sci. USA* **101**, 7082–7087 (2004).
44. Kaplan, E.L. & Meier, P. Nonparametric estimation from incomplete observations. *J. Am. Stat. Assoc.* **53**, 457–481 (1958).
45. Parmar, M. & Machin, D. *Survival Analysis: A Practical Approach* 115–142 (Wiley, New York, 1995).
46. Lynch, M. & Walsh, B. *Genetics and Analysis of Quantitative Traits* 51–80 (Sinauer Associates, Sunderland, Massachusetts, 1998).
47. Province, M.A. A single, sequential, genome-wide test to identify simultaneously all promising areas in a linkage scan. *Genet. Epidemiol.* **19**, 301–322 (2000).
48. Cox, D.R. & Snell, E.J. *Analysis of Binary Data* 208–209 (Chapman & Hall, London, 1989).