A Novel Genetic Pathway for Sudden Cardiac Death via Defects in the Transition between Ventricular and Conduction System Cell Lineages

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Summary

HF-1b, an SP1-related transcription factor, is preferentially expressed in the cardiac conduction system and ventricular myocytes in the heart. Mice deficient for HF-1b survive to term and exhibit normal cardiac structure and function but display sudden cardiac death and a complete penetrance of conduction system defects, including spontaneous ventricular tachycardia and a high incidence of AV block. Continuous electrocardiographic recordings clearly documented cardiac arrhythmogenesis as the cause of death. Single-cell analysis revealed an anatomic substrate for arrhythmogenesis, including a decrease and mislocalization of connexins and a marked increase in action potential heterogeneity. Two independent markers reveal defects in the formation of ventricular Purkinje fibers. These studies identify a novel genetic pathway for sudden cardiac death via defects in the transition between ventricular and conduction system cell lineages.

Introduction

Sudden cardiac death is a leading cause of cardiovascular mortality, accounting for the loss of ~400,000 lives in the United States each year (Zipes and Wellens, 1998). In most cases, the underlying cause of death is the rapid onset of lethal ventricular arrhythmias in the setting of acquired heart disease. The onset of sudden cardiac death is multifactorial, and the elucidation of the precise molecular and cellular pathways responsible for this phenotype has been difficult due to the paucity of genetic insights into this complex human cardiac disease. The discovery of mutations in specific cardiac voltage-gated K⁺ channels in patients with long QT syndrome and their role in slowing cardiac repolarization has provided a potential mechanistic link with some of these acquired arrhythmias (for a review see Curran et al., 1999). However, studies of affected families have revealed that only 20% of the patients who harbor the disease genotype display sudden death (Rodent and Spooner, 1999), suggesting the requirement of additional pathways that must be present to produce the disease phenotype. Further evidence of the requirement of a “second hit” to produce lethal ventricular arrhythmogenesis has come from studies of genetically engineered mice that harbor mutations in the cardiac Na⁺ or K⁺ channels that produce long QT syndrome, prolonged cardiac repolarization, and associated arrhythmias, but not sudden cardiac death (Barry et al., 1998; Charpentier et al., 1998; Drici et al., 1998; London et al., 1998; Abbott et al., 1999; Kupershmidt et al., 1999). Currently, there are no accepted clinical criteria to unequivocally identify high-risk patients, and existing therapy for survivors of sudden cardiac death is palliative, primarily based upon implantable defibrillators. The development of biologically targeted therapy for this disease will first require the identification of new mechanistic pathways that can lead to sudden cardiac death in experimental systems. Presently, there are few reliable experimental systems in which spontaneous cardiac sudden death can be studied. While the mouse has been valuable as a tool to reveal new signaling pathways that drive complex features of dilated cardiomyopathy and heart failure (Coral-Vazquez et al., 1998; Chien, 1999; Hirota et al., 1999; Minamisawa et al., 1999), there have been no previous reports of genetically engineered mouse models of primary sudden cardiac death.

The establishment of organized, rapid cardiac conduction is dependent on the recruitment of cardiomyocytes into conduction system lineages (Gourdie et al., 1998). Synchronous generation of action potentials in distinct conduction system cell lineages is essential for orderly impulse propagation. A critical feature of ventricular arrhythmogenesis in cardiomyopathy and associated heart failure is the heterogeneity of action potential duration (APD), a significant clinical risk factor for cardiac sudden death (for a review see Marban, 1999). The mechanistic basis for this action potential heterogeneity is not understood, but conceptually, defects in the recruitment of conduction system cell lineages might be a contributing factor. In this manner, genes that guide and/or maintain the electrophysiological transition between ventricular and conduction cell lineages might play a critical primary role in the onset of lethal arrhythmias and sudden cardiac death, even in the absence of...
Figure 1. Gene Targeting Strategy of HF-1b Locus

(A) Diagram of the HF-1b endogenous locus, the targeting construct, and the resulting targeted HF-1b locus. Exons are denoted as solid black rectangles. Restriction sites are as follows: X = XhoI, H = HpaI, Xb = XbaI, N = NotI, and S = SalI.

(B) Representative Southern blot of genomic DNA from 13 ES clones; clones 1 and 12 show the mutant allele as a result of homologous recombination.

(C) Northern blot analysis of RNAs from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) HF-1b mice.

(D) Detection of wild-type (+/+) and mutant alleles by PCR analysis of genomic DNA from tail biopsies of progeny from a heterozygous cross. The genotypes and their corresponding PCR products are as follows: HF-1b−/− 5 250 bp; HF-1b+/− 5 354 bp and 250 bp; HF-1b+/+ 5 354 bp.

Results

Generation of HF-1b Null Allele Mice

HF-1b is a cardiac transcription factor related to the SP-1 gene family (Zhu et al., 1993). An earlier gene targeting study of HF-1b (or Sp4), where only the DNA binding domains were removed, revealed that HF-1b/Sp4 is essential for normal murine growth (Supp et al., 1996). Since this gene targeting strategy could potentially result in a hypomorphic and/or dominant-negative allele versus a true null allele, we introduced a lacZ reporter gene into the endogenous locus of HF-1b that eliminated the start codon (Figure 1A). Two targeted embryonic stem cell clones were used to establish mouse lines carrying the conoventricular boundary and in the interventricular septum with little or no detectable expression in the atrial chamber (Figure 2A). This predominantly ventricular restricted pattern is maintained from E13.5 pc (Figure 2B) up to E17.5 pc, when a gradient is established that reveals a higher level of HF-1b expression in the left ventricle.

Preferential Expression of HF-1b in Ventricular and Cardiac Conduction System Cell Lineages in the Developing Heart

During early embryogenesis, HF-1b is expressed predominantly in the developing neuroepithelium and the central nervous system with a uniformly low level of expression in the ventricular segment of the developing heart tube at E8.5 postcoitum (pc) (data not shown). By day 12.5 pc, HF-1b cardiac expression is restricted to the conotruncus, specifically at the lateral limits of the conoventricular boundary and in the interventricular septum with little or no detectable expression in the atrial chamber (Figure 2A). This predominantly ventricular restricted pattern is maintained from E13.5 pc (Figure 2B) up to E17.5 pc, when a gradient is established that reveals a higher level of HF-1b expression in the left ventricle.
A Novel Genetic Pathway for Sudden Cardiac Death

**Figure 2. Developmental Pattern of HF-1b Expression in Embryonic Mouse Heart as Determined by lacZ Staining**

(A) Heart from day 12.5 pc HF-1b embryo. (B) Hearts from day 13.5 pc wild-type, heterozygous, and homozygous HF-1b embryos. Transverse (C) and sagittal (D) sections from two different day 17.5 pc HF-1b^{+/-} embryos. (E and F) Serial transverse sections from an adult HF-1b^{+/-} mouse. lacZ staining in putative Purkinje cells (C, arrows), and in AV ring, His bundle, and bundle branches of the central conduction system (D, arrows). Asterisk in (D) indicates localized lacZ staining in the right atrial appendage.

versus the right ventricle (Figures 2C and 2D). The highest level of lacZ staining coincides with the developing conduction system. lacZ-positive cells were observed on the most anterior aspect of the interventricular septum and in a subpopulation of subendocardial cells extending down either side of the septum, particularly on the right ventricular side reminiscent of the branching bundles and Purkinje fibers of the ventricular conduction system (Figures 2C and 2D, arrows). lacZ-positive cells were also localized to a very discrete region in the right atrium (Figure 2D, asterisk). In the adult heart, the clusters of supravalvular cardiomyocytes with the most intensely blue lacZ staining coincided with the primary atrioventricular ring (Figure 2E, arrowhead) and were in greater abundance at the apex (Figure 2F) when compared to the base (Figure 2E) of the heart. Thus, HF-1b appears to be preferentially expressed in the ventricular muscle and in components of the cardiac conduction system.

**HF-1b-Deficient Mice Are Highly Susceptible to Inducible Ventricular Arrhythmias**

Even though the HF-1b deficiency did not result in embryonic lethality, the HF-1b-deficient animals displayed an increased incidence of postnatal mortality (by 6–8 months of age, 59%, or 20 out of 34 HF-1b^{-/-} mice died versus 0% for both heterozygous and wild type, i.e., 0/67 and 0/40 for +/- and +/+, respectively, in data compiled from 16 independent litters). The manner in which these homozygous mutants died was characterized as “sudden death,” since the animals were unexpectedly found dead within 24 hr of displaying relatively normal behavior and activity levels. Within one week of birth, the incidence of perinatal death for the HF-1b^{+/-} mice amounted to 11% (or 4/34). By one month, an additional 32% (or 11/34) of HF-1b^{+/-} mice were found dead. Of the mutant animals that survived past one month of age, 15% (or 5/34) also died unexpectedly around the age of 6–8 months despite having normal appearance and behavior the previous day.

HF-1b-Deficient Mice Display a Sudden Death Phenotype

A detailed analysis of cardiac electrophysiological function was undertaken in the surviving HF-1b mutant mice (Figure 3). Heart rate (HR, bpm) and PR interval (ms) were not significantly different in control (n = 10) and mutant (n = 13) mice, respectively (HR: control = 461 ± 51 versus +/- = 400 ± 39; PR: control = 36 ± 5 versus +/- = 38 ± 4). To gauge the relative inducibility of life-threatening ventricular arrhythmias, we developed an electrical stimulation protocol with a miniaturized ventricular plaque electrode designed to induce ectopic or reentrant rhythms. This approach involved performing...
Figure 3. Inducible and Spontaneous Ventricular Arrhythmias in HF-1b Mutant Mice
(A) Representative ECG of inducible ventricular tachycardia in HF-1b mutant mouse. Simultaneous ECC recordings (surface lead I and two epicardial leads, Epi1 and Epi2) for a HF-1b mutant that experienced ventricular tachycardia resulting from programmed ventricular stimulation with a single premature stimulus (S1).
(B) Summary of ECG data from HF-1b and wild-type mutant and mice obtained with implantable radio telemetry.

Continuous Telemetric Monitoring Reveals Spontaneous Sudden Cardiac Death Due to Widespread Conduction Defects and Ventricular Arrhythmogenesis in HF-1b−/− Mice

To determine if life-threatening arrhythmias contributed to the sudden death of the HF-1b mutant mice, implantable radio telemetry was used to continuously monitor electrocardiographical data from both wild-type and mutant mice (Figure 3B). A microradio transmitter was surgically implanted to allow transmission and online recording of electrocardiogram (ECGs) from conscious, freely moving, unanesthetized mice. To capture spontaneous arrhythmias, continuous ECG recordings were acquired and stored in full waveform format. Continuous recording of 11 HF-1b mutant animals was acquired (total accumulated time = 166 days) to qualitatively and quantitatively evaluate the arrhythmias and to obtain unequivocal ECG evidence for authentic, spontaneous sudden death events. Littermate control mice were also examined in parallel, revealing no ECG abnormalities in over 80 days of continuous recording. The incidence of sudden death was higher in HF-1b mutant mice (8 out of 11) when compared to their wild-type controls (0 out of 5). The cause of death in these HF-1b mutant mice was clearly related to the underlying rhythm disturbances. While no arrhythmias or conduction abnormalities were observed in wild-type or heterozygous controls, there were conduction defects in the HF-1b mutant mice that indicated physiological dysfunction at all levels of the conduction system, i.e., SA and AV node, His bundle, and distal Purkinje fibers. All of HF-1b mutant mice examined had intermittent sinus pauses (Figure 4A), while none were observed in the control mice. HF-1b mutant mice also show a high incidence of sinus bradycardia and AV block (Figures 4B and 4C) as well as spontaneous ventricular tachycardia (Figures 4D–4E). These data are most consistent with the arrhythmias observed in the setting of end stage heart failure that can be associated with idioventricular arrhythmias as

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<th>Genotype</th>
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<th>AV Blocks</th>
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Figure 3B: Summary of Electrocardiographic Data of HF-1b Mice Obtained with Implantable Radio telemetry

(A) Representative ECG of inducible ventricular tachycardia in HF-1b mutant mouse. Simultaneous ECC recordings (surface lead I and two epicardial leads, Epi1 and Epi2) for a HF-1b mutant that experienced ventricular tachycardia resulting from programmed ventricular stimulation with a single premature stimulus (S1).

(B) Summary of ECG data from HF-1b and wild-type mutant and mice obtained with implantable radio telemetry.
well as ventricular arrhythmias. The pattern of arrhyth-
mas at the time of death indicates a pattern often seen
in the setting of end stage heart failure where a mixture
of tachycardia and bradycardia degenerates into asyst-
ole as opposed to ventricular fibrillation (Figure 4E). How-
ever, since there was no associated evidence of hyper-
trophy, heart failure, or dilated cardiomyopathy, the
arrhythmias in these HF-1b mutant animals reflects a
primary electrophysiological defect as opposed to sim-
ply reflecting a secondary effect related to heart failure.

Marked Defects in Cardiac Conduction System
Cell Lineages in the HF-1b-Deficient Mice
To determine if the widespread arrhythmias reflected
structural defects in the cardiac conduction system, we
employed markers that differentiate the conduction tis-

tue from ventricular myocytes. During the first 3 weeks
of postnatal life, the connexins become localized in a
restricted manner to the intercalated discs as the adult
ventricular cells attain their rod shape and as the distal
Purkinje system becomes mature (Angst et al., 1997).
In the murine heart, connexin 40 (Cx40) has been shown
to be a sensitive marker for central and peripheral con-
duction system (Gourdie et al., 1993; Delome et al., 1995).
lacZ-positive areas in the adult HF-1b mutant hearts
(Figures 2E and 2F) were indeed part of the central con-
duction system since the same corresponding areas
also costained with Cx40 and β-galactosidase (Figures
5A–5C). To establish the role of HF-1b in defined regions
of the cardiac conduction system, we analyzed the Cx40
expression patterns in both wild-type and HF-1b mutant
mice. Cx40 immunolocalized to the appropriate compo-
nents of the conduction system, including the proximal
region of the AV node, His bundle, bundle branches
(data not shown), and in the peripheral Purkinje fibers
in both wild-type and mutant littermates (Figures 5D–5I).
However, there were distinct differences in the cellular
distribution of Cx40-containing gap junctional plaques
between wild-type and mutant mice, particularly in the
distal Purkinje cells (Figures 5D–5I). Cx40 expression,
as assessed by the number of Cx40-positive myocytes
per total Purkinje cells, demonstrated there were mark-
edly fewer cells expressing Cx40 in HF-1b mutant mice
(45%) when compared to their littermate controls (84%)
(Figure 5J). Furthermore, the distribution of Cx40 within
the cells themselves was altered in HF-1b mutant hearts.

Normally, there is a redistribution of Cx40 from the cyto-
sol to the cell membrane during early postnatal develop-
ment representing the formation of functional gap junc-
tion plaques at the cell membrane (Litchenberg et al.,

Figure 4. Representative ECGs from HF-1b
Mutant Mice Obtained with Implantable Ra-
dio Telemetry
(A) Sinus pause and sinus bradycardia in HF-
1b mutant mouse. AV block in HF-1b mutant
mouse, representative example of 2° (B) and
3° AV block (C). Spontaneous ventricular tachycardia in HF-1b mutant mouse (D).
Spontaneous ventricular tachycardia, asysto-
tole, and sudden death in an HF-1b mutant
mouse (E).
Figure 5. Immunolocalization of HF-1b and Connexin 40 in the Cardiac Conduction System

Colocalization of HF-1b (A) and Cx40 (B) in the AV node and branching bundle of an adult HF-1b mutant mouse (C). Expression pattern of connexin 40 (Cx40) in the distal Purkinje fibers of wild type (D and F) compared to HF-1b-deficient mouse (E and G). (H) and (I) are higher magnification views of the insets in (F) and (G). Note the more random distribution of Cx40 expression in the HF-1b null mutant. Alignment of Cx40 to cell borders is more easily identified in the wild-type animal (H, arrowheads) as compared to the mutant mouse (I, arrowheads).

Quantitation of Cx40 expression was performed by two different criteria: (1) by determining the relative number of Cx40-positive myocytes with clusters of Purkinje cells (J) and (2) by determining the relative intracellular distribution pattern of Cx40 protein either at the cell membrane (presumably functional gap junction plaques) or randomly distributed (nonfunctional) within the cell (K). Values are representative of two separate sets of wild-type and HF-1b mutant hearts. All Cx40-positive areas within 2–3 separate sections were analyzed for each animal. Note that in the HF-1b null heart, less cells within clusters of Purkinje cells were expressing Cx40 and those that were expressing Cx40 in mutant hearts had a more random distribution pattern within the cells.
2000). In wild-type mice, ~79% of Cx40-positive Purkinje cells displayed Cx40 immunostaining distributed at the cell borders in comparison to 46% of immunopositive sites located randomly within the cell (Figures 5F, 5H, and 5K). In contrast, mutant hearts showed only 32% of Cx40-positive staining at cell borders, while 68% displayed a random distribution (Figures 5G, 5I, and 5K). These results suggest that HF-1b-dependent downstream pathways may play a role in trafficking of the Cx40 protein to gap junctional sites. Interestingly, we have recently detected parallel differences in the cellular distribution pattern for Cx43, a gap junction protein that is predominately expressed in the working myocardium (L. W. N., R. S., V. T. B. N.-T., K. R. C., S. W. K., and R. G. G., unpublished data). Together, these findings are consistent with growing evidence supporting the role of altered connexin distribution in arrhythmogenesis (Kirchoff et al., 1998; Simon et al., 1998; van der Velden et al., 1998).

Recently, minK (or IsK or KCNE1), a small protein that heterodimerizes with KvLQT1 to generate a delayed rectifier K⁺ current in mammalian cardiac myocytes (Barhanin et al., 1996), has been shown to be a marker of the cardiac conduction system in the murine heart (Kupershmidt et al., 1999). Since the cardiac expression pattern of HF-1b is almost superimposable to that of minK during heart development, we analyzed minK expression via an RNase protection assay using RNA harvested from the intact ventricle of wild-type, heterozygous, and HF-1b mutant mice. While the transcript levels of both KvLQT1 and Kv1.5 remain relatively unchanged, there was a significant induction of minK in the ventricles of HF-1b mutant mice when compared to their littermate controls (Figures 6A and 6B).

To establish whether this increased minK mRNA level reflected the ectopic expression of minK outside of the conduction system per se in HF-1b mutant mice, the cellular distribution of minK was determined by in situ analyses in hearts at two distinct temporal windows, neonatal and adult (Figures 6C–6H). In wild-type neonatal hearts, minK expression was highly expressed in the atrioventricular junction and extended posteriorly in an organized pattern reminiscent of the cardiac conduction tissue along both sides of the interventricular septum (Figures 6C and 6E). In HF-1b mutant neonatal hearts, minK was detected in the atrioventricular junction at levels similar to those in the wild type. However, the organized expression in the ventricular septum seen in wild-type mice was replaced by a dispersed, intense hybridization signal throughout the septum (Figures 6D and 6F), reflecting a developmental defect in conduction system formation in HF-1b mutant mice. In the adult heart of wild-type mice, minK mRNA was downregulated in the adult heart to background levels (Figure 6G). In contrast, in the mutant adult hearts, downregulation of minK only occurred in the ventricular septum (negative results not shown), while the entire transmural myocardium displayed abundant expression of minK (compare Figures 6G and 6H), consistent with the earlier persistent, ectopic expression observed in the mutant neonatal hearts.

**HF-1b-Deficient Mice Display a Decrease in the Density of a Delayed Rectifier K⁺ Current**

Earlier work in normal adult mouse ventricular myocytes has shown that depolarizing voltage-clamp steps activate a number of outward K⁺ conductances (Zhou et al., 1998; Xu et al., 1999a); qualitatively, these can be described as a Ca²⁺-independent transient outward current and a rapidly activating, slowly inactivating delayed rectifier K⁺ current. Since action potential duration is regulated at least in part by repolarizing K⁺ currents, these conductances were measured under voltage-clamp. The superimposed current records in Figure 7C demonstrate that the rapidly activating, slowly inactivating delayed rectifier in myocytes from homozygotes was significantly decreased. In contrast, neither the transient outward K⁺ current nor the K⁺ current that regulates resting membrane potential (h₅) were changed significantly when control and HF-1b-deficient myocytes were compared (Figure 7C). The I–V plots in Figure 7D show that K⁺ current (measured at the peak and at the end of the 500 ms voltage-clamp steps) was reduced substantially at membrane potentials corresponding to the plateau and repolarization phases of the action potential. Taken together, these results suggest that a selective reduction in the density of the delayed rectifier (h₅) is largely responsible for the action potential prolongation and dispersion seen in HF-1b⁻/⁻ mutants (Zhou et al., 1998; Xu et al., 1999b).
Discussion

**HF-1b Pathways Play a Critical Role in the Electrophysiological Transition between Ventricular and Conduction System Cell Lineages**

Maintenance of normal cardiac rhythm in a mature heart requires the development of specialized pacemaker and conduction system that initiates and propagates the cardiac impulse in a uniform pattern, thus ensuring synchronous contraction and unidirectional blood flow. Conduction system cell lineages include the sinoatrial and atrioventricular nodes that contain pacemaker cells that determine the heart rate, as well as the His-Purkinje system that delivers the impulse to the ventricular myocardium, and is responsible for transmural activation in the left ventricle. Recently, retroviral lineage tracing studies in an avian system have provided compelling evidence that conduction cells are derived from cardiomyogenic precursors in the tubular heart (Cheng et al., 1999). This recruitment process is continuous throughout the early stages of heart development. Successful morphological and functional transition between myogenic and conduction system lineages is critical for establishing the graded changes in action potential duration, pacemaker activity, repolarization, and contractility phenotypes that are distinct features of ventricular, SA nodal, AV nodal, and Purkinje cell lineages. Each of these cells displays a distinct subset of genes that confer the specific electrophysiological phenotype in the central conduction, distal conduction, and ventricular muscle cells. In the ventricular chamber of the mouse, connexin 40 is localized to the myocardial component of the conduction system. In contrast, connexin 43 is predominantly expressed throughout the ventricular myocardium, although coexpression of both Cx40 and Cx43 occurs in some distal Purkinje fiber networks (Gourdie et al., 1993). minK, a subunit of one type of heteromeric potassium channel that regulates repolar-
Figure 7. HF-1b/SP4 Mice Have Prolonged and Heterogeneous Action Potential Durations and a Decrease in the Density of a Delayed Rectifier K⁺ Current

(A) Representative action potentials recorded from ventricular myocytes isolated from control (+/+ and HF-1b⁻/⁻ animals. Stimulation frequency was 5 Hz. Dashed line indicates zero membrane potential. Time and membrane potential calibrations are the same for both action potentials.

(B) Scattergram comparing action potential durations at 50% (APD₅₀) and 90% (APD₉₀) repolarization from control (n = 14) and HF-1b⁻/⁻ (n = 17) cells. The hatched bars indicate the mean ± SEM for each data group. Note that the difference in mean APD₅₀ between controls and HF-1b⁻/⁻ (10.6 ± 3 ms versus 25.3 ± 7.3 ms, respectively) was not statistically significant (p = 0.8, unpaired Student’s t test, with Welch’s correction). However, the difference in mean APD₉₀ (control = 32.5 ± 8.7 ms versus −/− = 68.3 ± 15.3 ms) was statistically significant (p = 0.006).

(C) Representative families of membrane currents from voltage-clamped ventricular myocytes from wild-type (+/+; left panel) and from HF-1b⁻/⁻ (right panel). Currents were activated by 500 ms voltage-clamp steps (from −120 mV to +60 mV, in 10 mV increments). Holding potential was −80 mV. Currents were normalized to cell membrane capacitance in each case: +/+ = 99.4 pF; −/− = 108 pF. Horizontal arrow to the left of each superimposed set of current records indicates the zero current level. Current density and time calibrations are identical for both sets of current records.

(D) Comparison of mean ± SEM current-voltage relationships for ventricular myocytes from +/+ and −/− mice. Peak current density versus membrane potential from 9 +/+ and 6 −/− cells (left panel). Isochronal current density measured at the end of the 500 ms voltage-clamp steps (right panel).

The present study provides several independent lines of evidence to support a critical role of HF-1b/SP4, a member of the SP-1 transcription factor gene family, in the electrophysiological transition between ventricular and conduction system cell lineages. As documented via a knockin of lacZ into the endogenous locus, the HF-1b gene displays a restricted pattern of expression within the ventricular chamber and is preferentially expressed in conduction system lineages, including the AV node, the atrioventricular ring, branching bundles in the interventricular septum, and the distal His-Purkinje fibers. The timing of onset of HF-1b appearance during cardiac development coincides with the process of the recruitment of myogenic precursors into the conduction system. Mice that harbor a complete deficiency in HF-1b fiber cells are unknown. Recently, perivascular derived factors have been implicated by retroviral lineage tracing studies that demonstrated that this transition occurs in close proximity to coronary arterioles.

The expression pattern of conduction system-specific markers are markedly abnormal with a decrease in the number of distal Purkinje fiber cells and an upregulation of a conduction system–specific marker, e.g., minK, in the ventricular chamber. Further evidence of defects in the electrophysiological phenotype of ventricular muscle cells includes a marked increase in action potential heterogeneity (prolonged action potentials in septal endocardial cells but not in apical epicardial cells), a selective decrease of the rapidly activating, delayed rectifier
K+ current and sudden cardiac death characterized by ventricular arrhythmias. Remarkably, all of the phenotypes in the HF-1b mice were restricted to features that control the electrophysiological phenotype of ventricular muscle and conduction system cells, with no effects on cardiac chamber morphogenesis, dilation, hypertrophy, or basal contractile function. Thus, a HF-1b-dependent pathway appears to selectively orchestrate the electrophysiological phenotype of ventricular muscle cells, which results in the defective formation of conduction system cell lineages.

HF-1b contains an SP-1-like C2H2 zinc finger DNA binding domain and has previously been shown to act as a transcriptional activator in ventricular muscle cells (Zhu et al., 1993). Since transgenic mice that conditionally overexpress HF-1b in the ventricular muscle do not display any conduction system defects at the physiological level (data not shown), it is unlikely that HF-1b is acting dominantly to promote the conversion of ventricular muscle cells to conduction system lineages. Clearly, other cues are required, and the possibility exists that HF-1b may be acting as a positive regulator for the activation of distinct panels of ion channels and connexins in both lineages. The development of mice that harbor lineage-restricted mutations in HF-1b with cre-lox technology (Chen et al., 1998; Hirota et al., 1999) should allow a critical examination of the mechanistic pathways and downstream target genes in the HF-1b pathway that controls the electrophysiological transition of ventricular to conduction system cells.

A Genetically Based Model of Cardiac Sudden Death due to Ventricular Arrhythmias in HF-1b-Deficient Mice
The development of genetically based mouse models to study the factors responsible for sudden cardiac death due to lethal arrhythmias has proven to be a significant challenge. The mouse model poses several problems arising from (1) its rapid heart rate, (2) a short refractory period, and (3) the small mass of the mouse heart, which has been proposed to confer resistance to ventricular fibrillation (Jalife, 1999). The difficulty is compounded by differences in the ionic currents that underlie the action potential between mouse and larger mammalian species and the technological difficulties of the long-term, continuous, and accurate acquisition of the electrocardiographic monitoring required to capture the sudden death event. Mice that harbor mutant channel genes identical to those found in the human long QT studies do not display a sudden cardiac death phenotype, although arrhythmias can sometimes be detected (Folco et al., 1997; Barry et al., 1998; London et al., 1998). Similarly, a knockout of the minK gene, a critical component of the potassium channel that is defective in a subset of long QT patients, does not produce any evidence of arrhythmias or lethality (Charpentier et al., 1998; Drici et al., 1998). To date, there have been no previous reports of genetically based mouse models of authenticated sudden cardiac death. The results of the present study support the concept that the mouse can be engineered to be a valid model of cardiac sudden death, and the technology exists to accurately quantitate complex rhythm disturbances, even in animals at an early age with spontaneous heart rates in excess of 500 beats per minute. The insights gained from the monitoring data suggest that it will indeed be feasible to identify modifiers of this complex electrophysiological phenotype in a manner analogous to what has already been accomplished with regard to contractility and heart failure phenotypes (Chien, 1999; Hirota et al., 1999; Minamisawa et al., 1999). It will become of particular interest to interbreed HF-1b−/− mice with strains that harbor modifications in targets that have been proposed to suppress or promote the sudden death phenotype.

Defects in the Transition between Ventricular and Conduction System Cell Lineages: A New Mechanistic Pathway for Cardiac Sudden Death
Although sudden cardiac death is a major manifestation of heart disease, understanding of the mechanism that leads to its onset is very limited at present. Recently, the discovery of heritable mutations in potassium and sodium channel genes that can confer a risk for sudden death due to their effects on prolonging the QT interval and the action potential duration were reported (Curran et al., 1999; Roden and Spooner, 1999). However, only 20% of family members harboring the disease gene ultimately display the cardiac sudden death phenotype. In addition, sudden cardiac death in its most common form is associated with cardiomyopathy and/or heart failure in the absence of ion channel mutations per se. These considerations strongly suggest that additional pathways to induce sudden death must exist. The discovery of this “second hit” required to produce sudden death would be of importance in understanding the pathways that control normal cardiac rhythm and in identifying new therapeutic targets to intervene in this disease. Currently, cardiac sudden death is largely untreated via drug-based approaches, and implantable defibrillators are the only currently accepted form of therapy.

The present study provides clear evidence that defects in the pathways that guide and/or maintain the recruitment of ventricular cells into the conduction system lineage may underlie the onset of serious life-threatening ventricular arrhythmias and sudden death. In the absence of HF-1b, there is a selective dysregulation of a panel of genes that are involved in the divergent electrophysiological phenotypes of these distinct cell types. This produces a “confused” electrophysiological identity of both ventricular and conduction system lineages characterized by action potential prolongation, dispersion, and heterogeneity, i.e., the anatomic substrate for lethal ventricular arrhythmias. As a result, stochastic events eventually lead to the onset of sudden cardiac death due to lethal ventricular arrhythmias that are classically found in the setting of severe end stage

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Table 1. Spatial Dispersion of Action Potential Duration

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<tr>
<td>Septal Endocardial</td>
<td>67.2 ± 8.0 (13)</td>
<td>92.9 ± 6.5 (15)</td>
</tr>
<tr>
<td>Apical Epicardial</td>
<td>53.4 ± 4.9 (15)</td>
<td>48.9 ± 12.2 (8)</td>
</tr>
</tbody>
</table>

*APD (ms) measured at 90% repolarization.
*p < 0.009 control septal versus −/− septal cells.
*p < 0.001 −/− septal versus −/− apical cells.
heart failure. Since the HF-1b mice show no evidence of heart failure per se, the HF-1b pathway can act as a primary pathway for cardiac sudden death, thereby raising the question as to whether defects in the HF-1b pathway may contribute to the increased incidence of sudden death and lethal ventricular arrhythmias seen in the setting of acquired forms of dilated cardiomyopathy and heart failure in humans. This hypothesis should now be testable, and if confirmed, the identification of the downstream and upstream targets in the HF-1b pathway via cardiovascular genomic strategies (Chien, 2000) may ultimately lead to the development of more biologically targeted therapeutic strategies for lethal ventricular arrhythmias and sudden cardiac death.

Experimental Procedures

Generation of HF-1b-Deficient Mice
The targeting construct was made from a 10 kb genomic fragment of HF-1b isolated from a 129SvJ mouse genomic library. Out of 120 G418-resistant ES clones, eight were identified as homologous recombinants based on Southern blot hybridization analysis. Two of the eight positive clones were microinjected into J1 129SvJ blastocysts that were subsequently transferred to pseudopregnant female mice. Chimeric male mice were crossed with Black Swiss breeders.

Histochromical Analysis
Whole-mount enzyme histochemical detection of the β-galactosidase activity in developing embryos (Ross et al., 1996) and immunohistochemical analysis of connexin 40 (Gourdie et al., 1993) was performed using previously described protocols. All immunohistochemical analyses were analyzed by laser scanning confocal microscopy.

Electrocardiographical Analyses of HF-1b-Deficient Mice
A custom, miniaturized in vivo electrophysiological monitoring system and programmed stimulation protocol were used in these studies. A custom-designed linear electrode array, which consisted of 3 bipolar pairs (interelectrode distance 1 mm), was placed on the anterior-ventricular surface of each heart to deliver stimuli and record electrophysiological data.

Telemetric Analysis of Electrocardiograms from HF-1b Mice
Data were acquired by using an implantable radio frequency transmitter (Data Sciences, St. Paul, MN, 10ETA-F20) with subcutaneous leads surgically placed in the conventional lead II position. Precise and stabilized placement of the ECG leads were critical to allow continuous acquisition of the ECG waveforms over a long period of time with relatively low signal-to-noise ratios. Hardware modifications to provide unlimited memory space were necessary to allow continuous, off-line acquisition of electrocardiographic data in the waveform format from multiple instrumented animals.

Electrophysiological Recordings of Isolated Ventricular Cardiomyocytes
Preparation of adult ventricular myocytes and electrophysiological assessments were performed as previously described (Fiset et al., 1997; Ward and Giles, 1997).

In Situ Hybridization and RNase Protection Analyses
In situ hybridization with Sph-digested antisense minK probe (EST clone NM009842) was performed as previously described (Ruiu-Lozano et al., 1998). Mouse riboprobes used in RNase protection assays were transcribed from cloned cdNA fragments generated by PCR amplifications with specific oligonucleotide primers: (1) Kv1.5 (AL22216): forward, 5′-ccggtgttcgATGAGATCTCCCTCGTG; reverse, 5′-ccggtgttcgAGCCCTCGTGTGTT; (2) Kv1.2 (ATU7008): forward, 5′-ccggtgttcgGAGACCTTCAACCGG; reverse, 5′-ccggtgttcccGCGATACCTTAGCGG; (3) minK (GenBank AX92485): forward, 5′-ccggtgttcgATGAGCTGCGCCAAATT; reverse, 5′-ccggtgttcccGCTGAGAC TTCGAGC.

Acknowledgments
We thank F. Abdel-Wahab, C. Kondo, B. Poon, J. Anderson, J. Kleint, J. D. Tran, and X. H. Gao for their expert assistance. This work was supported by the National Institutes of Health, the Jean Le Ducq Foundation, and an Endowed Chair of the AHA to K. R. C., an MRC Group Award and the Heart and Stroke Foundation of Alberta Research Chair to W. R. G., and the AHA to S. W. K. and P. R. L.

Received September 18, 1998; revised June 30, 2000.

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complexes consisting of truncated Kv1.1 potassium channel poly-peptides and native Kv1.4 and Kv1.5 channels in the endoplasmic reticulum. J. Biol. Chem. 272, 26505–26510.


