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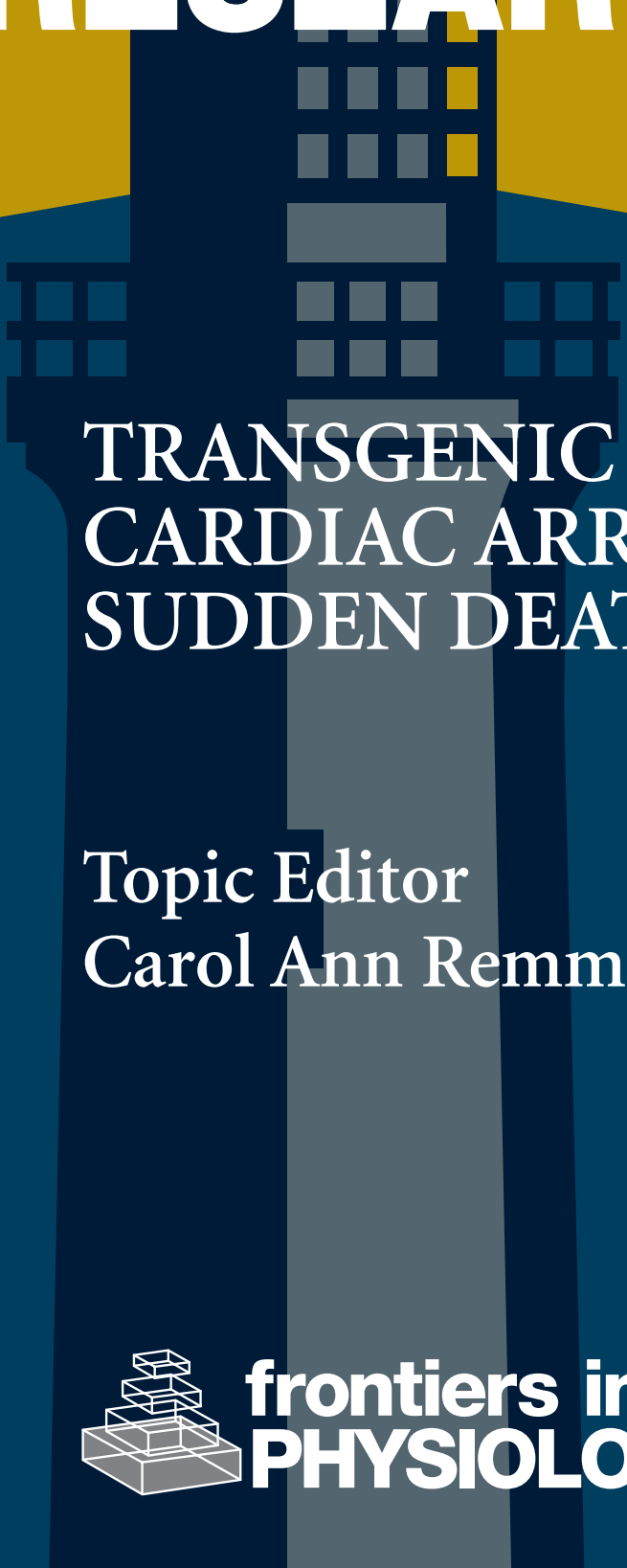

TRANSGENIC MODELS OF CARDIAC ARRHYTHMIAS AND SUDDEN DEATH

Topic Editor
Carol Ann Remme

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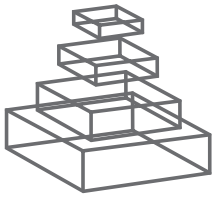

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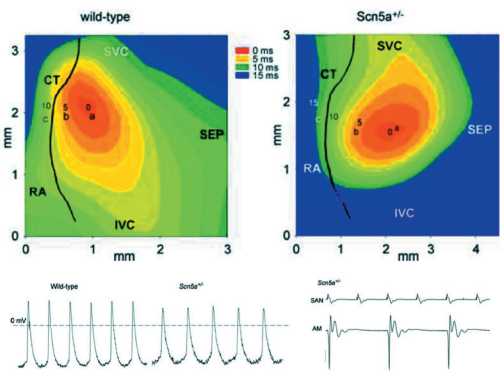
TRANSGENIC MODELS OF CARDIAC ARRHYTHMIAS AND SUDDEN DEATH

Topic Editor:
Carol Ann Remme, University of Amsterdam, Netherlands



SAN pacemaking and conduction in WT and Scn5a+/− mice. Figure taken from Huang CL-H, Lei L, Matthews GDK, Zhang Y and Lei M (2012) Pathophysiological mechanisms of sino-atrial dysfunction and ventricular conduction disease associated with SCN5A Deficiency: insights from mouse models. *Front. Physio.* **3**:234. doi: 10.3389/fphys.2012.00234

Cardiac arrhythmias secondary to hereditary genetic disorders are increasingly recognized as a cause for sudden cardiac death in the general population. Mutations in a large number of genes encoding ion channels and proteins involved in cell-cell coupling have been identified in affected patient populations. Furthermore, genetic defects in other proteins directly or indirectly influencing cardiac electrophysiological properties have been shown to be involved. In-depth studies of the mechanisms underlying the observed electrophysiological abnormalities are often limited in patients carrying these mutations. Transgenic mouse models incorporating the genetic defect in question have been successful in studying genotype-phenotype correlations and have provided important insight into the underlying electrophysiological, biophysical, and molecular mechanisms. In this Research Topic Issue, an overview is presented of the knowledge and insight obtained from various transgenic models of cardiac electrophysiology so far, and how further research in this field may be of additional benefit for the future identification, risk-stratification, and treatment of patients with inherited cardiac arrhythmias and sudden death.

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EDITORIAL
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Transgenic models of cardiac arrhythmias and sudden death

Carol Ann Remme*

Department of Clinical and Experimental Cardiology, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands
**Correspondence: c.a.remme@amc.uva.nl*

Edited by:
Ruben Coronel, Academic Medical Center, Netherlands

Reviewed by:
Ruben Coronel, Academic Medical Center, Netherlands

Cardiac arrhythmias are associated with a wide range of clinical problems, ranging from relatively benign (a)symptomatic heart rhythm alterations to life-threatening ventricular arrhythmias associated with sudden cardiac death. In most cases, atrial and ventricular arrhythmias are acquired, i.e., secondary to cardiac (structural) pathologies, including valvular disease, heart failure, cardiomyopathy, coronary artery disease, and myocardial infarction. Mechanisms underlying arrhythmogenesis in these conditions have been extensively investigated in the last decades (Janse, 2004; Rubart and Zipes, 2005). In a subset of patients, however, cardiac arrhythmias occur secondary to congenital or inherited alterations in electrophysiological properties of cardiomyocytes. Cardiac arrhythmias secondary to hereditary genetic disorders are increasingly recognized as a cause for sudden cardiac death in the general population (Perrin and Gollob, 2013). Mutations in a large number of genes encoding either ion channels, proteins involved in cell–cell coupling, or other proteins directly or indirectly influencing cardiac electrophysiology have been identified in affected patients (Amin et al., 2010; George, 2013). Widespread genetic testing has in some cases allowed for development of genotype-specific treatment strategies (Hofman et al., 2010; Ackerman et al., 2011). However, in-depth studies of the mechanisms underlying the observed electrophysiological abnormalities are often limited in patients, and *in vitro* studies in heterologous expression systems may not adequately reflect the endogenous cardiomyocyte environment. As reviewed in this Research Topic of *Frontiers in Cardiac Electrophysiology*, transgenic mouse models incorporating the genetic defect in question may be successful in studying genotype-phenotype correlations and may provide important insight into the underlying electrophysiological, biophysical, and molecular mechanisms.

The availability of genetically engineered transgenic mice has allowed for detailed investigation of the *in vivo*, *ex vivo*, and *in vitro* electrophysiological consequences of human mutations associated with arrhythmias. However, as reviewed by Kaese and Verheule, certain cardiac electrophysiological properties are inherently different between mouse and man (Kaese and Verheule, 2012). The authors conclude that transgenic mouse models may nevertheless successfully mimic human arrhythmia syndromes although observations regarding arrhythmia mechanisms may only be extrapolated to the human situation with caution. Grubb and co-workers provide an overview of studies on mice deficient for the potassium channel interacting protein KChIP2, a component of the transient outward potassium current (I_{to}) (Grubb et al., 2012). These studies have provided increased insight into the multiple actions of this promiscuous accessory protein, and KChIP2 is now known to modulate numerous potassium channels in addition to L-type calcium channels and possibly sodium channels (Grubb et al., 2012). The cardiac conduction system is another area where genetic defects in ion channels have been associated with arrhythmias and sudden cardiac death. Hyperpolarization-activated Cyclic Nucleotide-gated (HCN) channels, which are responsible for the pacemaker funny current I_f, are located specifically within the conduction system. A range of transgenic mouse models of different HCN isoforms has shed light on the role of I_f on pacemaking and atrioventricular conduction, as reviewed by Bucchi et al. (2012) and Aránega et al. (2012). In other models, genetic defects in certain ion channels widely expressed throughout the myocardium were found to have specific electrophysiological consequences within the specialized conduction system, which may promote arrhythmias (Aránega et al., 2012). In particular, mice with genetic alterations in the *Scn5a* gene encoding the cardiac sodium channel have provided insight into the role of this channel in both sinus node dysfunction and progressive cardiac conduction disease, as detailed by Huang et al. (2012). The various available *Scn5a* transgenic mouse models (in addition to mice lacking specific sodium channel auxillary β-subunits) have furthermore been instrumental in clarifying the biophysical alterations underlying Long QT syndrome type 3, Brugada syndrome, and cardiac conduction disease, inherited conditions associated with sodium channel mutations (Derangeon et al., 2012). Transgenic mice also allow for in-depth investigation of the interaction between (abnormalities in) cardiogenesis and arrhythmias. This is exemplified by Franco and colleagues who review the role for the transcription factor PITX2 in cardiac (in particular pulmonary vein) development and its link with atrial arrhythmias including atrial fibrillation (Franco et al., 2012). The many existing mouse models of atrial fibrillation and their usefulness for studying the pathways and mechanisms underlying this complex arrhythmia is further discussed by Riley et al. (2012). Defects in cytoskeletal signaling pathways have also been associated with atrial fibrillation, as shown by studies in mice with a heterozygous deficiency for the adapter protein Ankyrin-B (Smith et al., 2012). *Ankyrin-B*$^{+/-}$ mice have further provided fundamental insight into the role of ankyrins in sinus node disease and ventricular arrhythmias (Smith et al., 2012). Finally, the use of murine transgenic and targeted models of desmosomal proteins associated with

arrhythmogenic right ventricular cardiomyopathy (ARVC) has been instrumental in investigating disease etiology and progression in addition to the complex interaction between cell adhesion defects and arrhythmogenesis in this inherited syndrome (Lodder and Rizzo, 2012).

Apart from transgenic mouse models, novel techniques are increasingly recognized as useful in studying electrical roles of novel identified genes, and electrophysiological effects of specific mutations in the native cardiomyocyte environment. Zebrafish (*Danio rerio*) are relatively easy to study and genetically modify, but crucial differences exist between zebrafish and human cardiac electrophysiology (Verkerk and Remme, 2012). Nevertheless, ion channel disorders related to repolarization disorders have been successfully modeled in zebrafish; their applicability in studying depolarization disorders and calcium-related arrhythmias is however as yet unclear (Verkerk and Remme, 2012). More recently, it has been shown that human-induced pluripotent stem cell-derived (iPSC) cardiomyocytes may recapitulate disease phenotype in Mendelian cardiac rhythm disorders (Hoekstra et al., 2012). Although the interpretation of electrophysiological data from iPSC-derived cardiomyocytes should be done with caution (due to their immature phenotype), they are considered a promising tool for studying pathophysiology, genotype-phenotype relationship, and pharmacology in cardiac arrhythmia syndromes (Hoekstra et al., 2012).

In summary, the collection of 12 articles presented in this Research Topic provides an overview of the knowledge and insight obtained from various transgenic and targeted models of cardiac electrophysiology, and how further research in this field may be of additional benefit for the future identification, risk-stratification, and treatment of patients with inherited cardiac arrhythmias and sudden death.

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Impact of KChIP2 on cardiac electrophysiology and the progression of heart failure

Søren Grubb, Kirstine Calloe and Morten B. Thomsen*

Danish National Research Foundation Centre for Cardiac Arrhythmia, Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark

Edited by:
Carol Ann Remme, University of Amsterdam, Netherlands

Reviewed by:
Dirk Snyders, University of Antwerp, Belgium
Arun Sridhar, GlaxoSmithKline, UK

****Correspondence:***
Morten B. Thomsen, Danish National Research Foundation Centre for Cardiac Arrhythmia, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej 3b, Building 12.5.36, DK-2200 Copenhagen, Denmark.
e-mail: mbthom@sund.ku.dk

Electrophysiological remodeling of cardiac potassium ion channels is important in the progression of heart failure. A reduction of the transient outward potassium current (I_{to}) in mammalian heart failure is consistent with a reduced expression of potassium channel interacting protein 2 (KChIP2, a K_V4 subunit). Approaches have been made to investigate the role of KChIP2 in shaping cardiac I_{to}, including the use of transgenic KChIP2 deficient mice and viral overexpression of KChIP2. The interplay between I_{to} and myocardial calcium handling is pivotal in the development of heart failure, and is further strengthened by the dual role of KChIP2 as a functional subunit on both K_V4 and $Ca_V1.2$. Moreover, the potential arrhythmogenic consequence of reduced I_{to} may contribute to the high relative incidence of sudden death in the early phases of human heart failure. With this review, we offer an overview of the insights into the physiological and pathological roles of KChIP2 and we discuss the limitations of translating the molecular basis of electrophysiological remodeling from animal models of heart failure to the clinical setting.

Keywords: potassium, ion channels, heart failure, ventricular arrhythmia, knockout mice, action potential, repolarization, sudden death

INTRODUCTION

The normal pumping action of the mammalian heart is critically dependent on the cardiac electrophysiological function. Within a heartbeat, the single cardiac cell generates an action potential in consequence of sequential depolarizing and repolarizing currents. The exact function of the multitude of ion channels and other transporters of electrical charges determines the morphology of the action potential. Owing to diversities in properties and expression profiles of ion channels in different regions in the heart, in different species and in different physiological and pathological settings, the action potential waveform is highly variable (Carmeliet, 1999; Nerbonne and Kass, 2005; Nattel et al., 2007; Ter Keurs and Boyden, 2007).

The human action potential has a spike-and-dome morphology, with a rapid early repolarizing phase or notch that reflects the transient outward potassium current, I_{to}. The magnitude of the notch determines the membrane potential for the plateau phase of the action potential, and thus establishes the driving force for Ca^{2+} entry through the L-type Ca^{2+} channel triggering calcium-induced calcium release and contraction of the myocyte (Greenstein et al., 2000; Sah et al., 2003). In rodents, I_{to} is the major repolarizing force abbreviating the action potential considerably in adaptation to the fast heart rates. Reduced I_{to} amplitude in non-rodent mammals is associated with desynchronized Ca^{2+} release from intracellular stores (Harris et al., 2005; Wasserstrom et al., 2009) and suboptimal cardiac performance secondary to uncoordinated calcium-induced calcium release and contraction (Sah et al., 2002, 2003; Cordeiro et al., 2004).

The landmark finding of reduced I_{to} in heart failure (Beuckelmann et al., 1993) spurred an intense interest into function and regulation of this current. An et al. (2000) reported how I_{to} was modulated by K^+-channel interacting proteins (KChIPs). Since the initial characterization of KChIPs, several additional functions of KChIPs have been described, including tuning of several ionic currents and gene-transcription regulation (Buxbaum, 2004). In the present review, we outline the basic molecular biology and cardiac electrophysiology of KChIP2 and integrate these findings in a discussion of the role of KChIP2 in cardiac plasticity, hypertrophic remodeling, arrhythmia, and failure.

KChIP STRUCTURE AND EXPRESSION

The KChIPs belong to the family of small cytosolic calcium-binding proteins and consists of four different KChIP isoforms ranging between 216 and 270 amino acids. They all have a reasonably conserved core region of 185 amino acids and a heterogeneous N-terminal part (An et al., 2000; Holmqvist et al., 2002). All KChIPs have calcium-binding EF hands which appear important for modulation K_V-channels (An et al., 2000; Li et al., 2005) but not for Ca_V-channels (Thomsen et al., 2009c). Myristoylation of KChIP1 (Burgoyne and Weiss, 2001) and palmitoylation of KChIP2 (Takimoto et al., 2002) play important roles in post-translational modification to improve plasma membrane localization. It is well established that KChIP1–3 functionally increase peak K_V4 current, slow channel inactivation and accelerate recovery from inactivation (An et al., 2000; Lundby et al., 2010); whereas KChIP4 eliminates inactivation (Holmqvist et al., 2002). Furthermore, KChIP2 has been reported to increase $I_{Ca,L}$ (Thomsen et al., 2009c), and suggested to decrease $K_V1.5$ cell surface expression (Li et al., 2005) and facilitate I_{Na} (Deschenes et al., 2008).

All four KChIP mRNAs are found in the brain; however only KChIP2 is expressed in the heart (An et al., 2000; Kuo et al., 2001; Rosati et al., 2001). KChIP expression in cardiac autonomic nerves

has not been reported. The cardiac KChIP2 gene generates three protein isoforms of 220, 252, and 270 amino acids via alternative splicing of exons 2 and 3 (Kuo et al., 2001). Immunocytochemistry has localized KChIP2 to the sarcolemma and the nucleus (Deschenes et al., 2002). Cardiac expression of KChIP2 is absent in embryonic and neonatal stages; however developmental upregulation potentially suggests a physiological role for KChIP2 once a transmural gradient of I_{to} is appearing (Kuo et al., 2001; Plotnikov et al., 2004). KChIP2 is expressed heterogeneously across the canine and human left and right ventricular free wall with higher concentrations in epicardium compared to endocardium, whereas no transmural gradient of expression is found for $K_V4.3$ (Rosati et al., 2001, 2003; Calloe et al., 2009b). This finding has led to the suggestion that KChIP2 is responsible for the I_{to} gradient; whereas other studies failed to show a transmural KChIP2 protein gradient in canine and human hearts (Deschenes et al., 2002), arguing against the role of KChIP2 in the generation of the transmural I_{to} gradient. More recently, studies of the human heart have shown strong KChIP2 expression in the epicardium based on both mRNA and protein levels (Gaborit et al., 2007; Soltysinska et al., 2009). In mice, transmural $K_V4.2$ protein expression parallels the $I_{to,f}$ gradient; whereas $K_V4.3$ and KChIP2 protein levels were uniformly distributed in an early study (Guo et al., 2002). More recently, laser capture microdissection of the murine LV has revealed a transmural gradient of both $K_V4.2$ and KChIP2 mRNA (Teutsch et al., 2007).

KChIP2 AND ION CHANNELS

K_V4

KChIP2 is a cytosolic protein when expressed in absence of K_V4; however co-expression of K_V4 and KChIP2 leads to co-localization at the cell surface (An et al., 2000; Bahring et al., 2001). Biochemically, KChIP2 co-immunoprecipitates with $K_V4.2$ and $K_V4.3$ in mouse ventricular homogenates (Guo et al., 2002). Crystallographic, electron microscopic and electrophysiological experiments have shown that KChIPs physically interact with the distal part of N-terminus of K_V4 subunits, forming a cross-shaped octamer, where two interaction sites on each K_V4 subunit are important for KChIP2-mediated channel trafficking and gating (Bahring et al., 2001; Kim et al., 2004; Pioletti et al., 2006). KChIP2 decreases the turnover rate of $K_V4.2$ by stabilizing the KChIP2–$K_V4.2$ complex at the cell surface. This stabilization is lost with deletion of the N-terminal part of $K_V4.2$ (Foeger et al., 2010). KChIP2 protein levels are decreased in the absence of $K_V4.2$, despite unaltered mRNA levels, suggesting post-translational downregulation of KChIP2 in the absence of the pore-forming subunit (Guo et al., 2005). In addition, both $K_V4.2$ and $K_V4.3$ mRNA and protein expression are augmented in hearts from KChIP2$^{-/-}$ mice as well as in cultured neonatal rat cardiomyocytes overexpressing KChIP2, suggesting regulation of the pore-forming subunits at gene and protein levels (Thomsen et al., 2009b; Jin et al., 2010).

The rodent outward potassium currents (**Figure 1**) involved in action potential repolarization can broadly be divided into transient outward (I_{to}) and delayed rectifier (I_K) currents (for a review, see Nerbonne et al., 2001), which can be further separated into four components, $I_{to,fast}$ ($I_{to,f}$, $K_V4.2$/$K_V4.3$ + KChIP2), $I_{to,slow}$ ($I_{to,s}$, $K_V1.4$), $I_{K,slow1}$ (I_{Kur}, $K_V1.5$), and $I_{k,slow2}$ (I_{SS}, $K_V2.1$). In order to dissect these current components, several studies have used electrophysiology combined with pharmacology on cardiomyocytes of wild-type (WT) or transgenic mice (Himmel et al., 1999; Brouillette et al., 2004; Brunet et al., 2004; Liu et al., 2011). $I_{to,f}$ and $I_{to,s}$ both have a fast activation and a fast inactivation and are under normal conditions very similar, however they can be distinguished by the rate of recovery from inactivation (Wickenden et al., 1999a; Patel and Campbell, 2005). $I_{to,f}$ recovers from inactivation in the order of milliseconds, whereas $I_{to,s}$ recovers much slower. In order to inhibit $I_{to,f}$, an inactivating pre-pulse to −40 mV for 50 ms can be applied, which captures $I_{to,f}$ in a closed state (Brouillette et al., 2004); alternatively *Heteropoda* toxin can be used, which selectively blocks K_V4 channels in low-micromolar concentrations (Sanguinetti et al., 1997; DeSimone et al., 2011; Liu et al., 2011). For evaluating $I_{K,slow1}$, the sensitivity toward 4-aminopyridine can be exploited, because $I_{K,slow1}$ is sensitive in the micromolar range while $I_{to,f}$, $I_{to,s}$, and $I_{K,slow2}$ are sensitive in the millimolar range (Guo et al., 1999). Furthermore, in order to dissect $I_{K,slow2}$, an inactivating pre-pulse in combination with micromolar 4-aminopyridine can be used (Brouillette et al., 2004). Several studies have failed to find $I_{to,s}$ in the free wall of the mouse ventricle and only located it in myocytes from the interventricular septum (London et al., 1998; Guo et al., 1999).

Electrophysiologically, KChIP2 increases K_V4-mediated current amplitude and modulates channel gating by slowing the fast time constant of inactivation at depolarizing potentials and accelerating recovery from inactivation (Decher et al., 2001; Deschenes et al., 2002; Patel et al., 2002). The binding of KChIP2 to K_V4 is calcium-independent; however the effect of KChIP2 on slowing the fast time constant of inactivation at depolarizing potentials is dependent on calcium-binding to three of the four EF hands on KChIP2 (Deschenes et al., 2002; Patel et al., 2002). Interestingly, when K_V4 and KChIP2 are co-expressed in HEK293 the current inactivation is much slower than the native I_{to} in human and canine cardiomyocytes (Deschenes et al., 2002), which could be explained by the presence of additional subunits in the native cardiac cell. Dipeptidyl-peptidase-like proteins (DPPX) have been suggested as part of the missing link since a ternary complex of $K_V4.3$, KChIP2, and DPPX produced currents closely resembling $I_{to,f}$ in mammalian expression systems (Radicke et al., 2005; Lundby et al., 2010). Additionally, KCNE2–4 have all been implicated in regulation of the K_V4-mediated current, suggesting a diverse macromolecular complex governing I_{to} (Zhang et al., 2001; Lundby and Olesen, 2006; Lundby et al., 2010).

The development of the KChIP2 deficient mouse provided the first clues to the role of KChIP2 in generating the native I_{to} (Kuo et al., 2001). Voltage-clamp recordings of KChIP2$^{-/-}$ cardiomyocytes revealed a complete loss of $I_{to,f}$ defined as the portion of the potassium currents sensitive to an inactivating voltage prepulse to −40 mV. Moreover, administration of *Heteropoda* toxin to KChIP2$^{-/-}$ cardiomyocytes had no effect on potassium current amplitude (Thomsen et al., 2009b). *In vivo*, the loss of $I_{to,f}$ translated to an elevated and earlier J wave of the murine ECG, normal QT intervals and ventricular effective refractory periods, and susceptibility to pacing-induced polymorphic ventricular tachyarrhythmias (Kuo et al., 2001). Furthermore, we showed that

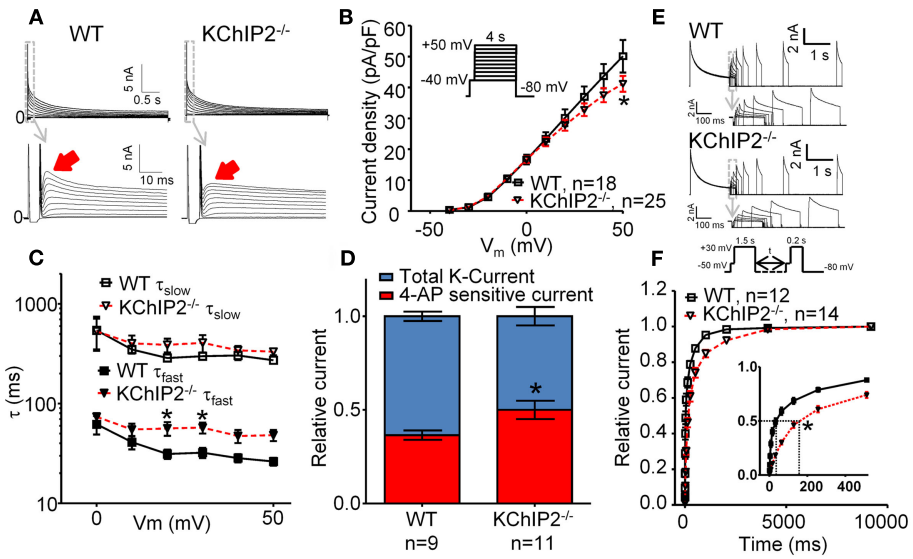

FIGURE 1 | Whole-cell voltage-clamp recordings of outward K-currents recorded in disaggregated left ventricular cardiomyocytes from wild-type (WT) versus KChIP2$^{-/-}$ mice. Cardiomyocyte isolation and patch-clamp solutions (37°C) were made according to (Brouillette et al., 2004) with minor modifications. Cadmium (300 μM) was added to inhibit L-type Ca^{2+} currents. **(A)** Representative current traces showing that KChIP2$^{-/-}$ cardiomyocytes lack a fast decaying component of the outward K-current (solid arrows). **(B)** Peak current density as a function of test potential, however, was not significantly changed at any voltage (inset, voltage protocol). **(C)** The decay of the outward current (0–700 ms) was fitted to a second-order exponential function to obtain time-constants (τ) of inactivation for positive test potentials. No differences between WT and KChIP2$^{-/-}$ were found for the slow component; however, the fast component of the current decay was significantly slower for KChIP2$^{-/-}$ myocytes, confirming the loss of a transient component. **(D)** In order to dissect $I_{K,slow1}$ from $I_{to,f}$, the current sensitive to 100 μM 4-AP was determined. The 4-AP sensitive fraction (red) of the peak current (blue, normalized to 1) was increased in KChIP2$^{-/-}$ compared to WT, indicating increased $I_{K,slow1}$. **(E)** Recovery from inactivation was addressed by a double-pulse voltage-clamp protocol with varying interpulse intervals (see inset). Representative current traces from WT and KChIP2$^{-/-}$ cardiomyocytes are presented. **(F)** The mean recovery from inactivation is illustrated as the peak current during the 0.2-s test pulse relative to the 1.5-s base pulse. Recovery from inactivation was significantly slowed in KChIP2$^{-/-}$ compared to WT in interpulse intervals between 1 and 4000 ms. This is compatible with a larger fraction on the outward potassium currents attributable to $I_{K,slow}$ relative to $I_{to,f}$ in KChIP2$^{-/-}$ cardiomyocytes. Data are reported as mean ± SEM. Statistical significance was evaluated by ANOVA followed by Newman–Keuls' *post hoc* test where appropriate. $^*p < 0.05$. Comparable data have been published previously by Thomsen et al. (2009b).

transmembrane action potentials were comparable in ventricular multicellular preparations from KChIP2$^{-/-}$ and WT control mice paced at 2 Hz (Thomsen et al., 2009c), which was presumably secondary to an upregulation of a 4-aminopyridine-sensitive current that restored peak outward potassium current density in KChIP2$^{-/-}$ mice (**Figure 1**). Action potentials recorded from disaggregated right ventricular myocytes from KChIP2$^{-/-}$ mice are longer than those from WT mice and show reverse rate dependency (Kuo et al., 2001), potentially consequential of the slowed recovery from inactivation of the remaining outward potassium currents (**Figure 1**).

$Ca_V1.2$

KChIP2 binds to the N-terminal cytosolic domain of $Ca_V1.2$ in a calcium-independent manner. In heterologous expression systems, KChIP2 co-expression causes an increase in current mediated by $Ca_V1.2$, comparable to the effect of the calcium channel auxiliary β_2 subunit; however KChIP2 was not able to replace the β_2-mediated macromolecular ion channel trafficking to the cell membrane (Thomsen et al., 2009c). Also this functional modulation was independent of calcium-binding to the EF hands of KChIP2. Furthermore, the L-type calcium current is significantly smaller in disaggregated cardiomyocytes from KChIP2$^{-/-}$ mice compared to WT controls (**Figure 2**). This decrease in current density was observed in the presence of an intact trafficking pathway of $Ca_V1.2$ and increased transcriptional activity of the β_2 subunit (Thomsen et al., 2009a,c). Protein levels of $Ca_V1.2$ in the KChIP2$^{-/-}$ mice are increased, whereas acute gene silencing of KChIP2 in neonatal rat cardiomyocytes does not affect $Ca_V1.2$ protein expression (Deschenes et al., 2008). In addition, in neurons KChIP3 has been described as part of a macromolecular signaling complex together with Ca_V3 and K_V4 that confer physiological calcium modulation of K_V4-mediated currents (Anderson et al., 2010).

$Na_V1.5$

A structural and functional association of I_{to} and I_{Na} has been suggested based on data from neonatal rat cardiomyocytes (Deschenes et al., 2008). Genetic silencing of KChIP2 caused a decrease in protein contents of $K_V4.2$ and $K_V4.3$, presumably due to a trafficking defect. Interestingly, the authors showed a biochemical association between K_V4 and the auxiliary β_1 subunit of the sodium channel, and went on to demonstrate that KChIP2 silencing also dramatically reduced mRNA and protein levels of $Na_V1.5$ and the sodium channel β_1 subunit to a point where no I_{Na} could be recorded and no action potentials could be elicited (Deschenes et al., 2008).

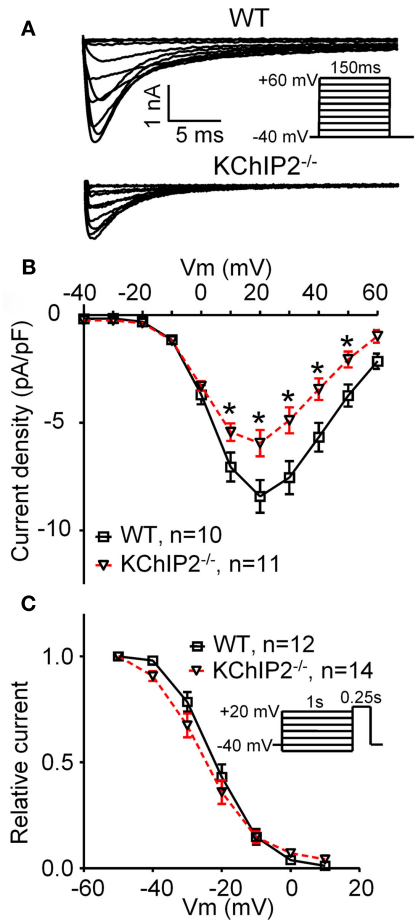

FIGURE 2 | L-type Ca^{2+} current in disaggregated cardiomyocytes from wild-type (WT) versus KChIP2$^{-/-}$. $I_{Ca,L}$ was recorded at 37°C in left ventricular cardiomyocytes as the current fraction sensitive to 300 μM Cd^{2+}, as described by Sah et al. (2002) with minor modifications. **(A)** Representative current traces of WT and KChIP2$^{-/-}$ (inset, voltage protocol). **(B)** Mean peak current densities show a decreased $I_{Ca,L}$ in KChIP2$^{-/-}$ cardiomyocytes. **(C)** No changes were found in the steady-state inactivation of $I_{Ca,L}$ in WT versus KChIP2$^{-/-}$ cardiomyocytes (inset, voltage protocol). Data are reported as mean ± SEM. Statistical significance was evaluated by ANOVA followed by Newman–Keuls' *post hoc* test where appropriate. *$p < 0.05$. Comparable data have been published previously by Thomsen et al. (2009c).

Reversibly, co-expression of KChIP2 with $Na_V1.5$ in a mammalian cell line augmented the sodium current, further substantiating the functional effect of KChIP2 on I_{Na} (Deschenes et al., 2008), suggesting a close structural and functional link of I_{to} and I_{Na}. These findings are not reproduced in mice with complete deletion of KChIP2 as they do not show embryonic lethality which would be expected if I_{Na} was reduced to the point the hearts were inexcitable (Kuo et al., 2001). The KChIP2$^{-/-}$ mice show a small, but statistically not significant, reduction of the upstroke velocity of action potentials, generally accepted to be an indication of I_{Na} availability (Thomsen et al., 2009b). Furthermore, mRNA levels for $Na_V1.5$ and β_1 are comparable in KChIP2$^{-/-}$ mice and WT controls (M Thomsen, unpublished data). The reason underlying the discrepant results is currently unknown, but could include an unidentified compensatory adaptation in the embryogenic development of the knockout mouse to counteract a reduction in I_{Na}.

$K_V1.5$

$K_V1.5$ governs the murine $I_{K,slow}$ and the human atrial-specific I_{Kur}. In HEK-293 cells, KChIP2 has been reported to reduce $K_V1.5$ current density without modifying the kinetics of the current (Li et al., 2005). Immunocytochemistry suggested that KChIP2 attenuate trafficking of $K_V1.5$ to the plasma membrane and EF-hand mutations abolished the effect of KChIP2. In the same study, mouse ventricular $K_V1.5$ and KChIP2 were found to co-immunoprecipitate (Li et al., 2005). However, co-expression of $K_V1.5$ with KChIP2 in *Xenopus laevis* oocytes revealed no difference in current levels in absence or presence of KChIP2 (Lundby et al., 2010). Additionally, both groups found no effect of KChIP2 on Kv1.4 currents (Li et al., 2005; Lundby et al., 2010). In KChIP2$^{-/-}$ mice, $K_V1.5$ mRNA levels were elevated in KChIP2$^{-/-}$ hearts, suggesting a potential KChIP2-mediated regulation of Kv1.5 on a transcriptional level.

KChIP2 AND ARRHYTHMIAS

Spontaneous ventricular arrhythmias are rarely seen in mice with genetic deletion of single potassium currents (Nerbonne et al., 2001). For example, the $K_V4.2^{-/-}$ mouse show complete elimination of $I_{to,f}$ but normal action potentials and ECG and no spontaneous arrhythmia (Guo et al., 2005). On the other hand, prolonged action potentials and QT intervals were reported in another mouse model with total elimination of $I_{to,f}$ ($K_V4.2$-W362F; Barry et al., 1998). Interestingly, overexpression of a dominant-negative $K_V4.2$ fragment generated a mouse line with reduced $I_{to,f}$, prolonged action potentials and hypercontractility that developed into heart failure within 3 months of age (Wickenden et al., 1999b).

The KChIP2$^{-/-}$ mouse had no spontaneous arrhythmia during continuous monitoring of conscious mice; however, it was possible to induce cardiac tachyarrhythmia by pacing the ventricles (Kuo et al., 2001). Presently, it is unclear whether the increased susceptibility to pacing-induced arrhythmias is secondary to decreased repolarizing potassium currents or decreased depolarizing calcium currents, or a combination of the two.

KChIP2 AND REMODELING

CARDIAC MEMORY

Cardiac memory describe a special form of cardiac plasticity observed as an altered T wave on the surface ECG (Rosenbaum et al., 1982). After a period of altered ventricular activation, due to pacing or arrhythmias, the T wave "remembers" the QRS complex from the paced or arrhythmic phase. The electrocardiographic T wave vector during sinus rhythm reflects the QRS complex vector during the preceding period of ventricular activation (Rosen, 2001).

Cardiac memory in dogs is associated with a decrease in I_{to} and a reduction in $K_V4.3$ mRNA and KChIP2 mRNA and protein (Yu et al., 1999; Patberg et al., 2003). $K_V4.2$, KChIP2, and the angiotensin-1 receptor co-localize and immunoprecipitate together in heart tissue (Doronin et al., 2004). Furthermore, when

ventricular pacing alters cardiac stretch, angiotensin-II is released (Sadoshima and Izumo, 1993). Activation of the angiotensin-1 receptor results in an internalization of the macromolecular complex and a decrease in I_{to} density. Secondly, during prolonged periods of ventricular pacing inducing cardiac memory, cyclic AMP response element binding protein (CREB) is downregulated via ubiquitination and proteosomal degradation (Ozgen et al., 2010). Transcriptional activity of KChIP2 is reduced by low levels of CREB as this fails to associate with cyclic AMP response elements in the KChIP2-gene promotor region, indirectly causing a further reduction in I_{to} (Patberg et al., 2005). In summary, altered ventricular pacing causes a rapid internalization if the K_V4 ion channel complex and a reduction in KChIP2 levels further reducing I_{to} (Rosen and Cohen, 2006). Angiotensin-1 receptor blockade do not suppress the latter pathway, whereas inhibition of $I_{Ca,L}$ attenuates cardiac memory and I_{to} downregulation, suggesting a role central for calcium handling in the development of cardiac memory (Plotnikov et al., 2003).

HYPERTROPHY

Hypertrophic enlargement of the ventricles involves complex cellular processes, and is typically divided into concentric hypertrophy due to pressure overload and eccentric hypertrophy due to volume overload. Hypertrophy is associated with growth of the individual cardiomyocyte which adds sarcomeres to the existing contractile apparatus, thus responding to the increased demand on the ventricle. In contrast, the failing heart cannot compensate sufficiently, and is impaired in its ability to sustain an adequate blood flow to the body. Among a plethora of changes, cardiac hypertrophy is accompanied by electrophysiological remodeling causing prolonged action potential durations (Gaughan et al., 1998; Kaab et al., 1998; Marionneau et al., 2008). Ion channel remodeling in cardiac hypertrophy and failure has been described recently in excellent reviews (Nattel et al., 2007; Nass et al., 2008; Aiba and Tomaselli, 2010). Here, we focus on KChIP2.

Modulating I_{to} affects action potential duration, intracellular calcium load and contractile force in a complex manner. In larger mammals, pharmacological activation of I_{to} lead to prolongation of the action potential in a concentration-dependent manner (Calloe et al., 2009a). Moreover, large increases in I_{to} to a point where $Ca_V1.2$ fails to activate, action potentials show a dramatic shortening (Greenstein et al., 2000; Sah et al., 2003; Calloe et al., 2011). Selective pharmacological inhibition of $I_{to,f}$ in species with a spike-and-dome morphology action potential is yet to be studied.

In rodents, I_{to} is part of the terminal repolarization and action potential prolongation is consistently seen in the absence of the current (Nerbonne et al., 2001). This action potential prolongation elevates cytosolic calcium levels and improves excitation–contraction coupling by mechanisms distinct from the effects of I_{to} modulation in larger mammals (Sah et al., 2003). Furthermore, an elevated calcium level activates the calcium-sensitive phosphatase calcineurin and initiates hypertrophic signaling (Kassiri et al., 2002; Zobel et al., 2002; Lebeche et al., 2004). Transcriptional regulation of many hypertrophic genes is regulated via NFAT by the activity of calcineurin (Gaughan et al., 1998; Zobel et al., 2002; Gong et al., 2006; Jin et al., 2010). It appears that an increased cytosolic calcium concentration during adrenergically induced hypercontractility activates calcineurin initiating the hypertrophic response. Hypertrophy is not observed in models with genetically reduced $I_{to,f}$ in the absence of action potential prolongation, potentially due to chronic upregulation of other repolarizing currents (Kuo et al., 2001; Guo et al., 2005). Interestingly, concentric hypertrophy in WT mice secondary to aortic banding is associated with prolonged action potentials, decreased $I_{to,f}$ and an increased protein level of the ion channel underlying $I_{K,slow1}$ (Marionneau et al., 2008), whereas angiotensin-II mediated hypertension causing cardiac hypertrophy leaves $I_{to,f}$ density and $K_V4.2$, $K_V4.3$, and KChIP2 protein contents unaltered.

Using KChIP2 overexpression to successfully prevent hypertrophy in rats showed that a KChIP2-mediated upregulation of I_{to} shortened action potentials and reduced myocardial shortening (Jin et al., 2010). In the same study, the authors showed that KChIP2 overexpression decreased calcineurin expression *in vitro*, suggesting that action potential shortening abrogates the hypertrophic response via the calcineurin pathway. Moreover, simultaneous overexpression of $K_V4.3$ and induction of concentric hypertrophy in rat hearts showed a robust increase in I_{to}, action potential abbreviation, reduced calcineurin expression and an attenuated hypertrophic response (Lebeche et al., 2004).

Transgenic mice overexpressing calcineurin have a reduced $I_{to,f}$, prolonged action potential duration and display cardiac hypertrophy, which could all be reversed by pharmacological inhibition of calcineurin (Dong et al., 2003, 2010). In young mice overexpressing calcineurin, a paradoxical increased $I_{to,f}$ was measured, secondary to elevated $K_V4.2$ expression (Gong et al., 2006). It was hypothesized that two regulatory pathways operated in parallel, one leading to positive regulation of $K_V4.2$ transcription via activation of calcineurin and NFAT translocation (Gong et al., 2006); and one that via NF-κB phosphorylation reduces $I_{to,f}$ via downregulation of KChIP2 and $K_V4.3$ (Panama et al., 2011).

HEART FAILURE

Heart failure is the condition, where the ability of the heart to adequately supply blood flow to meet the demand of the body is impaired. Whereas the underlying index events are often manageable if identified early, treatment options for heart failure are mainly palliative or merely slowing the relentless progression of symptoms (Dickstein et al., 2008). At end-stage heart failure, therapeutic options are heart transplantation or mechanical support devices. The lifetime risk of acquiring heart failure is estimated to 20% (Lloyd-Jones et al., 2002) and the 5-year mortality rate after diagnosis is 50% (Stewart et al., 2001).

Functional reduction of I_{to} has been observed in many animal models of heart failure (Nass et al., 2008) in addition to human samples from terminal heart failure transplants (Beuckelmann et al., 1993; Wettwer et al., 1994; Nabauer et al., 1996). $K_V4.3$ mRNA is reduced in human (Kaab et al., 1998) and canine heart failure (Akar et al., 2005). KChIP2 mRNA and protein is downregulated in human heart failure (Radicke et al., 2006; Soltysinska et al., 2009), although reports of unchanged KChIP2 levels are also available (Zicha et al., 2004). Heart failure secondary to pressure overload reduces peak potassium currents in mice, but does not completely abolish $I_{to,f}$ (Wang et al., 2007; Grubb et al., 2011).

Furthermore, preliminary studies show a reduction of non-$I_{to,f}$ currents in KChIP2$^{-/-}$ mice with heart failure, adding another layer of complexity to the electrophysiological remodeling seen in heart failure (Grubb et al., 2011).

LIMITATIONS

In order to translate findings in experimental models to useful information in a clinical setting, a clear understanding of species dependent differences is electrophysiology of rodents and larger mammals is imperative. Compatible with having a higher heart rate than larger mammals, rodents have a much shorter action potential and I_{to} is involved with total repolarization, whereas it constitutes the notch between the spike-and-dome in the action potential of larger mammals (for review, see Nerbonne et al., 2001). In rodents, a loss of I_{to} leads to a prolonged APD which results in a loss of temporal synchronization of the Ca^{2+} entry through $I_{Ca,L}$ due to channel inactivation instead of deactivation causing a longer Ca^{2+} entry and a stronger but less synchronized SR Ca^{2+} release. In larger mammals, instead of being responsible for $I_{Ca,L}$ deactivation, I_{to} provides a driving force for Ca^{2+} entry, and a decreased I_{to} therefore leads to an $I_{Ca,L}$ reduction and a desynchronized intracellular Ca^{2+}-transient (for review, see Sah et al., 2003). This is an important species dependent difference, and should be considered when using rodents as a model of hypertrophy or heart failure. Moreover, extrapolation of physiological data from rodent studies to clinical practice should always be performed with outmost caution and only with supporting results from additional experimental models and human trials.

The KChIP2$^{-/-}$ mouse does not show any apparent pathology; however, it is not uncommon that knockout animals lack phenotypic changes which may be due to a redundancy of genes that can compensate for the reduced repolarization reserve. The KChIP2$^{-/-}$ mouse is not an organ-specific knockout, which may have unspecific, peripheral effects compared to a cardiac-specific knockout mice, since many genes are expressed in several organs, including KChIP2 (An et al., 2000). Furthermore, the knockout of KChIP2 is congenital and non-inducible, which means that remodeling in the knockout animal could happen during development. This could be avoided by using an inducible organ-specific knockout or a tissue specific gene transfer by viral infection, which would also be preferable for studying the remodeling in hypertrophy or heart failure, since no pre-existing remodeling would exist.

SUMMARY AND CONCLUDING REMARKS

Substantial progress has been made in the mapping of the multiple characteristics and roles of KChIP2 in the heart. KChIP2 have been reported to modulate $K_V1.5$, $K_V4.2$, $K_V4.3$, $Ca_V1.2$, and $Na_V1.5$. Both $K_V4.2$ and $K_V4.3$ are intimately regulated by KChIP2, where peak current is increased, inactivation kinetics are slowed and recovery from inactivation is accelerated by the presence of KChIP2 (An et al., 2000). $Ca_V1.2$-conducted current is increased by KChIP2 via a potential interaction at the amino-terminal inhibitory module on the α_{1C} subunit of the L-type Ca^{2+} channel (Thomsen et al., 2009c). Conflicting reports regarding KChIP2-mediated modulation of $K_V1.5$ and $Na_V1.5$ exists and points toward areas where intensified research is required to resolve discrepancies and clarify our understanding of cardiac electrophysiology. Cardiac memory, hypertrophy, and failure are all associated with reductions in KChIP2 and it appears that overexpression of KChIP2 can prevent the development of hypertrophy in rats (Jin et al., 2010).

Several aspects remains poorly understood, however. Given the promiscuity of KChIP2, it is possible that additional binding partners will be identified, potentially in non-cardiac tissue. The physiological relevance of the many individual interactions needs to be tested and compared in health and disease. The finding that KChIP2 overexpression can prevent the development of the hypertrophic response may have implications for future clinical approaches to heart disease, although extrapolation from rodent to patient should be done with outmost caution. Excessive KChIP2 overexpression increasing I_{to} may in spite of an augmented inward $I_{Ca,L}$ lead to an early repolarization in mammals associated with a Brugada-like phenotype (Calloe et al., 2009a). Presently it is unknown whether KChIP2 holds regulatory control in the development of heart failure similar to its central role in the progress of cardiac hypertrophy. Furthermore, the antiarrhythmic properties of KChIP2 are still ill described, notwithstanding its initial report (Kuo et al., 2001). Reduced KChIP2 levels confer smaller $I_{Ca,L}$ potentially reducing the amplitude of early afterdepolarizations in events of current reactivation at long action potentials, reducing the risk of triggering ventricular arrhythmias. However, optimal KChIP2 levels contribute to maintaining minimal regional repolarization dispersion, thus reducing the substrate for arrhythmias. Nonetheless, KChIP2 is a multipronged modifier of a series of voltage-gated cardiac ion channels and has important physiological functions in health and in heart disease.

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Mouse models of *SCN5A*-related cardiac arrhythmias

Mickael Derangeon[1,2,3], Jérôme Montnach[1,2,3], Isabelle Baró[1,2,3] and Flavien Charpentier[1,2,3]*

[1] *INSERM, UMR 1087, l'Institut du Thorax, Nantes, France*
[2] *CNRS, UMR 6291, l'Institut du Thorax, Nantes, France*
[3] *Université de Nantes, Nantes, France*

Edited by:
Carol Ann Remme, University of Amsterdam, Netherlands

Reviewed by:
Toon Van Veen, University Medical Center Utrecht, Netherlands
Ming Lei, University of Manchester, UK

****Correspondence:***
Flavien Charpentier, INSERM, UMR1087, CNRS UMR6291, l'Institut du Thorax, IRT-UN, 8 quai Moncousu, BP 70721, 44007 Nantes Cedex 1, France.
e-mail: flavien.charpentier@inserm.fr

Mutations of *SCN5A* gene, which encodes the α-subunit of the voltage-gated Na^+ channel $Na_V1.5$, underlie hereditary cardiac arrhythmic syndromes such as the type 3 long QT syndrome, cardiac conduction diseases, the Brugada syndrome, the sick sinus syndrome, a trial standstill, and numerous overlap syndromes. Patch-clamp studies in heterologous expression systems have provided important information to understand the genotype-phenotype relationships of these diseases. However, they could not clarify how *SCN5A* mutations can be responsible for such a large spectrum of diseases, for the late age of onset or the progressiveness of some of these diseases and for the overlapping syndromes. Genetically modified mice rapidly appeared as promising tools for understanding the pathophysiological mechanisms of cardiac *SCN5A*-related arrhythmic syndromes and several mouse models have been established. This review presents the results obtained on these models that, for most of them, recapitulate the clinical phenotypes of the patients. This includes two models knocked out for Nav1.5 β1 and β3 auxiliary subunits that are also discussed. Despite their own limitations that we point out, the mouse models still appear as powerful tools to elucidate the pathophysiological mechanisms of *SCN5A*-related diseases and offer the opportunity to investigate the secondary cellular consequences of *SCN5A* mutations such as the expression remodeling of other genes. This points out the potential role of these genes in the overall human phenotype. Finally, they constitute useful tools for addressing the role of genetic and environmental modifiers on cardiac electrical activity.

Keywords: $Na_V1.5$, arrhythmia, conduction disease, long QT syndrome, Brugada syndrome, sinus node dysfunction

Mutations in the *SCN5A* gene, which encodes the α-subunit of the cardiac voltage-gated Na^+ channel $Na_V1.5$, underlie hereditary arrhythmic syndromes (so-called cardiac channelopathies). On one hand, gain-of-function mutations, that increase the late component of the Na^+ current (I_{Na}) and thus prolong the ventricular action potential, are responsible for the type 3 long QT syndrome (LQT3; Moss and Kass, 2005). On the other hand, loss-of-function mutations decrease I_{Na} and are responsible for cardiac conduction diseases (Schott et al., 1999; Tan et al., 2001; Probst et al., 2003), Brugada syndrome (Gussak et al., 1999), sick sinus syndrome (Benson et al., 2003), and atrial standstill (Groenewegen et al., 2003). To complicate matters further, some *SCN5A* mutations can lead to more complex diseases associating different phenotypic traits such as, for instance, bradycardia, conduction disease, LQT3, and Brugada syndrome (so-called overlap syndromes; Bezzina et al., 1999; Kyndt et al., 2001; Grant et al., 2002; Rossenbacker et al., 2004; Smits et al., 2005; for review, see Remme et al., 2008). Finally, there is also an association between *SCN5A* genetic defects and susceptibility to dilated cardiomyopathy (DCM; McNair et al., 2004) and atrial fibrillation (Laitinen-Forsblom et al., 2006; Ellinor et al., 2008).

Although patch-clamp studies in heterologous expression systems have provided a great deal of information to understand the genotype-phenotype relationships of these diseases, these models could not clarify how loss-of-function-mutations can be responsible for such a large spectrum of diseases and the late age of onset or the progressiveness of some of them. They are even less adapted to overlap syndromes.

Genetically modified mice thus appeared as promising tools for understanding the pathophysiological sequence of cardiac *SCN5A*-related channelopathies and several mouse models have been established (**Table 1**). Fortunately, whereas some genetically modified mice related to K^+ channel dysfunction did not reach investigators' expectations because of major electrophysiological differences between mice and men (Nerbonne et al., 2001; Charpentier et al., 2004), mouse models of inherited *SCN5A* channelopathies, on the contrary, appeared to be informative.

MODELS FOR THE LQT3 SYNDROME

SCN5A-related long QT syndrome (LQT3; Wang et al., 1995) is less frequent but often more lethal than LQT1 and LQT2 (Priori et al., 2003). Like in other LQT syndromes, the abnormal prolongation of ventricular repolarization is associated with a susceptibility to a specific polymorphic ventricular tachycardia called *torsades de pointes* and ventricular fibrillation, leading to syncope and sudden death. Clinically, LQT3 is characterized by an increased duration of the ST segment with a late appearance of the T-wave (Moss, 2002). Bradycardia and pauses occurring at rest, and more particularly during sleep, are often at the origin of the arrhythmias. However, fatal tachycardia-induced arrhythmias have also been reported for a third of the patients (Schwartz et al., 2001). Two thirds of the *SCN5A* mutations found in LQT3 alter the fast inactivation process

Table 1 | Mouse models of *SCN5A*-related cardiac genetic diseases.

Mouse	Genetic modification	Human disease	ECG phenotype	Arrhythmias	Effect on I_{Na}	Original publication
Scn5a$^{\Delta/+}$	KI/heterozygote/ 150-5KPQ-1507 del	LQT3	QT prolongation	VT EADs *in vitro*	Increased late current	Nuyens et al. (2001), Head et al. (2005)
h*SCN5A*-N1325S	Tg/point mutation	LQT3	QT prolongation	VT EADs *in vitro*	Delayed inactivation increased late current	Tian et al. (2004)
Scn5a$^{\pm}$	KO/heterozygote	Brugada, PCCD	P wave, PR and QRS prolongation	Triggered VT (spontaneous in old)	≈50% decrease in peak current	Papadatos et al. (2002)
Scn5a$^{1798insD/+}$	KI/heterozygote/ insertion	Overlap syndrome (LQT3 – Brugada – CCD)	PR, QRS and QT prolongation	Sinus pauses EADs *in vitro*	≈40% decrease in peak current, increased late current	Remme et al. (2006)
SCN5A-D1275N	RMCE/hetero- and homozygote/ point mutation	Arrhythmogenic DCM	Bradycardia, P wave, PR and QRS prolongation	AT/AF, VT and sinus node dysfunction in homozygote	≈50% decrease in peak current (heterozygote)	Watanabe et al. (2011)
Scn1b$^{-/-}$	KO/hetero- and homozygote	LQT3	Bradycardia and QT prolongation (homozygote)	Not investigated	≈60% increase in peak and late current (homzygote)	Lopez-Santiago et al. (2007)
Scn3b$^{-/-}$	KO/homozygote	Brugada, CCD	Bradycardia, P wave and PR prolongation	Triggered VT spontaneous and triggered AT	≈50% decrease in peak current, negative shift of steady-state inactivation	Hakim et al. (2008)

AT, atrial tachycardia; AF, atrial fibrillation; DCM, dilated cardiomyopathy; del, deletion; EAD, early afterdepolarization; KI, knock-in; KO, knockout; LQT3, type 3 long QT syndrome; PCCD, progressive cardiac conduction defects; RMCE, recombinase mediated cassette exchange; VT, ventricular tachycardia; Tg, transgenic.

of the channel (Zimmer and Surber, 2008). For example, the first identified mutation, which leads to the deletion of three amino acids (1505-KPQ-1507) in the inactivation domain of $Na_V1.5$, results in a persistent inward Na^+ current (Wang et al., 1996) and prolongation of the action potential plateau phase (Moss and Kass, 2005).

SCN5A$^{\Delta 1505\text{-}1507KPQ/+}$ KNOCK-IN MOUSE

Nuyens et al. (2001) generated a knock-in mouse model lacking the same KPQ residues. Mice heterozygous for this mutation (*Scn5a*$^{\Delta/+}$) showed typical features of the LQT3 syndrome and spontaneously developed ventricular arrhythmias. Compared with wild-type mice, transmembrane action potentials recorded in ventricular preparations were markedly prolonged in *Scn5a*$^{\Delta/+}$ mice and showed a steeper "heart rate" versus "action potential duration" relationship (Nuyens et al., 2001). Action potential amplitude and upstroke velocity were not altered. Whole-cell patch-clamp recordings performed on cardiomyocytes confirmed the increase in late inward Na^+ current previously observed in heterologous expression system, which explains the action potential prolongation (Wang et al., 1996). Surprisingly, there was also a two-fold increase in the peak Na^+ current density, which could not be explained by an over-expression of $Na_V1.5$ or changes in the steady-state activation and inactivation of the current. However, this finding was not confirmed in a more recent study on the same model (Fredj et al., 2006) and the reason for this discrepancy remains to be clarified. Nuyens et al.'s (2001) study also revealed an acceleration of the recovery from inactivation, a property that might favor re-entrant arrhythmias. Indeed, intracardiac pacing with extra stimuli, short-long-short pacing sequences and abrupt accelerations of heart rate reproducibly induced polymorphic ventricular arrhythmias in *Scn5a*$^{\Delta/+}$ mice but not in wild-type mice. The *Scn5a*$^{\Delta/+}$ mice were also characterized by a paradoxical transient action potential prolongation upon abrupt acceleration of pacing rate that favored the occurrence of early after depolarizations. This prolongation was blocked by the Na^+ channel blocker mexiletine and the adrenergic agonist isoproterenol. It can be explained by the increase of both fast and slow components of the Na^+ current which causes a sudden increase of Na^+ load, and secondarily of Ca^{2+} load. As a consequence, during each action potential, the increased Ca^{2+} release from the sarcoplasmic reticulum activates a larger Na/Ca exchanger current which generates early after depolarizations. The autonomic modulation of cardiac electrical activity and arrhythmias of *Scn5a*$^{\Delta/+}$ mice has been recently investigated in more details both *in vivo* in freely moving mice and *ex vivo* (Fabritz et al., 2010). As expected from clinical investigations, cholinergic stimulation was shown to favor arrhythmias by inducing bradycardia. In contrast, β-adrenergic stimulation suppressed arrhythmias by shortening repolarization and increasing cardiac rate, while β-blockers had no effect on their own.

A second *Scn5a*$^{\Delta/+}$ mouse line, exhibiting the same phenotype as the previous line, was created (Head et al., 2005) and submitted to an extended pharmacological analysis. Electrophysiological studies performed on Langendorff-perfused whole hearts confirmed the anti-arrhythmic properties of mexiletine (Fabritz et al., 2003; Head et al., 2005) and showed that another Na^+ channel inhibitor, flecainide, could also prevent arrhythmias by preferentially inhibiting the late Na^+ current (Fredj et al., 2006), whereas β-blockers were inefficient (Head et al., 2005; Stokoe et al., 2007b;

Sabir et al., 2008). These results have been confirmed in freely moving mice (Fabritz et al., 2010). More recently, it was shown that a specific inhibition of the L-type Ca^{2+} channels with nifedipine was also efficient in preventing arrhythmias through the suppression of early after depolarizations (Thomas et al., 2007). Finally, nicorandil, an activator of the ATP-sensitive K^+ channel, also reduces arrhythmogenicity in *Scn5a*$^{\Delta/+}$ mice (Hothi et al., 2008), confirming previous results obtained with the canine wedge preparation pharmacological model of LQT3 (Shimizu and Antzelevitch, 2000). In contrast, quinidine was proarrhythmic (Sabir et al., 2008). *Ex vivo* studies also confirmed the key role played by bradycardia and increased dispersion of repolarization on the occurrence of spontaneous ventricular arrhythmias (Fabritz et al., 2003). Sudden rate accelerations initially and transiently increased the dispersion of repolarization due to early after depolarizations and action potential alternans (Fabritz et al., 2003; Hothi et al., 2008), as previously observed *in vivo* (Nuyens et al., 2001), and then secondarily suppressed and prevented ventricular tachycardia by decreasing dispersion of repolarization and suppressing early after depolarizations. Arrhythmogenesis, as assessed by programmed electrical stimulation on Langendorff-perfused hearts, has also been associated with abnormal patterns of myocardial activation and abnormally reduced transmural gradients of repolarization resulting from a larger increase in action potential duration in subepicardium than in subendocardium (Stokoe et al., 2007b). In addition, *Scn5a*$^{\Delta/+}$ mice exhibit larger differences between action potential duration and refractory period, so-called local critical interval (APD-ERP), in subepicardial regions than in wild-type mice. Those anomalies may support re-entrant events following premature excitation. More recently, Sabir et al. (2008) proposed that the increases of the slopes of restitution curves (plotting action potential duration versus the preceding diastolic interval; Gilmour, 2003) to values greater than one, rather than increased dispersion of repolarization, was associated with arrhythmogenicity. All these anomalies, including action potential alternans are corrected by nicorandil (Hothi et al., 2008).

Altogether, *Scn5a*$^{\Delta/+}$ mice recapitulate most of the LQT3 associated clinical symptoms. They confirmed previous studies suggesting that arrhythmias in LQT3 syndrome may result from reentries provoked by early after depolarizations and enabled to unveil the mechanisms of tachycardia-induced arrhythmias. Studies of *Scn5a*$^{\Delta/+}$ mice have also provided a rationale for the use of Na^+ channel blockers and pacing to prevent cardiac arrhythmias and sudden death in the context of LQT3, for which β-blockers have shown low efficacy. Finally, they suggest that Ca^{2+} channels inhibitors and ATP-sensitive K^+ channel activators might be interesting pharmacological approaches to prevent arrhythmias in this context.

In addition to early after depolarizations, the occurrence of delayed after depolarizations, resulting from the transient inward current (I_{ti}), was also clearly demonstrated in myocytes of *Scn5a*$^{\Delta/+}$ mice (Fredj et al., 2006). Most recently, Lindegger et al. (2009) showed that I_{ti} induction occurs under conditions of elevated sarcoplasmic reticulum Ca^{2+} content that is most probably related to the mutation-induced increase in late Na^+ current. Indeed, both I_{ti} and its underlying Ca^{2+} wave were suppressed by ranolazine, an antianginal agent well known to block the late Na^+ current (Hale et al., 2008). Whether delayed after depolarizations participate to spontaneous arrhythmic events in *Scn5a*$^{\Delta/+}$ mice, and of course in LQT3 patients, remains to be determined. Investigating the potential anti-arrhythmic effects of ranolazine in this model also remains to be performed.

Patients with LQT syndrome (El Yaman et al., 2008; Johnson et al., 2008; Zellerhoff et al., 2009), including LQT3 (Benito et al., 2008), may be at increased risk for atrial fibrillation. Therefore, the atrial phenotype of *Scn5a*$^{\Delta/+}$ mice was characterized. Using Langendorff-perfused hearts, Blana et al. (2010) showed that atrial action potential duration and effective refractory period were prolonged in *Scn5a*$^{\Delta/+}$ mice. Short runs of rapid pacing-induced a larger increase in post-pacing spontaneous atrial cycle length and action potential duration in *Scn5a*$^{\Delta/+}$ mice than in wild-type mice, leading to the occurrence of atrial tachycardia in *Scn5a*$^{\Delta/+}$ mice but not in wild-type mice. Extra stimuli triggered arrhythmias similarly in wild-type and *Scn5a*$^{\Delta/+}$ mice (in 30–40% of the mice). Contrasting results had been previously observed with the second mouse line suggesting that *Scn5a*$^{\Delta/+}$ mice are protected against pacing-induced atrial tachyarrhythmias when young (3 months; Dautova et al., 2009), whereas they exhibit an increased atrial arrhythmogenicity when old (1 year; Guzadhur et al., 2010). Without excluding the influence of variable experimental conditions, those discrepancies might also rely on the fact that the two lines have different genetic backgrounds, as we will discuss later for the *Scn5a*$^{1798insD/+}$ knock-in mouse. Both mouse lines, however, exhibit decreased heart rate and atrioventricular conduction (prolonged PR interval) versus wild-type mice.

Sinus node dysfunction has been reported in patients with *SCN5A*-related long QT syndrome whether these mutations only lead to an increased late Na^+ current (Moss et al., 1995; Wei et al., 1999) or lead to more complex biophysical alterations of the channel (Bezzina et al., 1999; van den Berg et al., 2001; Grant et al., 2002). Because QT prolongation is more pronounced at lower heart rates, bradycardia represents an important indirect factor in predisposition to lethal arrhythmias in LQT3 families (Schwartz et al., 1995). The mechanisms for LQT3-related bradycardia were investigated for the 1795insD mutation of $Na_V1.5$ (Veldkamp et al., 2003), a mutation inducing an overlap syndrome with bradycardia, conduction disease, LQT3 and Brugada syndrome (Bezzina et al., 1999). This mutation induces both a decrease in Na^+ peak current and the occurrence of a late Na^+ current (see dedicated section below for details). By combining current clamp studies and computational modeling, Veldkamp et al. (2003) suggested that both the decreased availability of Na^+ channels and the increase of late current accounted for bradycardia. However, the effect of action potential prolongation secondary to the increased late current appeared more prominent. The contribution of the late Na^+ current to sinus node dysfunction has been further confirmed on *Scn5a*$^{\Delta/+}$ mice (Wu et al., 2012). *Scn5a*$^{\Delta/+}$ mice exhibit sinus node dysfunction with episodes of bradycardia, sinus pauses, and longer sinus node recovery times. Sino-atrial preparations from *Scn5a*$^{\Delta/+}$ mice had lower intrinsic rate from the sinus node to the surrounding atrium than wild-type preparations. Computational studies confirmed that the decrease in sinus rate could be attributed to the increased late current. *Ex vivo* experiments on sino-atrial preparations also showed that conduction through the

sinus node to the surrounding atrium was decreased, a result that computer modeling could only explain by a decrease in Na^+ peak current amplitude, which has not been experimentally confirmed.

hSCN5A-N1325S TRANSGENIC MOUSE MODEL

The N1325S mutation of $Na_V1.5$, located in the intracellular region between segments 4 and 5 of domain III, also causes LQT3 syndrome (Wang et al., 1995). This mutation, like many similar mutations, leads to an increased late inward Na^+ current (Dumaine et al., 1996; Wang et al., 1996). A transgenic mouse with cardiac selective expression of human *SCN5A*-N1325S mutation under the control of the mouse α-myosin heavy chain promoter was established (Tian et al., 2004). Transgenic mice showed some of the typical features of the LQT3 syndrome such as prolonged QT interval, ventricular arrhythmias, and a high rate of sudden deaths due to documented polymorphic ventricular tachycardia and ventricular fibrillation. However, transgenic mice were also characterized by increased heart rate and shorter PR intervals that are not observed in LQT3 patients. Some of these features might result from over-expression of the channel rather than from the mutation itself since short PR interval was also observed in the control mouse over-expressing wild-type h*SCN5A* gene (Zhang et al., 2007).

Voltage-clamp experiments on cardiomyocytes isolated from wild-type and h*SCN5A*-N1325S mice confirmed that the N1325S mutation does not modify the peak current but delays the inactivation process and generates a late persistent current (Tian et al., 2004), which decreases with increasing pacing rates (Yong et al., 2007). Action potentials from h*SCN5A*-N1325S ventricular myocytes were longer than wild-type ones and exhibited early after depolarizations. As in $Scn5a^{\Delta/+}$ mice, mexiletine shortened ventricular action potential duration and prevented arrhythmias (Tian et al., 2004). Interestingly, action potential duration of transgenic but not wild-type myocytes paradoxically increased with pacing rate (Tian et al., 2004) and became highly variable with occurrence of repolarization alternans (Yong et al., 2007). Whether this phenomenon was only transient, as in $Scn5a^{\Delta/+}$ mice, or persisted during long periods of increased pacing rate is unclear. The Ca^{2+} channel blocker verapamil reduced these alternans, suggesting an involvement of intracellular Ca^{2+} deregulation in this phenomenon. Previous studies had shown that patients with long QT syndrome can exhibit T-wave alternans during sinus tachycardia (Cruz Filho et al., 2000; Viskin et al., 2004).

Like the $Scn5a^{\Delta/+}$ mouse model, the h*SCN5A*-N1325S mouse provided key information to our understanding of arrhythmias in LQT3 syndrome. For instance, it showed that the N1325S mutant dysfunction can explain both bradycardia- and non-bradycardia-related arrhythmias observed in patients carrying this mutation (Yong et al., 2007). However, the model might be taken with caution because of the limitations linked to the over-expression of the channel. Indeed, there is a clear relationship between the level of h*SCN5A*-N1325S expression and the extent of QT prolongation, i.e., by 23% with 1–2 copies and by 91% with 10 copies. Most recently, this model was shown to exhibit cardiomyocyte apoptosis, cardiac fibrosis, and contractile dysfunction during aging (Zhang et al., 2011). But signs of heart failure were markedly larger in the mouse line with 10 copies. Although heart failure is probably not related to *SCN5A* over-expression *per se*, because old mice over-expressing 10 times the wild-type channel remained normal, one should better consider the model over-expressing the least the mutant channel to extrapolate the results to the patients. In this second line, left ventricular fractional shortening was less decreased and only 30% of the mice showed features of DCM, a result more consistent with the scarcity of heart failure in LQT3 patients (Nguyen et al., 2008; Shi et al., 2008).

A MODEL FOR THE BRUGADA SYNDROME AND CONDUCTION DISORDERS: THE *Scn5a* KNOCKOUT MOUSE

The Brugada syndrome is a genetic disease which associates ST segment elevation in the right precordial leads V1 to V3 of the ECG, often with signs of conduction slowing, with a high risk of sudden cardiac death secondary to ventricular tachycardia or fibrillation (Gussak et al., 1999; Antzelevitch et al., 2005). In 20–30% of the patients, the disease is related to mutations in *SCN5A* leading to a complete or partial loss-of-function of $Na_V1.5$ (Tan et al., 2003). Loss-of-function mutations are also responsible for inherited Progressive Cardiac Conduction Defects (PCCD), also called Lev-Lenègre disease (Schott et al., 1999), or non-progressive conduction defects (Tan et al., 2001). More recently, PCCD has also been linked to gain-of-function mutations in *TRPM4* gene (Transient Receptor Potential cation channel, subfamily M, member 4), which encodes a Ca^{2+}-activated non-selective channel (Kruse et al., 2009; Liu et al., 2010). PCCD is a slowly evolving disease which progressively affects cardiac conduction leading ultimately to pacemaker implantation to prevent the risk of complete atrioventricular block and Stokes–Adams syncope. Inherited PCCD associates haploinsufficiency of *SCN5A* together with an additional yet unknown mechanism related to aging (Probst et al., 2003). Despite their major clinical differences, Brugada syndrome, and inherited conduction diseases share common features. First, both diseases can be caused by *SCN5A* haploinsufficiency. Second, Brugada patients with *SCN5A* mutations exhibit altered conduction whereas Brugada patients without *SCN5A* mutations do not (Smits et al., 2002). Finally, both Brugada syndrome and PCCD can be found in the same pedigree (Kyndt et al., 2001). In other families, patients carrying loss-of-function mutations can exhibit symptoms of Brugada syndrome, conduction disease, and sick sinus syndrome, either combined or not (Smits et al., 2005). Severe forms of sick sinus syndrome have also been found in patients with double inheritance of *SCN5A* loss-of-function mutations (Benson et al., 2003).

A mouse model with targeted disruption of *Scn5a* gene has been established (Papadatos et al., 2002). Homozygous knockout mouse embryos die during mid-gestation due to severe defects in ventricular morphogenesis whereas heterozygous ($Scn5a^{\pm}$) mice show normal survival. However, 8- to 10-week-old $Scn5a^{\pm}$ mice have several cardiac electrical defects such as decreased atrial and atrioventricular conduction and increased susceptibility to pacing-induced ventricular arrhythmias (Papadatos et al., 2002). Following this initial report, the model was further analyzed to investigate the potential pathophysiological mechanisms involved in the progressive evolution of *SCN5A*-related PCCD (Royer et al., 2005; van Veen et al., 2005). It was found that in addition to atrial and atrioventricular conduction, ventricular conduction was also

decreased. Moreover, ECG studies performed on mice ranging from 4 to 71 weeks of age showed that the ventricular phenotype of *Scn5a*$^{\pm}$ mice worsened with age. This feature was confirmed by activation mapping studies performed on Langendorff-perfused hearts. In young *Scn5a*$^{\pm}$ mice, conduction velocity was only affected in the right ventricle. In old mice, right ventricular conduction defect worsened and was in addition associated with conduction velocity defect in the left ventricle. This age-dependent deterioration of ventricular conduction was associated with the occurrence of fibrosis in ventricular myocardium. In some aspects, this phenotype resembles PCCD, although in the later fibrosis was found to be limited to the conduction system area (Lenègre and Moreau, 1963; Lev, 1964). However, it is worth noting that fibrosis has also been observed in patients with the Brugada syndrome (Coronel et al., 2005; Frustaci et al., 2005). More recently, it was shown that *Scn5a*$^{\pm}$ mice display variable degrees of conduction defects when young (10 weeks old): some mice have only mild prolongation of the QRS interval while others have more severe QRS prolongation associated with RR′ or SS′ patterns (Leoni et al., 2010). The extent of fibrotic remodeling was dependent on the severity of the initial conduction disorders: mice with larger QRS prolongation when young have the most severe fibrotic remodeling when they get older, suggesting that the ventricular activation pattern is a key element for triggering remodeling. Whatever their triggering mechanism, fibrosis, and redistribution of connexin 43 expression most likely contribute to the age-dependent degradation of ventricular conduction in the mouse model. Whether *SCN5A*-related inherited PCCD in human also results from a primary decrease in Na^+ current and a secondary progressive fibrosis with aging remains to be clarified. In this context, *Scn5a*$^{\pm}$ mice could be used for testing preventive therapies as an alternative to pacemaker implantation.

In their initial report on the *Scn5a*$^{\pm}$ mouse model, Papadatos et al. (2002) showed that programmed ventricular electrical pacing of Langendorff-perfused hearts induced ventricular tachyarrhythmias in two thirds of *Scn5a*$^{\pm}$ mice but not in wild-type mice and proposed that the model could be used for elucidating the mechanisms of arrhythmias in the context of *SCN5A*-related Brugada syndrome. Pathophysiological findings have implicated the right ventricular outflow tract as the primary location for electrical disorders in Brugada syndrome. Two hypotheses have been proposed to explain both ST segment elevation and occurrence of arrhythmias (Wilde et al., 2010). The first one, mainly based on experimental data obtained in arterially perfused canine right ventricular wedges, implicates alterations in transmural and regional repolarization gradients leading to localized losses of the subepicardial action potential dome and phase 2 reentries as the arrhythmogenic mechanism. The second one, mainly based on clinical studies, implicates abnormal conduction. To elucidate the arrhythmogenic mechanisms in *Scn5a*$^{\pm}$ mice programmed electrical stimulation, monophasic action potential (MAP) recording, and bipolar electrogram recording was performed on Langendorff-perfused hearts. These experiments showed that *Scn5a*$^{\pm}$ mice were characterized by increased electrogram duration with shortening extrasystoles intervals, especially in the right ventricular outflow tract, and increased transmural and regional heterogeneity of MAP duration and refractory periods in the right ventricle with the shortest in the right ventricular outflow tract (Stokoe et al., 2007a; Martin et al., 2010, 2011a,b). *Scn5a*$^{\pm}$ mice also exhibited increased incidence of MAP alternans. As a consequence, ventricular tachycardia occurred earlier in the right ventricular outflow tract (Martin et al., 2011b). All these mechanisms were potentiated by flecainide, a Na^+ channel inhibitor which is well described to favor ST segment elevation and arrhythmias in patients with the Brugada syndrome. Ventricular tachycardia also occurred spontaneously both *in vivo* (Leoni et al., 2010; Martin et al., 2011c) and *ex vivo* (Martin et al., 2011d), but with a much higher incidence under flecainide treatment (Martin et al., 2011c,d). Multielectrode array studies showed that the initiation of spontaneous arrhythmias resulted from a combination of conduction slowing and repolarization heterogeneities leading to lines of conduction blocks and unidirectional conduction (Martin et al., 2011d). Altogether these results seem to contradict the hypothesis that arrhythmias in the Brugada syndrome result from phase 2 re-entrant mechanisms. However, the main limitation of the mouse model is that the ventricular action potential lacks a plateau phase, and for the subepicardial action potential, a spike, and dome morphology, present in larger mammals and prerequisite for phase 2 reentries.

In addition, *Scn5a*$^{\pm}$ mice turned out to be useful for understanding the role of $Na_V1.5$ in sinus node normal function and the pathophysiology of sinus node dysfunction associated to *SCN5A* loss-of-function mutations (Benson et al., 2003; Smits et al., 2005). Indeed, *Scn5a*$^{\pm}$ mice also display moderate bradycardia that can be associated with occasional sino-atrial blocks in older animals (Lei et al., 2005). Lei et al. showed that small central sino-atrial node cells, which do not express $Na_V1.5$, exhibited normal intrinsic pacemaker rate. In contrast, larger peripheral sino-atrial node cells, which normally express $Na_V1.5$, exhibited reduced intrinsic pacemaker rate associated to reduced expression of $Na_V1.5$. These data were consistent with the slower sino-atrial conduction and frequent sino-atrial conduction blocks observed in *Scn5a*$^{\pm}$ sino-atrial node preparations compared to wild-type although they exhibited normal activation patterns. A computer model successfully reproduced these findings and implicated I_{Na} in action potential propagation through the sino-atrial node, from sino-atrial node to atria, and in modifying heart rate through a coupling of sino-atrial node and atrial cells. More recently, this same team has shown that both aging and *Scn5a* disruption affect sino-atrial node function through electrical remodeling and TGF-β_1-mediated fibrosis, with the most severe phenotype observed in old *Scn5a*$^{\pm}$ mice (Hao et al., 2011). Interestingly, this *in vivo* sino-atrial node dysfunction appears less severe than expected from *ex vivo* experiments and computer simulations seems to be due to an increase in sympathetic regulation of sino-atrial node with age, partly explained by an over-expression of β_1-adrenoceptors (Hao et al., 2011).

AN OVERLAP SYNDROME MODEL: THE *Scn5a*$^{1798INSD/+}$ KNOCK-IN MOUSE

In 1999, a *SCN5A* mutation (1795insD) associated with an overlap syndrome of cardiac Na^+ channelopathies was described in a large Dutch family. The patients' ECG presented features of bradycardia, conduction disease, LQT3, and Brugada syndrome (Bezzina et al., 1999). The analysis of the 1795insD mutation

biophysical properties led to contrasting results depending on the heterologous expression system used. In *Xenopus oocytes*, the mutation reduced the Na^+ current through a negative shift in voltage-dependence of steady-state inactivation but did not produce the persistent current commonly observed in LQT3 (Bezzina et al., 1999). In contrast, in HEK293 cells, 1795insD mutation disrupted fast inactivation leading to sustained inward Na^+ current that could prolong repolarization at slow heart rates and, at the same time, increased slow inactivation, delaying recovery of Na^+ channel availability between stimuli and reducing the Na^+ current under conditions mimicking fast heart rates (Veldkamp et al., 2000). Computer modeling showed that this dual mechanism could indeed explain the overlap syndrome, depending on the heart rate (Clancy and Rudy, 2002). Because of these discrepancies and because the co-inheritance of other genetic variants besides the 1795insD mutation as a cause of the phenotype complexity in the family could not be ruled out, Remme et al. (2006) generated a knock-in mouse carrying the mouse equivalent (1798insD) of the human *SCN5A*-1795insD mutation. Homozygous mice are not viable. Heterozygous *Scn5a*$^{1798insD/+}$ mice recapitulate many of the pathological features of the patients including sinus node dysfunction, conduction slowing, and QT prolongation at slow rates. Patch-clamp experiments performed on ventricular *Scn5a*$^{1798insD/+}$ cardiomyocytes have shown that action potential duration was longer than in wild-type cardiomyocytes and that this prolongation was more pronounced at slow pacing rates. Also, at fast pacing rates, action potential upstroke velocity, which reflects Na^+ channel availability, was reduced in *Scn5a*$^{1798insD/+}$ mice compared to wild-type. However, the potential biophysical mechanisms thought to explain decreased availability of Na^+ channels in heterologous expression systems were not confirmed in *Scn5a*$^{1798insD/+}$ mice. Indeed, rather than changes in the voltage-dependence of activation and inactivation or slow inactivation properties, dysfunctional mutant channels at the membrane or ineffective trafficking of mutant channels to the membrane more likely explained the marked decrease of Na^+ current in *Scn5a*$^{1798insD/+}$ mice. In addition, a small persistent inward Na^+ current was found in *Scn5a*$^{1798insD/+}$ cardiomyocytes, explaining the prolongation of action potential duration and QT interval. These studies constitute another example of the limitations of heterologous expression systems and illustrate the benefits of genetically engineered mouse models. In summary, the phenotypic characterization of this mouse model has demonstrated that a single *SCN5A* mutation is sufficient to cause an overlap syndrome of cardiac Na^+ channel diseases.

This unique model also represented a useful tool to investigate the impact of genetic and environmental modifiers on cardiac conduction and repolarization. Indeed, also genetic background has often been proposed to affect the phenotypic consequences of ion channel mutations, the identification of genetic modifiers of disease severity in genetically inherited arrhythmias is rare (Scicluna et al., 2008). In order to identify genetic modifiers of conduction disease, Remme et al. (2009) studied the effect of the *Scn5a*-1798insD mutation in mice of two distinct inbred genetic backgrounds. They showed that the phenotype severity of mice carrying the *Scn5a*$^{1798insD/+}$ mutation varies depending on the genetic background. This variability is associated with differential expression of a large number of genes including some encoding ion channels. In particular, ventricular expression levels of $Na_V1.5$ regulatory β4-subunit (encoded by *Scn4b* gene) were markedly reduced in the mouse strain with the most severe conduction defects, leading to a shift of $Na_V1.5$ channel activation curve toward more positive voltages and consequently to a further decrease of I_{Na}. Most recently, these authors went a step further and applied linkage analysis to a F2 cross resulting from the two strains carrying the *Scn5a*-1798insD mutation to identify quantitative trait loci (QTL) for heart rate, ECG parameters, and susceptibility to arrhythmias (Scicluna et al., 2011). This first genetic mapping analysis for cardiac electrical traits in mice identified 3 QTL linked with heart rate (chromosome 4), PR interval (chromosome 3), and QRS interval (chromosome 7) at baseline. Additional significant linkage to chromosome 4 locus was observed for post-flecainide heart rate and ventricular arrhythmias. Unexpectedly, numerous sex-specific QTLs were also identified. Finally, QTL for ECG parameters and arrhythmias coincided at five chromosomal locations suggesting pleiotropic effects at these loci. Interestingly, identified QTLs coincided with chromosomal locations of genes found differentially expressed between the two parental strains in the prior study (Remme et al., 2009). This considerably reduces the number of candidate genes responsible for these QTLs. Nevertheless, additional experiments are still needed to reduce linkage intervals and identify the causal genes. In spite of their success, genome-wide association studies (GWAS) performed in large human populations identified only a very small fraction of heritability of ECG traits and often failed to identify the causal genes. In this context, QTL analysis studies performed in mice could provide independent information pointing to the right genes (Dina, 2011).

A MODEL FOR *SCN5A*-RELATED DILATED CARDIOMYOPATHY: THE *Scn5a*-D1275N MOUSE

The D1275N *SCN5A* mutation has been related to different cardiac diseases. It was first reported in a family affected by atrial standstill, mild conduction disease, and atrial enlargement. However, all affected members also carried a variant in the connexin 40 promoter (Groenewegen et al., 2003). Subsequently, D1275N mutation was reported in a family affected by DCM, sinus node dysfunction, atrial and ventricular tachyarrhythmias and conduction disorders (McNair et al., 2004; Olson et al., 2005; Ge et al., 2008). More recently, it was also reported in a family with atrial tachyarrhythmias, conduction disease, and ventricular enlargement, but without impaired contractility, as opposed to the previous family (Laitinen-Forsblom et al., 2006). Finally, this mutation was also identified in a patient with atrial flutter, atrial standstill, conduction disease, and sinus node dysfunction (Watanabe et al., 2011). Despite these phenotypic differences, patch-clamp studies that have compared wild-type and D1275N channels in heterologous expression systems have not shown major differences in the biophysical properties of the mutant channel (Groenewegen et al., 2003; Gui et al., 2010; Watanabe et al., 2011).

To address this discrepancy, Watanabe et al. (2011) used recombinase mediated cassette exchange to engineer a mouse model expressing the human D1275N mutant channel and compare it to the mouse model expressing the human wild-type channel

(Watanabe et al., 2011). The D1275 allele was associated with slow heart rate and prolonged atrial, atrioventricular and ventricular conduction in a gene-dose-dependent manner, i.e., D1275N homozygous mice had a more severe phenotype. Interestingly, conduction disease increased with age. In addition, sinus node dysfunction, second degree atrioventricular block, atrial tachycardia/fibrillation, and polymorphic ventricular tachycardia occurred in homozygous D1275N mice. Patch-clamp studies of isolated cardiomyocytes demonstrated that D1275N mutation causes a severe decrease in peak I_{Na} by 54% in heterozygous mice and 78% in homozygous mice in relation to a decreased sarcolemmal expression of $Na_V1.5$. Moreover, homozygous D1275N mice exhibited an increased late Na^+ current, leading to prolonged action potential duration. Finally, the mutant was also associated with a gene-dose-dependent reduction of contractile function and DCM. These results are in line with the clinical findings described in the mutation carriers.

Scn1b KNOCKOUT MOUSE

The cardiac Na^+ channel is a heterotrimer composed of $Na_v1.5$, the pore-forming α-subunit, and two auxiliary β-subunits (Meadows and Isom, 2005): a non-covalently linked β1-subunit ($Na_V\beta1$ or β3) and a disulfide-linked subunit ($Na_V\beta2$ or β4). In a genetic screening of 282 probands with Brugada syndrome and 44 with conduction disease, none of whom had mutations in *SCN5A* gene, three mutations in the gene encoding $Na_V\beta1$ (*SCN1B*) were identified in three kindred (Watanabe et al., 2008). Two mutations were located in a newly described alternately spliced transcript, $Na_V\beta1B$ (Qin et al., 2003). Both transcripts are expressed in human heart and to a greater extent in Purkinje fibers than in ventricular myocardium (Watanabe et al., 2008). The E87Q mutation, located in the extracellular immunoglobulin loop of both β1- and β1B-subunits, was identified in a family affected by conduction disease. The two other mutations, which resulted in a β1B-subunit truncated after the amino-acid tryptophan-179, were identified in a family affected with both Brugada syndrome and conduction disease and in a kindred affected with conduction disease. Electrophysiological studies showed that peak I_{Na} amplitude was lower when $Na_v1.5$ was co-expressed with mutant $Na_V\beta1$- and $Na_V\beta1B$-subunits versus wild-type subunits. This result was consistent with the patients' phenotypes.

The cardiac electrophysiological phenotype of a mouse model invalidated for *Scn1b* markedly differed from the phenotype of the patients presented above (Lopez-Santiago et al., 2007). Compared to wild-type mice, *Scn1b* knockout mice exhibited lower heart rates and prolonged QT intervals, without any signs of conduction disease. Surprisingly, loss of $Na_V\beta1$ expression resulted in an increase in both peak and persistent Na^+ current while channel gating and kinetics were unaffected. This increase in current was most likely due to the increased expression of $Na_v1.5$ observed in these mice. Immunostaining studies revealed no alterations in the localization of Na^+ channel α- or β-subunits. Action potentials were prolonged, supporting the increased QT interval. These results are consistent with the observation that the D1790G mutation of *SCN5A*, which affects the interaction of $Na_V1.5$ with $Na_V\beta1$, also leads to a long QT phenotype (Benhorin et al., 1998; An et al., 1998). Once again, studies performed in heterologous expression systems led to contrasting results. Indeed, the D1790G mutation was first found to shift negatively steady-state inactivation of the Na^+ current without increasing its late component (An et al., 1998). The action potential prolongation was attributed to alterations of calcium-sensitive exchange and ion channel currents (Wehrens et al., 2000). By contrast, a second study showed that the mutation-induced a marked prolongation of the late Na^+ current, a result more consistent with the long QT phenotype (Baroudi and Chahine, 2000). Interestingly, the phenotype of *Scn1b* knockout mice resembles that found in a family of patients carrying a mutation on *SCN4B*, the gene encoding the β4-subunit. These patients exhibit a markedly prolonged QT interval (LQT10; Medeiros-Domingo et al., 2007) due to a large increase of the late Na^+ current amplitude, as in LQT3 patients.

Scn3b KNOCKOUT MOUSE

In order to understand better the role of $Na_V\beta3$ subunit on $Na_V1.5$ function, Hakim and co-workers generated a mouse model lacking the *Scn3b* gene (*Scn3b*$^{-/-}$ mouse; Hakim et al., 2008). They showed that the lack of $Na_V\beta3$ induced a ≈30% decrease in cardiac Na^+ peak current amplitude associated with a shift of the steady-state inactivation curve toward negative potentials while other I_{Na} biophysical parameters were not altered, thus confirming previous studies in mammalian heterologous expression system (Ko et al., 2005). As a consequence, *Scn3b*$^{-/-}$ mice exhibited decreased ventricular conduction, shortened ventricular action potential duration, and refractory period and increased incidence of arrhythmias under programmed electrical stimulation in *ex vivo* Langendorff-perfused hearts (Hakim et al., 2008). Arrhythmogenic incidence was reduced by flecainide and quinidine (Hakim et al., 2010b), in contrast to what had been observed previously for flecainide in *Scn5a*$^{\pm}$ mice (Martin et al., 2011c,d). Like *Scn5a*$^{\pm}$ mice, *Scn3b*$^{-/-}$ mice also exhibited sinus node dysfunction and decreased atrial and atrioventricular conduction, sometimes leading to atrioventricular block (Hakim et al., 2010a). Finally, *Scn3b*$^{-/-}$ mice were more prone than wild-type mice to develop atrial tachycardia and fibrillation upon burst pacing in *ex vivo* Langendorff-perfused hearts. Altogether, these studies suggested that *Scn3b* gene could also be involved in cardiac channelopathies in human.

This hypothesis has been confirmed. Indeed, *SCN3B* mutations have been identified in patients with either Brugada syndrome (Hu et al., 2009), idiopathic ventricular fibrillation (Valdivia et al., 2010) or lone atrial fibrillation (Olesen et al., 2011). All the mutations lead to a loss-of-function of $Na_V\beta3$ and consequently a decrease in cardiac Na^+ current.

CONCLUSION

Most mouse models of *SCN5A*-related cardiac channelopathies recapitulate most of the diverse clinical phenotypes observed in patients. However mouse models also have their own limitation as a phenotype observed in mice may not be encountered in patients. For example, the *Scn1b* knockout mouse shows prolonged ventricular repolarization, a feature that is not seen in patients with *SCN1B* loss-of-function mutations. These discrepancies relate to either species differences or differences between a heterozygous point mutation and complete invalidation of a gene on one allele.

Mouse models also turned out to be powerful tools to elucidate the pathophysiological mechanisms of *SCN5A*-related diseases. For instance, the *Scn5a*$^{1798insD/+}$ mouse clearly demonstrated that a single *SCN5A* mutation is sufficient to cause an overlap syndrome. This mouse also illustrated the limitations of heterologous expression systems in the elucidation of the biophysical consequences of the mutation on the channel function.

Finally, mouse models of $Na_V1.5$ (or $Na_V1.5$-associated proteins) dysfunction offer the unique opportunity to investigate the secondary cellular consequences of *SCN5A* mutations such as the expression remodeling of other genes, which is hardly accessible in the hearts of patients, but that might participate to the overall phenotype and explain some of the differences among patients. The most exiting benefit of mouse models is that they raise new working hypotheses such as the putative link between $Na_V1.5$ loss-of-function and occurrence of fibrosis in the context of PCCD, thereby providing new potential therapeutic approaches. One limitation, though, is that remodeling might be partly species dependent. They also constitute useful tools for future studies addressing as yet unanswered questions, such as the role of genetic and environmental modifiers on cardiac conduction and repolarization.

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Cardiac conduction system anomalies and sudden cardiac death: insights from murine models

Amelia Aránega*, Angel J. De La Rosa and Diego Franco

Department of Experimental Biology, Faculty of Experimental Sciences, University of Jaén, Jaén, Spain

Edited by:
Carol Ann Remme, University of Amsterdam, Netherlands

Reviewed by:
Carmen Valenzuela, Instituto de Investigaciones Biomédicas CSIC-UAM, Spain
Marina Cerrone, NYU School of Medicine, USA

****Correspondence:***
Amelia Aránega, Department of Experimental Biology, Faculty of Experimental Sciences, University of Jaén, Paraje de las Lagunillas s/n, 23071 Jaén, Spain.
e-mail: aaranega@ujaen.es

The cardiac conduction system (CCS) is composed of a group of myocardial tissues that control and coordinate the heart. Alterations in the CCS – especially in the His–Purkinje system, have been identified as a major cause of lethal arrhythmias. Unstable arrhythmias secondary to channelopathies significantly increase the risk of sudden cardiac death (SCD). SCD is a major contributor to mortality in industrialized countries, and most cases of SCD in the young are related to inherited ion channel diseases. In this paper, we review a series of studies with murine transgenic models that revealed that some arrhythmias are associated with the CCS and may lead to SCD

Keywords: cardiac conduction system, ion channel, sudden death

INTRODUCTION

Sudden cardiac death (SCD) occurs when an apparently healthy person unexpected dies, which occurs within a very short time interval – usually 1 h after symptom onset. Although sudden death in infants, children, and young adults is relatively rare – with an incidence of one to five cases per 100,000 population per year, up to 7,000 asymptomatic children die every year in the USA. In almost half of the cases no warning signs or symptoms were detected. Although structural cardiovascular abnormalities explain most cases of SCD in young people, the cause of death remains unexplained after autopsy in 10–30% of cases (Kauferstein et al., 2009). Cases of unexplained SCD in young people are mostly due to inherited ion channel diseases such as long QT syndrome (LQTS), short QT syndrome (SQTS), Brugada syndrome, and catecholaminergic polymorphic ventricular tachycardia (CPVT) (Krous et al., 2004; Basso et al., 2009).

The cardiac conduction system (CCS) generates and conducts electrical signals (action potentials) throughout the heart to trigger and coordinate the heartbeat. The electrical activity of the heart behaves like the electrical activity of any other biological tissue, and is ultimately dependent on the expression of ion channels in the myocytes forming the CCS. Ion channel expression defines the characteristic action potentials generated by these specialized myocytes, and is clearly different from that in the working myocardium (Boyett, 2009). Interestingly, several recent reports have pointed out that ion channel expression remodeling within the different CCS components may be responsible for the bradycardia and the increase in atrioventricular block with aging (Muir et al., 2005, Abd Allah et al., 2007). There is growing evidence that the different components of the CCS (e.g., Purkinje fibers network) play a pivotal role in both the initiation and perpetuation of several arrhythmias such as ventricular fibrillation (VF; Nogami, 2001). In addition, recent mapping studies have shown that Purkinje fibers can initiate VF in some patients with pro-arrhythmogenic syndromes: Brugada syndrome, LQTS (Haïssaguerre et al., 2003). Despite the significant role of ion channels in the electrical activity of CCS, their influence and contribution to CCS dysfunctions are still poorly understood.

ELECTRICAL CONFIGURATION OF THE CARDIAC CONDUCTION SYSTEM

The heart achieves coordinated contraction of atrial and ventricular chambers thanks to the precise timing of firing of the CCS; it is a specialized complex and heterogeneous network of cells that initiate and allow propagation of action potentials through the heart (Gourdie et al., 2003). The normal cardiac impulse in vertebrates' heart originates in the pacemaker cells of the sinoatrial node (SAN) located in the right atrium (Anderson et al., 1983). The impulse is then conducted through the atrium to the atrioventricular junction, where impulses are delayed through specialized slow conducting cardiomyocytes in the atrioventricular node (AVN). This delay is necessary to allow time for the atrial chambers to fully contract and pump blood across AV valves before the occurrence of ventricular contraction (Hatcher and Basson, 2009). After AVN delay, the electrical signal is propagated to the ventricles along bundles of specialized conduction tissue to the distal Purkinje fibers, which ramify among the contractile myocardium. The tips of the Purkinje fibers are electrically coupled to muscle cells and the working myocytes are longitudinally connected via gap junctions, thereby initiating a coordinated contraction of the ventricles (Pennisi et al., 2002).

It is interesting to remark that the SA node is a complex and irregular structure. There is regional heterogeneity in the SAN in mammals in terms of cell morphology, pacemaker activity, action potential configuration and conduction, ionic current densities (INa ICa, L, Ito, Iks, If), gap junction protein expression (Cx40,

Cx43, Cx45), and autonomic regulation (Hall et al., 2004; Herfst et al., 2004). Moreover, although this bundle is located in the AV junction area, its molecular phenotype shows most of the characteristics of the working ventricular myocardium, displaying a high conduction velocity compared to the slower conducting AV nodal tissue (Franco and Icardo, 2001).

For instance, in ventricular and atrial myocytes and Purkinje fibers, the upstroke of the action potential (phase 0) is rapid and results from the activation of voltage-gated Na^+ (Nav) channels. However, phase 0 is markedly slower in the pacemaker cells of the SAN and AVN suggesting that Nav channels do not play a relevant role in depolarization (Baruscotti et al., 2005). These findings are consistent with the heterogeneity found in Na^+ channel expression within the CCS (Domínguez et al., 2005, 2008).

Phase 0 of the action potential in Purkinje fibers and in atrial and ventricular myocytes is followed by a transient repolarization (phase 1), reflecting Nav channel inactivation and the activation of the fast transient voltage-gated outward K^+ current (Ito, f). This transient repolarization or "notch," which can be quite prominent in Purkinje and ventricular cells, influences the height and duration of the action potential plateau (phase 2). Membrane depolarization also activates voltage-gated Ca^{2+} (Cav) currents; Ca^{2+} influx through L-type Cav channels during the phase 2 plateau is the main trigger for excitation-contraction coupling in the working myocardium. Thus, Ca^{2+} entering through L-type Cav triggers a relatively larger release of Ca^{2+} from the sarcoplasmic reticulum (SR) via intracellular Ca^{2+} release channels (ryanodine receptors, RyRs). The increase in $[Ca^{2+}]i$ promotes cell contraction by Ca^{2+} binding to contractile proteins (Moosmang et al., 2001). In SAN and AVN cells, activation of (L-type) Cav channels also contributes to action potential generation, particularly in cells expressing low levels of functional Nav channels (Moosmang et al., 2001).

The driving force for K^+ efflux is high during the plateau phase of the action potential in the ventricular and atrial myocardium; when Cav channels become inactive, outward K^+ currents predominate resulting in repolarization (phase 3), and bringing membrane voltage back to the resting potential. In contrast to Nav and Cav currents, there are multiple types of voltage-gated K^+ (Kv) currents, as well as non-voltage-gated, inwardly rectifying K^+ (Kir) currents that contribute to myocardial action potential repolarization: the greatest functional diversity is among Kv channels.

At least two types of transient outward currents – Ito, f and Ito, s – and several components of delayed rectification – including IKr [IK(rapid)], IKs [IK(slow)], and IKur [IK(ultrarapid), among others] – have been identified. However the timing and voltage-dependent properties of the different Kv currents identified in different regions of the heart are remarkably similar. This suggests that the same (or very similar) molecular entities contribute to the generation of each of the Kv channels in different cells. Relative Kv channel expression levels in cardiac cells vary according to the region of the heart (i.e., atria, ventricles); this heterogeneity significantly contributes to the observed regional differences in action potential waveforms (Stieber et al., 2003; Aiba and Tomaselli, 2010).

CARDIAC CONDUCTION SYSTEM, ARRHYTHMOGENESIS, AND SUDDEN CARDIAC DEATH: CONTRIBUTIONS FROM MURINE MODELS

In the CCS, ion channels are specialized and differ from those in the working myocardium, as they provide the electrical activity required for its proper function. Although changes in the expression and/or configuration of ion channels and connections within the conduction system may have fatal consequences – they can generate arrhythmogenic processes – the role of ion channels in CCS dysfunction remodeling is not yet well understood. For instance, it is noteworthy that in an aging heart, there are deleterious changes in CCS remodeling that are a contributing factor to the relatively high prevalence of cardiac arrhythmias, which increases the risk of SCD in the aging population (Dobrzynski et al., 2007). Furthermore, it is well known that athletes have a lower heart rate than non-athletes, and sinoatrial node remodeling in athletes can become pathological and result in a sick sinus syndrome (Baldesberger et al., 2008). Although there is growing evidence supporting that molecular remodeling of electro-generating, propagating, and coupling channels, as well as CCS structural remodeling may be behind cardiac arrhythmias (Thomas et al., 2007), the mechanism by which CCS remodeling takes place is still unknown.

With the development of transgenic animal technology, a growing number of experimental mouse/rat models have emerged. However, as the cardiac electrophysiological characteristics of mice are significantly different from those of humans, these models have substantial limitations – e.g., the duration of cardiac action potential is very short in mice; there is no plateau phase, and the heart rate is extremely high (resting heart rate: 600 bpm) in mice. Despite these differences, over the past 15 years, genetically engineered mouse models have been developed to elucidate the role of different ion channels in arrhythmogenic pathologies. The results obtained suggest that CCS components play a major role in the generation of cardiac arrhythmias. In this paper, we describe the main mouse models with ion channel alterations in which a CCS dysfunction leads to an arrhythmogenic substrate (**Table 1**).

MOUSE MODELS OF ION CHANNELS REMODELING LINKED TO NODE TISSUE DYSFUNCTION

A number of mouse models have been developed to elucidate the role of different ion channels in node tissue and in the generation of arrhythmias. For instance, different mouse transgenic models have been developed with the aim of identifying the relationship between Hcn channel alterations and pacemaker activity. The homozygous embryo mice lacking the exon 4 of the Hcn4 gene – which encodes ion channel pore and transmembrane segment six – died in the uterus, thus indicating that Hcn4 is essential for the normal development of the heart (Stieber et al., 2003). In contrast, the role of If in adult mice is controversial. As Hcn4 knockout mice fail to develop a normal heart, it has not been until recently that we have elucidated whether If has the same prominent role in adult as in embryonic mice.

Accordingly, Herrmann et al. (2007) developed an adult mouse lacking Hcn4 channels in a temporally controlled manner. Deletion of Hcn4 in adult mice eliminated most of sinoatrial If and resulted in a cardiac arrhythmia characterized by recurrent sinus pauses. However, these mutants showed no impairment in heart

Table 1 | Mouse transgenic models with ion channel deficiency and CCS dysfunction.

Mouse model	Ion channel deficiency/location	Arrhythmogenic phenotype
Hcn4−/− mice (Stieber et al., 2003)	Hcn4/systemic	Died *in utero*
Hcn4-inducible-knockout mice (Herrmann et al., 2007)	Hcn4/whole heart	Recurrent sinus pauses
Hcn4-KiT mice (Hoesl et al., 2008)	Hcn4/CCS	Normal cardiac rhythm
ciHcn4-KO (Baruscotti et al., 2011)	Hcn4/whole heart	Severe bradycardia and AV block
Scn5a+/− mice (Hao et al., 2011; Martin et al., 2011)	Nav1.5/systemic	Reduced sinoatrial node automaticity/polymorphic ventricular tachycardia
R176Q/+ knock-in mice (Mathur et al., 2009)	Ryr2 mutation/systemic	Sudden cardiac death
RyR(R4496C) mice (Cerrone et al., 2005, 2007)	Ryr2 mutation/systemic	CPVT/DAD activity in Purkinje fibers

rate acceleration during sympathetic stimulation, thus revealing that, in adult mice, Hcn4 does not appear to be the only mechanism involved in the adrenergic stimulation of sinus activity.

Moreover, conduction system-specific deletion of Hcn4 channels using the KiT transgene did not result in bradycardia (Hoesl et al., 2008). This supports the notion that Hcn4 channels mainly provide the depolarizing currents necessary to counterbalance an increase in repolarizing currents in the CCS. On the contrary, in an inducible and cardiac-specific Hcn4 knockout (ciHcn4-KO) mouse model, ablation of Hcn4 consistently led to the progressive development of severe bradycardia (∼50% reduction of original rate) and AV block, eventually leading to heart arrest and death in about 5 days. This outcome suggests that cardiac Hcn4 channels are essential for normal heart impulse generation and conduction in adult mice; in addition, these results support the notion that dysfunctional Hcn4 channels can be a direct cause of rhythm disorders, which lead to sudden death events (Baruscotti et al., 2011). These contradictory results might be partially due to the nature of the mouse models used in these studies, as they were designed to lack a Hcn channel within the whole heart, and/or within both the SAN and AVN (**Table 1**). The development of Cre deleter mouse lines – which can render sinoatrial-specific Hcn channel deletion – will help to better understand the role of Hcn channels in pacemaker activity.

Knockout mouse models for other key cardiac ion channels are also useful tools for evaluating how a misexpression of specific ion channels can generate an arrhythmogenic substrate. Thus, Nav1.5 deficiency in heterozygous Scn5a knockout [Scn5a(+/−)] mice resulted in reduced SAN automaticity, which suggests that the loss of expression/function of sodium currents can severely impair action potentials in pacemaker cells. Moreover, downregulation of a wide range of other ion channels and related transcripts in Scn5a(+/−) mice suggest that impaired Scn5a expression may lead to significant electric remodeling in the sinus node (Hao et al., 2011).

In addition, the contribution of transcription factors to proper CCS embryonic development is being widely explored. Previous studies have described that the T-box transcription factor Tbx3 is predominantly expressed in developing and mature pacemakers [SAN and AV canal (AVC) and node], as well as in the AVB and branches. Tbx3 is sufficient to induce the formation of ectopic pacemakers in the atrial myocardium during its development (Hoogaars et al., 2004, 2007). These data suggest a role for Tbx3 in mammalian cardiac development and CCS function. More recently, the generation of a unique series of Tbx3 hypomorphic and conditional mouse mutants (Frank et al., 2011) revealed that the development of the CCS structure and function is extremely sensitive to Tbx3, to the extent that a decrease in Tbx3 below a critical level causes lethal embryonic arrhythmias; these arrhythmias are accompanied by disrupted expression of multiple ion channels such as voltage-gated sodium channels (Scn7a) and Connexin-30.2. Furthermore, surviving Tbx3 mutants are at an increased risk for sudden death, thus suggesting that TBX3 may be a candidate gene for human arrhythmia syndromes.

MOUSE MODELS OF ION CHANNEL REMODELING LINKED TO PURKINJE SYSTEM DYSFUNCTION

Catecholaminergic polymorphic ventricular tachycardia is an inherited disease leading to arrhythmias and SCD. The autosomal dominant form has been associated with ryanodine receptor gene (RyR2) mutations, leading to increased spontaneous Ca^{2+} release from the SR (Priori et al., 2001). Mice heterozygous for the R4496C mutation (RyR2/RyR2R4496C) recapitulate the human phenotype of CPVT by developing ventricular tachycardia and/or VF under adrenergic stimulation (Cerrone et al., 2005). More recently, epicardial and endocardial optical mapping analyses have demonstrated that arrhythmias in RyR2/RyR2R4496C mice are mediated by delayed afterdepolarization (DAD)-induced triggered activity occurring in Purkinje fibers; this reveals that the His–Purkinje system is an important source of focal arrhythmias in CPVT (Cerrone et al., 2007). Some authors have suggested that RYR2 mutations may cause "arrhythmogenic right ventricular dysplasia type 2" (ARVD2), a condition characterized by fibro-fatty degeneration of the right ventricle and by the presence of ventricular arrhythmias. The generation of a knock-in mouse model with the R176Q mutation (a human Ryr2 mutation previously identified in a 15-year-old male with ARVD2) led us to better understand the relationship between Ryr2 mutations and ARVD2. During the experiment, R176Q/+ knock-in mice displayed an increased incidence of sudden unexpected death during the first weeks of life, and electrophysiological recordings displayed cardiac arrhythmias in R176Q/+ mice associated with SCD (Mathur et al., 2009). In another study, Kang et al. (2010) crossed RyR2R4496C/+ CPVT mutant mice with Cntn2-EGFP BAC transgenic mice (which express a fluorescent reporter gene in cells of the CCS). Interestingly, Kang's study revealed that the RyR2(R4496C/+) Purkinje cells display a greater propensity to develop abnormalities in intracellular

Ca^{2+} handling than ventricular myocytes. This proarrhythmic behavior was enhanced by disease-causing mutations in the RyR2 Ca^{2+} release channel and greatly exacerbated by catecholaminergic stimulation, with the development of arrhythmogenic triggered beats.

Finally, very recent experiments carried out using the *Scn5a*+/− mouse model – which has been shown to closely reproduce many of the key features of Brugada syndrome (a genetic channelopathy strongly linked to SCD) in humans – have revealed that *Scn5a*+/− mice display lines of conduction block across the RV resulting from premature ventricular beats led to the formation of reentrant circuits and polymorphic ventricular tachycardia (Martin et al., 2011). Some authors have reported that the Purkinje system plays a major role in the development of VF in patients with LQTS and Brugada syndrome (Haïssaguerre et al., 2003). Consequently, the results obtained suggest that the Purkinje system might play a significant role in the outset of the electrophysiological abnormalities found in *Scn5*+/− mice.

CONCLUDING REMARKS

There is growing evidence that ion channel remodeling within the CCS may play a crucial role in the generation of cardiac arrhythmias associated with SCD. A number of mouse transgenic models have been developed to analyze the role of the different ion channels in the development of specific arrhythmias. Studies with these animal models have revealed that the arrhythmias associated with changes in ion channel expression/function within the heart are mainly caused by an impaired CCS component. Therefore, an understanding of the role of ion channels within the CCS will lead to the development of new treatments for cardiac diseases such as sick sinus syndrome, AV block, and tachycardia which origin is in the CCS.

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Transgenic insights linking Pitx2 and atrial arrhythmias

Diego Franco*, Ana Chinchilla and Amelia E. Aránega

Department of Experimental Biology, University of Jaen, Jaen, Spain

Edited by:
Carol Ann Remme, University of Amsterdam, Netherlands

Reviewed by:
Larissa Fabritz, Hospital of the University of Münster, Germany
Bas Boukens, Academic Medical Center, Netherlands

****Correspondence:***
Diego Franco, Department of Experimental Biology, University of Jaen, 23071 Jaen, Spain.
e-mail: dfranco@ujaen.es

Pitx2 is a homeobox transcription factor involved in left–right signaling during embryogenesis. Disruption of left–right signaling in mice within its core nodal/lefty cascade, results in impaired expression of the last effector of the left–right cascade, Pitx2, leading in many cases to absence or bilateral expression of Pitx2 in lateral plate mesoderm (LPM). Loss of Pitx2 expression in LPM results in severe cardiac malformations, including right cardiac isomerism. Pitx2 is firstly expressed asymmetrically in the left but not right LPM, before the cardiac crescent forms, and subsequently, as the heart develops, becomes confined to the left side of the linear heart tube. Expression of Pitx2 is remodeled during cardiac looping, becoming localized to the ventral portion of the developing ventricular chambers, while maintaining a distinct left-sided atrial expression. The importance of Pitx2 during cardiogenesis has been illustrated by the complex and robust cardiac defects observed on systemic deletion of Pitx2 in mice. Lack of Pitx2 expression leads to embryonic lethality at mid-term, and Pitx2-deficient embryos display isomeric hearts with incomplete closure of the body wall. However, whereas the pivotal role of Pitx2 during cardiogenesis is well sustained, its putative role in the fetal and adult heart is largely unexplored. Recent genome-wide association studies have identified several genetic variants highly associated with atrial fibrillation (AF). Among them are genetic variants located on chromosome 4q25 adjacent to PITX2. Since then several transgenic approaches have provided evidences of the role of the homeobox transcription factor PITX2 and atrial arrhythmias. Here, we review new insights into the cellular and molecular links between PITX2 and AF.

Keywords: Pitx2, atrial fibrillation, microRNAs, ion channel, electrophysiology

Cardiac arrhythmia can be defined as a deviation from the normal heart rate and/or rhythm under physiological circumstances which are thus reflected on specific ECG patterns (Bellet, 1971). Atrial arrhythmias may be regular, as in the case of flutter or monomorphic tachycardia, or irregular, as in the case of fibrillation or polymorphic tachycardia (Gussak and Antzelevitch, 2008). We focus herein on atrial fibrillation (AF), the most prevalent type of arrhythmia in humans. AF is characterized by uncoordinated atrial activation with consequent weakening of atrial mechanical function with intact AV conduction (Gussak and Antzelevitch, 2008). AF prevalence ranges from 1% in young adults to >10% of those over 80 years old (Fuster et al., 2001) and is frequently associated with hypertension, coronary disease, valvular diseases, and cardiomyopathies (Kannel et al., 1998), yet in 10–20% of AF patients, no underlying disease is found (Brand et al., 1985), i.e., idiopathic or lone AF. Great insights have been gained concerning the cellular and molecular mechanisms that underlie AF (Schotten et al., 2011; Wakili et al., 2011). Enhanced automaticity in one or several rapidly depolarizing foci leads to AF (Haissaguerre et al., 1998), being frequently located within the superior pulmonary veins, and more rarely within the superior vena cava or coronary sinus (Moe and Abildskov, 1959). Alternatively, multiple wave fronts propagate within the atria, resulting in derived wavelets that self-perpetuate (Arnar et al., 2006). However, the molecular substrates of AF remain obscure.

Although AF is the most prevalent type of arrhythmia, its genetic etiology remains elusive. The first AF genetic locus was reported by Brugada et al. (1997) identifying linkage to chromosome 10q22–q24 and soon thereafter Chen et al. (2003) identify a mutation in KCNQ1. Mutations in KCNQ1 have been subsequently confirmed (Ellinor et al., 2007) as well as in other ion channels including KCNE2, KCNE3, KCNJ2, KCNA5, and SCN5A (Ellinor et al., 2004; Mc Nair et al., 2004; Yang et al., 2004; Xia et al., 2005). Surprisingly, mutations in ion channels would underlie less 1% of AF cases.

The advent of new genetic strategies, in particular genome-wide association studies (GWAS), has shed new light on the genetic bases of arrhythmogenic syndromes and the identification of novel disease-causing genes (Larson et al., 2007). GWAS have identified several genetic variants highly associated with AF, which are located at 9p21, 1q21, 16q22, and 4q25, respectively. On chromosome 9p21, no candidate gene has been linked to AF, and thus its relevance awaits further experimental and functional evidence (Larson et al., 2007). Distinct genetic variants at 1q21 are linked to KCNN3, a potassium channel involved in atrial repolarization (Ellinor et al., 2011) and IL6R, a gene involved in inflammatory response (Schnabel et al., 2011), respectively. Genetic variants at 16q22 are linked to ZFHX3, a zinc finger homeobox transcription factor involved in liver gene expression (Gudbjartsson et al., 2009). However, in both cases 1q21 and 16q22 risk variants, their role in AF remains obscure. Finally, genetic variants on chromosome

4q25 which are strongly associated with lone AF in three distinct populations of European descent (Gudbjartsson et al., 2007) map adjacent to PITX2, which is known to have a critical function in establishing left–right asymmetry (Gage et al., 1999; Lin et al., 1999) and plays a critical role in cardiac development (Campione et al., 1999, 2001; Franco and Campione, 2003) and thus supporting the notion that impaired function of PITX2 might underlie AF (Gudbjartsson et al., 2009).

Pitx2 is a homeobox transcription factor involved in left–right signaling during embryogenesis (Gage et al., 1999; Lin et al., 1999). Disruption of left–right signaling in mice within its core nodal/lefty cascade (Blum et al., 1999; Hamada et al., 2001; Bamforth et al., 2004) results in impaired expression of Pitx2, the last effector of the left–right cascade, being absent or bilateral expressed in lateral plate mesoderm (LPM). Loss of Pitx2 expression in LPM results in severe cardiac malformations, including transposition of the great arteries, double-outlet right ventricle, and right cardiac isomerism (Gage et al., 1999; Lin et al., 1999). However, whereas the pivotal role of Pitx2 during cardiogenesis is well sustained, its putative role in the fetal and adult heart is largely unexplored.

The Pitx2 gene encodes three distinct isoforms, Pitx2a, Pitx2b, and Pitx2c, through alternative splicing and distinct promoter usage (Schweickert et al., 2000). Pitx2c is the most prominent isoform in the developing mouse heart and only weak and transient expression of Pitx2a is observed during early embryonic (E9.5–E12.5) stages, whereas Pitx2b displays progressively decreasing expression in embryonic and fetal stages (D. Franco and A. Aranega, unpublished data). In man, a fourth isoform, PITX2D is also expressed, which acts as a dominant negative isoform (Cox et al., 2002). PITX2 mutations are linked to Axenfeld–Rieger syndrome, which is characterized by abnormal morphogenesis of the anterior segment of the eye, variable degree of maxillary hypoplasia, skeletal abnormalities, and abdominal defects (Hjalt and Semina, 2005). In rodents and avian, Pitx2 displays a highly dynamic expression profile during cardiogenesis. Pitx2 is initially expressed asymmetrically in the left but not right LPM, before the cardiac crescent forms, and subsequently, as the heart develops, becomes confined to the left side of the linear heart tube (Campione et al., 1999, 2001; Franco and Campione, 2003). Pitx2 expression is later remodeled during cardiac looping, becoming confined to the ventral portion of the forming ventricular chambers, while keeping left-sided atrial expression (Campione et al., 2001). With further development, Pitx2 is down-regulated in the ventricular chambers, while high and robust expression of Pitx2 is maintained in the atrial chambers as well as in discrete components of the inflow tract, including the left atrial chamber, left superior caval vein, the pulmonary veins, and the interatrial septum (Franco et al., 2000; Kahr et al., 2011).

The significance of Pitx2 during heart development is illustrated by the complex and robust cardiac defects observed on Pitx2 defective mouse models. Systemic deficiency of Pitx2 leads to embryonic lethality at mid-term, leading to severe cardiovascular defects such as double-outlet right ventricle and right atrial isomerism as well as incomplete closure of the body wall (Gage et al., 1999; Lin et al., 1999; Liu et al., 2001, 2002). Isoform-specific Pitx2 mutants revealed that Pitx2c is the most relevant isoform during cardiogenesis, since lack of Pitx2c expression, but not Pitx2a or Pitx2b, recapitulate the morphogenetic defects similar to systemic Pitx2-deficient mice (Liu et al., 2001). More recently, with the advent of tissue-specific conditional mutants, our understanding of the role of Pitx2 during cardiogenesis has greatly expanded. Ai et al. (2006) revealed that lack of Pitx2 function in the secondary heart field, a subpopulation of cardiovascular progenitor cells emanating from the medio-lateral cardiogenic mesoderm (Kelly et al., 2001), leads to developmental defects reminiscent of systemic Pitx2c null mutants. In addition, further evidence on the role of Pitx2 within the secondary heart field has been reported at the arterial (Nowotschin et al., 2006; Yashiro et al., 2007) and venous (Galli et al., 2008) poles of the heart. Importantly, Pitx2 function is not exclusive related to cardiac precursor cells but is also relevant in differentiated cardiomyocytes as reported by Tessari et al. (2008). Using αMHC-Cre driver mice, these authors demonstrated that functional impairment of Pitx2 in cardiomyocytes leads to cytoarchitectural disarray in ventricular myocytes and to abnormal left–right expression of *Bmp10* in the atria. Unexpectedly, αMHCCrePitx2 mutants do not display atrial isomerism, suggesting that left/right atrial morphological identity is acquired before αMHC-Cre activation. Overall, these data demonstrate a developmental role of Pitx2 with precursor cells as well as in cardiomyocytes, including importantly, morphological structures prone to trigger atrial arrhythmias, yet no evidence of such electrophysiological defects was reported in these studies. Moreover, although it is clearly established that Pitx2 plays a role in pulmonary vein development (Mommersteeg et al., 2007), a frequent AF foci, Pitx2 driven alterations occur in the setting of complex cardiac abnormalities such as atrial isomerism, a condition which is not linked to atrial arrhythmias in men. Therefore, additional evidence is required to fill the gap between Pitx2 function in the embryo and the onset of AF.

Genetic variants at 4q25, close to the Pitx2 locus, are highly associated with AF (Gudbjartsson et al., 2007), providing the conjecture thus that Pitx2 dysfunction might be to AF. Linkage of 4q25 AF risk variants have been consistently reported within distinct cohorts, highlighting thus the reproducibility of these findings (Chinchilla et al., 2011; Henningsen et al., 2011; Kiliszek et al., 2011; Schnabel et al., 2011; Delaney et al., 2012; Liu et al., 2012; Olesen et al., 2012). A twofold question arises from this hypothesis, first is whether the 4q25 genetic variants can modulate Pitx2 expression, a question that remains unanswered, and secondly, whether Pitx2 impairment can lead to AF. Reports in human AF right and left atrial biopsies demonstrated that PITX2 expression is severely diminished in AF patients as compared to patients with no clinical history of AF (Chinchilla et al., 2011), validating thus the hypothesis that PITX2 is impaired in AF patients. In addition to the human data, several independent experimental studies have provided evidences for this second issue, using subtle distinct experimental models. Wang et al. (2010) have demonstrated that Pitx2 haploinsufficiency predisposes to AF in electrically stimulated adult mice with structurally normal hearts. These authors reported a developmental link in that Pitx2 inhibits the sinoatrial node gene program in the embryonic left atrium, by regulating Shox2 and Nkx2.5 expression. When Pitx2 function is partially (heterozygous) or completely (null) impaired in the embryonic

(E12.5) mouse heart, Shox2, as well as several other sinoatrial node markers such as Hcn4 and Tbx3, are ectopically expressed in the forming inflow tract of the heart. These findings are further underscored by the recent study of Ammirabile et al. (2012) using a myocardiac specific conditional Pitx2 mouse model (cardiac troponin T Cre deletor mice). Thus, these observations provide a link between the embryonic function of Pitx2 and AF.

Kirchhof et al. (2011) have recently described impaired gap and tight junction expression in microarray analysis of the left atria of adult Pitx2c heterozygous mutant mice which display no overt structural and/or morphological alterations. These data provide a putative link to AF since mutations in GJA5 (Cx40) are associated with AF (Gollob et al., 2006) and further implicate impaired Pitx2 function at fetal and/or adult stages in predisposition to AF. In line with these findings, recent work in our laboratory using a Pitx2 over-expression model of embryonic stem cell-derived cardiomyocytes (Lozano-Velasco et al., 2011) has shown that Pitx2 does not modify the expression of contractile proteins such as actin and myosin heavy and light-chain isoforms, thus suggesting that Pitx2 does not impair the contractile properties of cardiomyocytes, but importantly, Pitx2 mis-expression impairs connexin-40 expression.

In addition, our recent work using atrial-specific conditional Pitx2 mouse mutants, in which right-sided sinoatrial node formation is intact, also supports a role of Pitx2 impairment on the onset of left-sided electrophysiological defects (Chinchilla et al., 2011) which are prone substrates for AF. Voltage-gated sodium and inward rectifying K+ (Kir2.1, Kir2.2, and Kir2.3) ion channels are abnormally expressed in the atrial myocardium both in fetal and adult stages of atrial-specific conditional Pitx2 mouse mutants, while cardiac morphogenesis is not compromised. Importantly, inward rectifying K+ channel de-regulation, but not sodium, is mediated by impaired miR-1 expression in atrial-specific conditional Pitx2 mutant mice. As a consequence, in these mice, the action potential amplitude is decreased, while the resting membrane potential is more depolarized. Further evidence on the de-regulation of ion channels by Pitx2 impairment has been recently reported also by Kirchhof et al. (2011) using mRNA microarray analyses in Pitx2c heterozygous adult mutant mice. Thus, these data reveal a role for Pitx2 controlling ion channel expression, and thus a link to atrial arrhythmogenesis, as summarized in **Figure 1**.

CONCLUDING REMARKS AND PERSPECTIVE

We have highlighted herein our current knowledge on the role of Pitx2 in the developing and adult heart as well as on the state-of-the-art link between PITX2 and AF. Several studies have shown that Pitx2 is essential for pulmonary vein development (Mommersteeg et al., 2007), a highly recurrent pro-arrhythmogenic region in humans. Experimental evidence has shown that Pitx2 is important for transcriptional regulation in the secondary heart field (Ai et al., 2006), derivatives of which will form the venous pole of the heart. Similarly, additional evidence has revealed a role of Pitx2 in the regulation of cell-to-cell communication proteins and essential ion channels, which modulate critical aspects of the cardiac action potential, as well as a role repressing the sinoatrial node gene program (Wang et al., 2010; Chinchilla et al., 2011; Kirchhof et al., 2011). Overall, these data strongly implicate Pitx2 as an upstream regulator of pro-arrhythmogenic events. However, there are several missing links. The first one is how the identified genetic variants alter Pitx2 expression and to date, this point

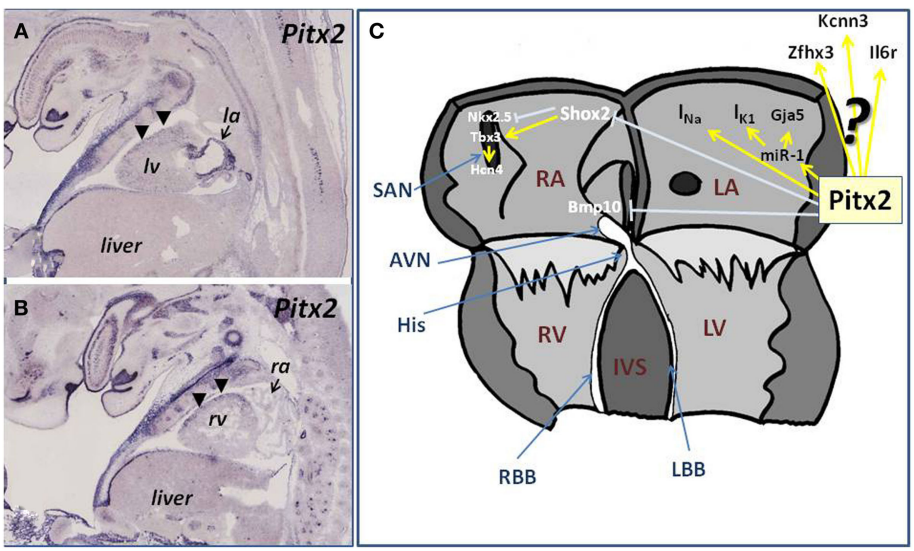

FIGURE 1 | (A,B) Illustrations of Pitx2 expression in the developing fetal embryo (E14.5) in longitudinal histological sections. **(A)** Illustrate the expression of Pitx2 at the level of the left ventricle (lv) and left atria (la), showing the robust expression of Pitx2 in the developing left atrial chamber, whereas **(B)** illustrates the expression of Pitx2 at the level of the right ventricle (rv) and right atrium (ra). Observe the marked contrast of expression between the right atrium (low) and the left atrium (high) as revealed by the arrows. Note also the dorso-ventral difference of Pitx2 expression at the ventricular level [arrowheads, **(A,B)**]. **(C)** Schematic representation of the current state-of-the-art role of Pitx2 in the developing and adult atrial chambers linked to atrial arrhythmogenesis. Pitx2 controls miR-1 expression in the left atrial chamber, providing thus a regulatory mechanism for I_{K1} and Gja1 function. In addition Pitx2 regulates I_{Na} while inhibits the expression right atrial (Bmp10) and sinoatrial (Shox2) markers in the left atrium, which in turn play key regulatory roles on cardiomyocyte cell cycle and pacemaker activity, providing additional links to atrial arrhythmias.

remains unanswered. Secondly, it remains elusive how altered Pitx2 expression would modify conductive elements leading to the onset of AF (although new insights have progressively being gained; **Figure 1**), in particular whether the impaired conductive elements might be deregulated already from embryonic stages, as suggested by Wang et al. (2010) and Ammirabile et al. (2012) or exclusively in the fetal/adult heart as reported by Kirchhof et al. (2011) and Chinchilla et al. (2011). On the basis of the developmental role of Pitx2, it might be postulated that impaired Pitx2 in the developing heart might be a predisposing factor for AF. However, an early defect in Pitx2 function would be expected to have severe consequences for cardiac embryogenesis, and thus it is more likely that fetal or adult dysfunction might underlie Pitx2 predisposition to AF. In addition, it is unclear if Pitx2 deficiency might also lead to impaired formation and/or function of other components of the ventricular cardiac conduction system such as the AV node and the left and right AV bundle branches. Morphological analysis of the ventricular conduction system in the left–right mutant mouse model *iv/iv* suggests that left/right cues can influence AV node formation (Franco and Icardo, 2001). Nonetheless, further studies are required to demonstrate a putative role of Pitx2 in ventricular conduction system deployment.

Interestingly, in addition to reports linking 4q25 risk variants to AF, new GWAS have identified several other genetic variants highly associated with AF, which are located at 9p21, 1q21, and 16q22, respectively. On chromosome 9p21, no candidate gene has been linked to AF, and thus its relevance awaits further experimental and functional evidence (Larson et al., 2007). However, genetic variants at 1q21 are linked to KCNN3 (Ellinor et al., 2011) and IL6R (Schnabel et al., 2011), respectively whereas risk genetic variants at 16q22 are linked to ZFHX3 (Gudbjartsson et al., 2009). To date it remains elusive the putative involvement of these genes in the pathophysiology of AF. A putative scenario might be that PITX2 might modulate these genes. Thus, further insights are required to explore both the links between all GWAS associated genes as well as the cellular and molecular mechanisms that might underlie a functional role between KCNN3, IL6R and ZFHX3, and AF, yet this aspect remains to be elucidated. Thus, new exciting dates are ahead right now.

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Pathophysiological mechanisms of sino-atrial dysfunction and ventricular conduction disease associated with *SCN5A* deficiency: insights from mouse models

Christopher L.-H. Huang[1,2], Lily Lei[2], Gareth D. K. Matthews[1,2], Yanmin Zhang[1,3] and Ming Lei[3]*

[1] *Physiological Laboratory, Department of Biochemistry, University of Cambridge, Cambridge, UK*
[2] *Murray Edwards College, University of Cambridge, Cambridge, UK*
[3] *Institute of Cardiovascular Sciences, University of Manchester, Manchester, UK*

Edited by:
Carol Ann Remme, University of Amsterdam, Netherlands

Reviewed by:
Flavien Charpentier, Institute National de la Santé et de la Recherche Médicale, France
Wayne Rodney Giles, The University of Calgary, Canada

****Correspondence:***
Ming Lei, Institute of Cardiovascular Sciences, University of Manchester, Core Technology Facility Building, 46 Grafton Street, Manchester M13 9NT, UK.
e-mail: ming.lei@manchester.ac.uk

Genetically modified mice provide a number of models for studying cardiac channelopathies related to cardiac Na^+ channel (*SCN5A)* abnormalities. We review key pathophysiological features in these murine models that may underlie clinical features observed in sinus node dysfunction and progressive cardiac conduction disease, thereby providing insights into their pathophysiological mechanisms. We describe loss of Na^+ channel function and fibrotic changes associated with both loss and gain-of-function Na^+ channel mutations. Recent reports further relate the progressive fibrotic changes to upregulation of TGF-β1 production and the transcription factors, *Atf3*, a stress-inducible gene, and *Egr1*, to the presence of heterozygous *Scn5a* gene deletion. Both changes are thus directly implicated in the clinically observed disruptions in sino-atrial node pacemaker function, and sino-atrial and ventricular conduction, and their progression with age. Murine systems with genetic modifications in *Scn5a* thus prove a useful tool to address questions concerning roles of genetic and environmental modifiers on human *SCN5A* disease phenotypes.

Keywords: *SCN5A*, progressive cardiac conduction disease, sinus node dysfunction, mouse genetic models

INTRODUCTION

The *SCN5A* gene directs synthesis of cardiac-type, voltage-dependent, Na^+ channels ($Na_v1.5$), abundant in the heart. The simultaneous and transient openings of large numbers of voltage-gated Na^+ channels mediate the rapid inward current responsible for the rising phase of the cardiac action potential (AP). This is fundamental to initiation, propagation, and maintenance of the normal cardiac rhythm that ensures synchronized atrial and ventricular contraction and therefore the normal heartbeat. *SCN5A* mutations are associated with cardiac arrhythmic syndromes ranging from chronic bradyarrhythmias to acute life-threatening tachyarrhythmias. These include the congenital long QT syndrome subtype 3 (LQT3; Wang et al., 1995), Brugada syndrome (BrS; Chen et al., 1998; Brugada et al., 2000), isolated cardiac conduction disease (CCD; Schott et al., 1999), sinus node dysfunction (SND; Benson et al., 2003), and sudden infant death syndrome (SIDS; Otagiri et al., 2008). Over the past decade, mouse models with genetic modifications in *SCN5A* have provided invaluable insights into these cardiac disorders. This review summarizes recent studies on $Na_v1.5$ disorders and their bearing on cardiac conduction properties.

THE ROLE OF VOLTAGE-GATED NA^+ CHANNELS IN CARDIAC PACEMAKER FUNCTION

The last two decades have seen significant progress in our understanding of the molecular structure of voltage-gated Na^+ channels (reviews Goldin, 2001, 2002; Catterall et al., 2003). These channels comprise pore-forming α-subunits, with molecular weights of ∼260 kDa, and associated auxiliary β-subunits with molecular weights ∼36 kDa (Goldin, 2002; Catterall et al., 2003). Expression of the α-subunit alone is sufficient for functional Na^+ current expression, but the presence or absence of β-subunits modifies the kinetics and voltage-dependence of channel gating. The α-subunits are organized into four homologous domains (I–IV). Each contains six transmembrane α-helices (S1–S6) and includes an additional pore loop located between the S5 and S6 helices. The various α-subunit isoforms are differentially expressed in different tissues and have distinct pharmacological properties (Goldin, 2002; Catterall et al., 2003).

Recent investigations also demonstrate that in addition to $Na_v1.5$, different cardiac tissues co-express a range of different α-subunit isoforms. These include several neuronal, $Na_v1.1$, $Na_v1.3$, and $Na_v1.6$, isoforms primarily expressed in brain (Maier et al., 2002; Lei et al., 2004). This pattern is exemplified by the SA node, which shows a complex co-expression of multiple Na_v isoforms. It contains both $Na_v1.5$ and $Na_v1.1$ with $Na_v1.5$ absent in the central nodal cells (Lei et al., 2004). Nav1.3 is also present in mouse (Maier et al., 2002) but not rabbit or rat SA node (Baruscotti et al., 1997; Maier et al., 2002). More recent, competitive RT-PCR and immunohistological, studies have demonstrated Nav1.1, Nav1.2, and Nav1.5 expression in canine SA and atrioventricular (AV) nodes (Haufe et al., 2005).

Recent studies have also clarified the functional roles of these distinct cardiac and neuronal-type Na^+ channels in the SA node. First, it is likely that the tetrodotoxin (TTX)-sensitive neuronal ($Na_v1.1$) Na^+ current is involved in pacemaker function in the

SA node. Nanomolar TTX concentrations known to only inhibit neuronal Na^+ current slowed down pacemaker rates of intact mouse hearts by ∼65% (Maier et al., 2002) isolated SA nodes by ∼22% and isolated SA nodal pacemaker cells by ∼15% (Lei et al., 2004). Furthermore, studies using the AP clamp technique demonstrated that the neuronal Na^+ current could be activated within the voltage ranges of the pacemaker potential (Lei et al., 2004).

In contrast, the TTX-resistant cardiac ($Na_v1.5$) Na^+ current is likely to be important in AP propagation and conduction through the SA node. Thus, measurements of SA node conduction times demonstrated that block of both TTX-sensitive and TTX-resistant Na^+ current by μM TTX slowed or even blocked AP conduction through the SA node periphery, from the leading pacemaker site in the center of the SA node to the surrounding atrial muscle. However, block of the TTX-sensitive Na^+ current alone by 10 or 100 nM TTX did not produce such effects (Lei et al., 2004).

Together these findings implicate both neuronal ($Na_v1.1$) and cardiac ($Na_v1.5$) Na^+ channels in pacemaker activities as well as AP conduction through the SA node and from the SA node to surrounding atrial muscle. Their demonstration of physiological roles for $Na_v1.5$ in SA node function underpins our understanding of the SND attributed to genetic defects in $Na_v1.5$ channel, as discussed in the sections below.

SCN5A MUTATIONS AND HUMAN CARDIAC CONDUCTION DISEASES

SINUS NODE DYSFUNCTION

Sinus node dysfunction is associated with abnormal impulse formation and propagation in the SA node. It presents clinically as sinus bradycardia, sinus pause or arrest, atrial chronotropic incompetence, and SA node exit block (Freedman, 2001). It affects ∼1 in 600 cardiac patients older than 65 years and is responsible for ∼50% of the million permanent pacemaker implants per year worldwide (de Marneffe et al., 1993; Dobrzynski et al., 2007). Thus far, 13 human Nav1.5 (hNav1.5) mutants have been associated with familial SND (reviews: Lei et al., 2007, 2008). In expressed heterologous systems, these fall into three groups respectively generating peak Na^+ currents that were comparable to those observed for wild-type hNav1.5 (e.g., L212P, P1298L, DelF1617, and R1632H), significantly reduced but nevertheless detectable (e.g., E161K, T220I, and D1275N) or undetectable (e.g., T187I, R878C, G1408R, and the truncated variants W1421X, K1578fs/52, and R1623X; Gui et al., 2010). Their comparisons with the corresponding clinical outcomes suggest that *SCN5A* haploinsufficiency markedly reducing Na^+ current can result in SND. However, disease phenotypes can be accompanied by other cardiac excitation disorders: further factors besides Na^+ current reduction and aging may also contribute to development of SND phenotypes (Leoni et al., 2010).

PROGRESSIVE CARDIAC CONDUCTION DISEASE

Progressive cardiac conduction disease (PCCD), also known as Lev–Lenegre disease (Schott et al., 1999), presents as a progressive prolongation of electrocardiographic cardiac conduction parameters. PCCD is most commonly seen in the elderly, and has been attributed to senile degeneration of the conduction system. Hereditary PCCD has been attributed to loss-of-function *SCN5A* mutations that reduce Na^+ current by decreasing sarcolemmal expression of channel proteins, causing an expression of non-functional channels, or altering channel gating properties through delayed activation, earlier inactivation, enhanced slow inactivation, or slowed recovery from inactivation (Tan et al., 2001; Probst et al., 2003; Zimmer and Surber, 2008). Moreover, a single *SCN5A* mutation may cause either an isolated PCCD or an overlap syndrome with PCCD co-existing with BrS (Zimmer and Surber, 2008). This suggests that in addition to reductions in Na^+ current sufficient to slow conduction in PCCD or BrS, other factors contribute to the development of the typical right-precordial ST segment elevation in BrS. Further reports describe overlaps between gain- and loss-of-function phenotypes associated with a single C-terminal 1795insD gene mutation yielding ECG features of bradycardia, conduction disease, LQT3, and BrS (Bezzina et al., 1999).

MOUSE MODELS OF CARDIAC CONDUCTION DISEASES ASSOCIATED WITH *SCN5A* MUTATIONS

A mouse model containing a disrupted $Na_v1.5$ gene, *Scn5a*, was developed a decade ago (Papadatos et al., 2002). Homozygotes show intrauterine lethality with severe defects in ventricular morphogenesis. $Scn5a^{+/-}$ heterozygotes show normal survival but a number of electrophysiological defects including impaired atrioventricular conduction, delayed intramyocardial conduction, increased ventricular refractoriness, and ventricular tachycardia with characteristics of re-entrant excitation. Single whole-cell patch clamp studies of isolated adult $Scn5a^{+/-}$ ventricular myocytes demonstrated a ∼50% reduction in Na^+ conductance. Such mice thus provide a unique and valuable model system for studying physiological effects of cardiac Na^+ channelopathies.

LOSS OF $Na_v1.5$ CHANNEL FUNCTION AND SINUS NODE DYSFUNCTION

Long-term telemetric ECG recordings demonstrated that $Scn5a^{+/-}$ mice retain physiological circadian variations in heart rates but replicated the depressed mean rates and persistent SA block observed in patients with SND (Asseman et al., 1983). Isolated hearts similarly showed a sinus bradycardia, slowed SA conduction, and sino-atrial exit block. Isolated $Scn5a^{+/-}$ SA node and atrial preparations (**Figure 1**) showed slowed SA conduction and frequent SA conduction block. Patch clamp analyses of $Scn5a^{+/-}$ SA node cells demonstrated similar steady-state activation and inactivation properties but reduced maximum Na^+ currents (∼30%) compared to WT (Lei et al., 2005). These studies together implicate the *Scn5a* Na^+ channel in both pacemaker and conduction functions of the mouse SA node, and in the clinical consequences of SND.

LOSS OF $Na_v1.5$ CHANNEL FUNCTION AND PROGRESSIVE CARDIAC CONDUCTION DISEASE

$Scn5a^{+/-}$ mice have been further investigated for potential pathophysiological mechanisms involved in the progressive evolution of *SCN5A*-related PCCD (Royer et al., 2005; van Veen et al., 2005). Atrial, atrioventricular, and ventricular conduction velocities were all prolonged to extents that increased with age. This

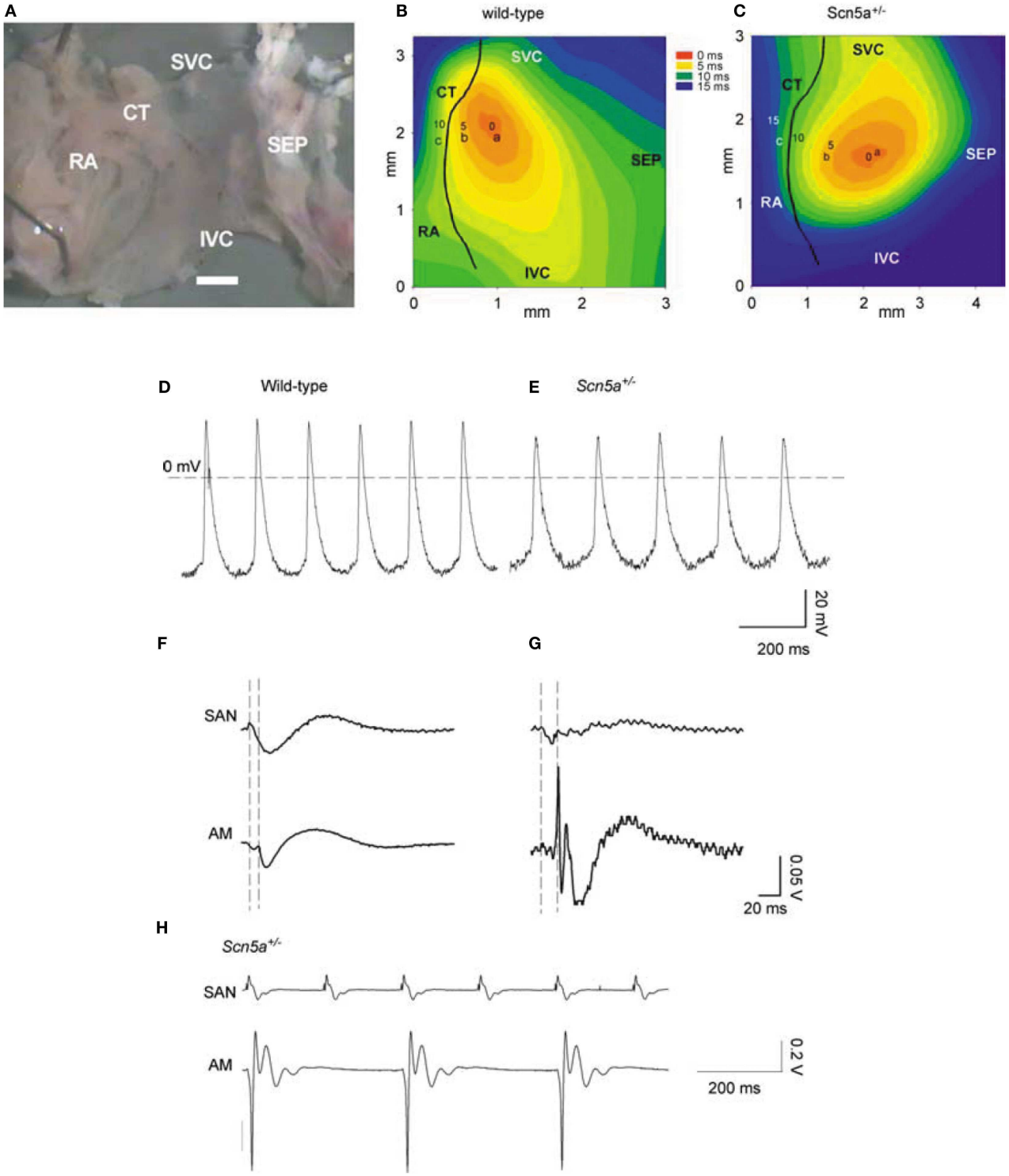

FIGURE 1 | SAN pacemaking and conduction in WT and *Scn5a*$^{+/-}$ mice. (A) Example of SAN preparation used for electrical mapping. Scale bar, 200 μm. **(B,C)** Activation sequence in SAN of WT and *Scn5a*$^{+/-}$. **(D,E)** Action potentials recorded from sites near the center of the SAN in **(A,B)** from WT (left) and *Scn5a*$^{+/-}$ (right) SAN preparations. **(F,G)** SAN conduction in WT and *Scn5a*$^{+/-}$ preparations. Extracellular potentials from sites a [leading pacemaker site in the center of the SAN; see **(B,C)**] and c (atrial muscle, AM) shown. Vertical dashed lines indicate the time of initiation of the AP at the leading pacemaker site (left) and the arrival of the AP in atrial muscle (right). **(H)** Simultaneous SAN and atrial muscle recordings showing sino-atrial conduction block in *Scn5a*$^{+/-}$ SAN, never observed in WT. SEP, septum; SVC, superior vena cava; IVC, inferior vena cava; CT, crista terminalis; and RA, right atrial appendage.

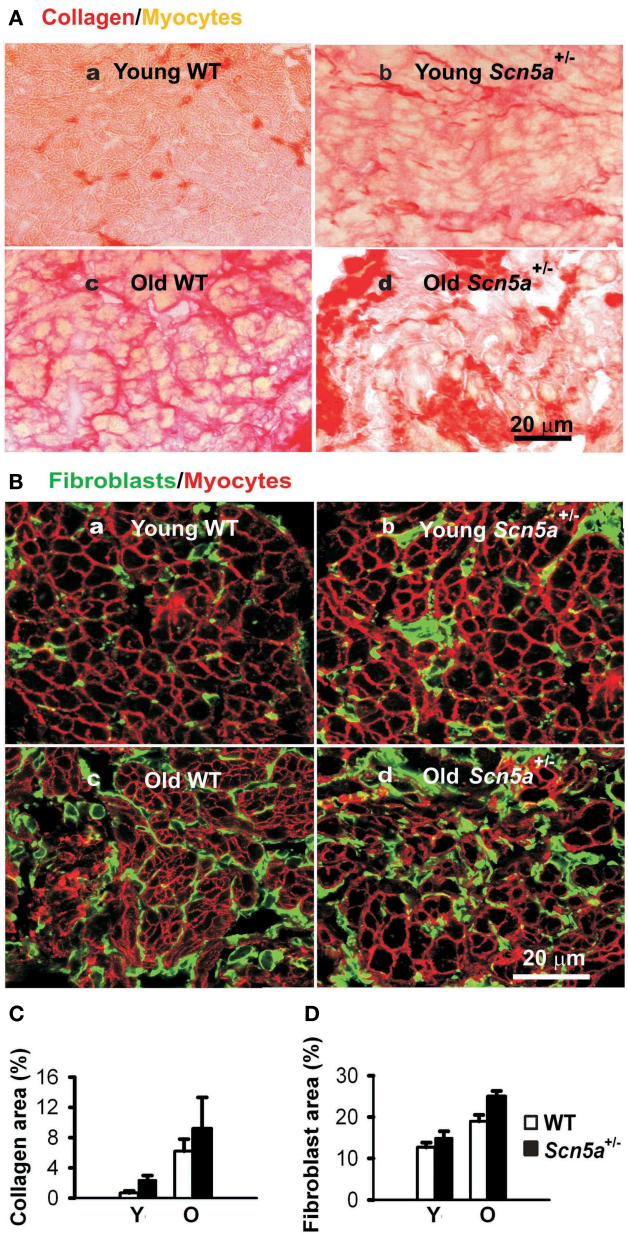

FIGURE 2 | Characterization of remodeling of extracellular matrix in the SAN. (A) Picosirus red collagen staining in SAN section from each of four groups of mice. **(B)** Vimentin immunostaining for fibroblasts in SAN tissue sections. **(C)** Collage quantification: area of picosirus red-stained tissue expressed as a percentage of the field of view. **(D)** Fibroblast quantification with area of vimentin staining expressed as a percentage of the field of view. Y, young mice; O, old mice. Two-way ANOVA: $P < 0.05$, *Scn5a*$^{+/-}$ vs. WT mice; $P < 0.001$, young vs. old mice.

feature was confirmed by activation mapping studies performed in Langendorff-perfused hearts. In young *Scn5a*$^{+/-}$ mice, conduction velocity was only affected in the right ventricle. In old mice, the right ventricular conduction defect worsened and was also associated with conduction velocity defects in the left ventricle. This age-dependent deterioration of ventricular conduction was associated with the occurrence of fibrosis in the ventricular myocardium (Jeevaratnam et al., 2011).

GAIN OF $Na_v1.5$ FUNCTION AND SINUS NODE DYSFUNCTION

SA node function in mice heterozygous for a knock-in, gain-of-function KPQ-deletion (*Scn5a*$^{+/\Delta}$) also appears to recapitulate major features of SND reported in LQT3 patients, suggesting phenotypic overlaps with *loss*-of-function Na^+ channel mutations. Abrupt accelerations in heart rate or premature beats thus caused AP lengthening, early after depolarizations, and triggered arrhythmias (Nuyens et al., 2001). Electrophysiological characterizations of SA node function in intact *Scn5a*$^{+/\Delta}$ mice and *in vitro* sinoatrial preparations were compared with features of cellular SA node and two-dimensional tissue models exploring consequences of *Scn5a*$^{+/\Delta}$ mutations. Whilst showing prolonged electrocardiographic QT and QTc intervals expected for LQT3, *Scn5a*$^{+/\Delta}$ mice showed frequent episodes of sinus bradycardia, sinus pause/arrest, and increased sinus node recovery times, suggesting compromised pacemaker activity, as well as depressed intra-atrial, atrioventricular node, and intraventricular conduction. Isolated SA preparations correspondingly showed lower mean intrinsic heart rates and slower conduction both within the SA node and from SA node to surrounding atrium. Modeling studies reconstructed such findings through a combination of augmented tail and reduced peak Na^+ currents attributable to downregulation of a Na^+ channel that itself shows increased tail currents as a result of a gain-of-function mutation (Wu et al., 2012).

Veldkamp et al. (2003) also studied the effects of the 1795insD Na^+ channel mutation on SA pacemaker function using experimental models in HEK-293 cells and computer simulation. They demonstrated late Na^+ currents from the 1795insD channels whose magnitude varied between 0.8 ± 0.2 and $1.9 \pm 0.8\%$ of peak Na^+ current. AP clamp experiments confirmed an existence of 1795insD channel activity during SA node pacemaker function. Computational models for the SA node AP then incorporated late Na^+ current and the negative shift in voltage-dependence of inactivation. Such negative shifts decreased the sinus rate by decreasing the diastolic depolarization rate, whereas the presence of late Na^+ current decreased the sinus rate by AP prolongation, despite a concomitant increase in diastolic depolarization rate. This suggested that Na^+ channel mutations displaying a late Na^+ current or a negative shift in inactivation may account for the bradycardia seen in LQT3 patients, whereas SA node pauses or arrest may result from failure of SA node cells to repolarize under conditions of extra net inward current (Veldkamp et al., 2003).

GAIN OF $Na_v1.5$ FUNCTION AND CONDUCTION FAILURE

The overlap patterns indicated above involving gain- and loss-of-function phenotypes can also involve atrial and ventricular conduction. 1795insD hearts combine a preferential right ventricular conduction slowing accompanied by reduced peak sodium current densities, and AP upstroke velocities with AP prolongation, slowed Na^+ current decays but normal activation and inactivation characteristics, and increased persistent inward currents compared to WT (Remme et al., 2006). Increased atrial arrhythmic tendencies in aging gain-of-function *Scn5a*$^{+/\Delta}$ are accompanied by reductions in conduction velocity that could result from downregulation

of Nav1.5 in contrast to its increased expression in WT hearts (Guzadhur et al., 2010).

RECENT NEW INSIGHTS INTO THE MOLECULAR BASIS OF *SCN5A* MUTATIONS AND HUMAN CARDIAC CONDUCTION DISEASES

Recent studies have explored for possible relationships between the pathogenesis of SND, aging processes, and *Scn5a*-disruption, as well as their possible interactions in the *Scn5a*$^{+/-}$ mouse model (Hao et al., 2011). These associated both electrical remodeling and tissue degeneration, detected as a TGF-β_1-mediated fibrosis, with altered pacemaker and conduction function in SND. Such changes occurred with both *Scn5a*-disruption and aging. A combination of both factors produced the most severe phenotype. Thus, *ex vivo* SA node preparations isolated from their autonomic inputs then showed increased cycle lengths and sino-atrial conduction times. These changes accompanied alterations in the extent of fibrosis assessed by collagen and fibroblast levels, and ion channel and regulatory gene transcriptional remodeling. Aging and *Scn5a*-disruption correspondingly resulted in interacting up-regulatory effects on levels of the key modulator of fibrosis, TGF-β_1, and the fibroblast marker, vimentin. The latter changes were also greatest in the old *Scn5a*$^{+/-}$ hearts (**Figure 2**). Altered expression of TGF-β_1 and vimentin transcripts are associated with increased collagen and fibroblast abundance indicating an occurrence of interstitial fibrosis. The fibrosis could potentially slow conduction both within the SA node and from the SA node to the surrounding atrium. Its occurrence parallels previous reports associating the *Scn5a*$^{+/-}$ condition and similar, ventricular, fibrotic changes (Royer et al., 2005; van Veen et al., 2005) as well as associating age-dependent SND and fibrosis (Benditt et al., 1990) although such features were not observed in all studies (Alings et al., 1990; Alings and Bouman, 1993). In implicating $Na_v1.5$ deficiency in such changes these findings additionally suggest novel regulatory roles for $Na_v1.5$ in cellular biological processes extending beyond its electrical function.

The above findings add to studies that similarly showed that heterozygous *Scn5a* inactivation in mouse produces ventricular rearrangements and fibrosis with aging (Royer et al., 2005; van Veen et al., 2005; Leoni et al., 2010). They also demonstrated aging resulted in an upregulation of two transcription factors, *Atf3*, a stress-inducible gene, and *Egr1*, an early growth response gene, particularly in *Scn5a*$^{+/-}$ mice (Royer et al., 2005; van Veen et al., 2005). Furthermore, the variable $Na_v1.5$ protein expression from the WT allele correlates with the extent of PCCD in the *Scn5a*$^{+/-}$ mouse model (Leoni et al., 2010). This study (Leoni et al., 2010) divided 10-week-old *Scn5a*$^{+/-}$ mice into two electrocardiographic subgroups showing either severe or mild ventricular conduction defects. These phenotypic differences persisted with aging. At 10 weeks, the Na^+ channel blocker ajmaline produced similar prolongations of QRS intervals in both groups of *Scn5a*$^{+/-}$ mice. However, the effect of ajmaline was greater in the severely affected subgroup in old mice (>53 weeks). Ventricular tachycardia developed in 5- to 10-week-old severely but not mildly affected *Scn5a*$^{+/-}$ mice. Such findings therefore matched clinical observations in patients with *SCN5A* loss-of-function mutations with either severe or mild conduction defects. The severely but not mildly affected old *Scn5a*$^{+/-}$ mice also showed extensive cardiac fibrosis. Finally, the severely affected *Scn5a*$^{+/-}$ mice had similar $Na_v1.5$ mRNA but lower $Na_v1.5$ protein expression, moderately smaller Na^+ currents, and reduced AP upstroke velocities than the mildly affected *Scn5a*$^{+/-}$ mice.

CONCLUSION

Mice have become powerful tools for clarifying the pathophysiological consequences of *SCN5A* mutations in *SCN5A*-related cardiac disorders. Mice harboring *SCN5A* mutations related to PCCD and SND convincingly recapitulate the clinical phenotypes of PCCD and SND in patients, and provide insight into the mechanisms of *SCN5A* deficiency-associated cardiac conduction diseases. They should constitute useful tools for future studies addressing as yet unanswered questions, such as the role of genetic and environmental modifiers on *SCN5A* disease phenotypes.

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Defects in cytoskeletal signaling pathways, arrhythmia, and sudden cardiac death

Sakima Smith[1,2], Jerry Curran[1], Thomas J. Hund[1,2,3] and Peter J. Mohler[1,2,4]*

[1] Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University Medical Center, Columbus, OH, USA
[2] Division of Cardiovascular Medicine, Department of Internal Medicine, The Ohio State University Medical Center, Columbus, OH, USA
[3] Department of Biomedical Engineering, The Ohio State University Medical Center, Columbus, OH, USA
[4] Department of Physiology and Cell Biology, The Ohio State University Medical Center, Columbus, OH, USA

Edited by:
Carol Ann Remme, University of Amsterdam, Netherlands

Reviewed by:
Hugues Abriel, University of Lausanne, Switzerland
Heather S. Duffy, Beth Israel Deaconess Medical Center, Harvard Medical School, USA

***Correspondence:**
Peter J. Mohler, Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University Medical Center, 473 W. 12th Avenue, 110G DHLRI, Columbus, OH 43210, USA.
e-mail: peter.mohler@osumc.edu

Ankyrin polypeptides are cellular adapter proteins that tether integral membrane proteins to the cytoskeleton in a host of human organs. Initially identified as integral components of the cytoskeleton in erythrocytes, a recent explosion in ankyrin research has demonstrated that these proteins play prominent roles in cytoskeletal signaling pathways and membrane protein trafficking/regulation in a variety of excitable and non-excitable cells including heart and brain. Importantly, ankyrin research has translated from bench to bedside with the discovery of human gene variants associated with ventricular arrhythmias that alter ankyrin–based pathways. Ankyrin polypeptides have also been found to play an instrumental role in various forms of sinus node disease and atrial fibrillation (AF). Mouse models of ankyrin-deficiency have played fundamental roles in the translation of ankyrin-based research to new clinical understanding of human sinus node disease, AF, and ventricular tachycardia.

Keywords: ankyrin, spectrin, arrhythmia, cytoskeleton, mouse model

INTRODUCTION

Ankyrin polypeptides are a family of adapter proteins that link integral membrane proteins with the submembranous actin/spectrin-based cytoskeleton. Ankyrin-R was identified in the late 1970s as a critical link between several anion exchanger isoforms and β-spectrin in red blood cells (Bennett, 1979). Over 30 years later, ankyrins are now widely regarded as key players in the formation of protein complexes including ion channels and transporters, cell adhesion molecules (CAMs), signaling proteins, and cytoskeletal elements. In heart, ankyrin-associated protein complexes organize specialized membrane-domains with distinct electrical and structural properties in cardiac sinus node, atrial, and ventricular cardiomyocytes. With particular emphasis given to ankyrin-B and ankyrin-G, this review provides important insight into the role of ankyrins in cardiac physiology as well as providing information on the link between ankyrin dysfunction and human arrhythmias based primarily on data garnered from mouse models.

ANKYRIN STRUCTURE AND FUNCTION

ANK1, *ANK2*, and *ANK3* genes encode three classes of ankyrin polypeptides (ankyrin-R, ankyrin-B, and ankyrin-G, respectively). While ankyrin-R expression is primarily limited to erythrocytes, neurons, and skeletal muscle, both ankyrin-B and ankyrin-G are widely expressed (Bennett and Chen, 2001; Cunha and Mohler, 2006). Although each gene is alternatively spliced to produce protein products of variable size, canonical ankyrin gene products include 210 kD ankyrin-R, 220 kD ankyrin-B, and 190 kD ankyrin-G (**Figure 1**; Bennett and Chen, 2001; Cunha and Mohler, 2006). Ankyrin genes are large, and complex splicing events determine cell-specific binding partners and function. For example, *ANK1* contains over 40 exons resulting in gene products ranging from −30 to +200kD (Bennett and Chen, 2001). The *ANK2* gene spans over 560 kb with >50 exons and diverse splice forms ranging from −50 to 440 kD in brain, heart, skeletal muscle and thymus (Bennett and Chen, 2001). *ANK3* encodes numerous ankyrin-G isoforms broadly expressed in epithelial tissue, kidney, skeletal and cardiac muscle, and brain (Bennett and Chen, 2001). While the specific role of alternatively spliced ankyrin isoforms is unknown, it is likely that variability in expression contributes to organization and maintenance of distinct subcellular domains in eukaryotic cells.

Canonical ankyrins are comprised of four functional domains: the membrane-binding domain (MBD), the spectrin-binding domain (SBD), the death domain (DD), and the C-terminal domain (CTD; **Figure 1**; Hashemi et al., 2009). The MBD mediates the majority of ankyrin interactions with integral membrane proteins including voltage-gated Na^+ and K^+ channels, the Na/Ca (NCX) exchanger, the Na^+/K^+ ATPase (NKA), the ammonium transporter, the K_{ATP} channel, the inositol 1,4,5 trisphosphate receptor (IP3R), and the anion exchanger (**Table 1**; Bennett and Healy, 2009; Kline et al., 2009). Furthermore, the MBD interacts with CAMs including CD44, E-cadherin, and L1-CAMs. Ankyrin membrane-domains are multivalent for integral membrane protein binding, and therefore have the ability to coordinate large membrane-associated protein complexes. For example, in cardiomyocytes, a single ankyrin-B polypeptide can form a ternary membrane protein complex including the NCX, NKA, and IP3R (Mohler et al., 2005). While there is a homology in the MBD across members of the ankyrin family, each ankyrin possesses a unique set of binding partners (e.g., ankyrin-G and voltage-gated

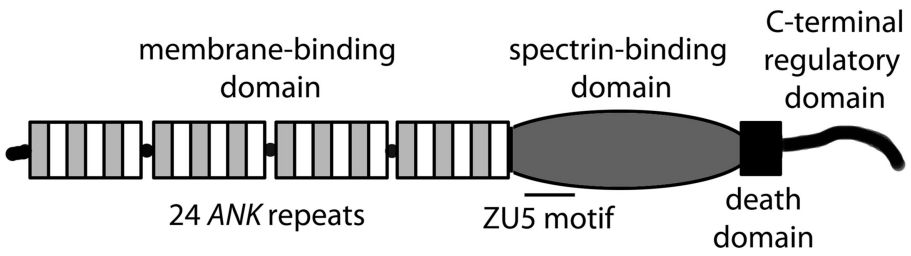

FIGURE 1 | Canonical ankyrin (R, B, G) domain organization.

Table 1 | Select ankyrin (R, B, G) interacting proteins and site of protein binding.

Interacting protein	Ankyrin	Ankyrin domain (if known)
ION CHANNELS/TRANSPORTERS		
Na/Ca exchanger	B	MBD
Voltage-gated Nav channel	G	MBD
Na/K ATPase	B,G	MBD
Anion exchanger (1, 2, 3)	R	MBD
Kir6.2	B	MBD
IP3 receptor	B	MBD
Kv7	G	MBD
Ammonium transporter	R, G	
ADHESION MOLECULES		
Cadherin (N, E)	G	
L1-CAMs	R, B, G	MBD
CD44	R	MBD
CYTOSKELETON		
Beta-spectrin	R, B, G	SBD
Obscurin	R, B, G	CTD
MEMBRANE TRAFFICKING		
Clathrin	R	MBD
EHD3	B	MBD
Tubulin	R, B	MBD
Dynactin-4	B	SBD
MISCELLANEOUS		
PP2A	B	SBD
Hdj1	B	CTD
Fas	G	CTD

MBD, membrane-binding domain; SBD, spectrin-binding domain; CTD, C-terminal domain.

Na^+ and K^+ channels, ankyrin-B and NCX, NKA, IP3R). The ankyrin SBD links ankyrin to the actin-based cytoskeleton via β-spectrin isoforms (Mohler et al., 2004c) thereby conferring structural stability to larger protein networks. Apart from structural capacities, the SBD has also been demonstrated to bind other proteins including the regulatory subunit (B56alpha) of the PP2A family (Bhasin et al., 2007). Lastly, the DD and CTD together comprise the ankyrin "regulatory domain." This domain regulates the binding of ankyrin isoforms to large muscle proteins including obscurin (Kontrogianni-Konstantopoulos et al., 2003; Cunha and Mohler, 2008). However, beyond intermolecular interactions, this domain associates with the ankyrin-BMBD (Abdi et al., 2006). Relevant for this review, the ankyrin-B "regulatory domain" is the site of the majority of human gene variants linked with arrhythmia phenotypes.

ANKYRIN-B AND VENTRICULAR TACHYCARDIA

Defects in ankyrin have been linked to a variety of human diseases, and play an integral role in cardiovascular disease and arrhythmia. In heart, a growing number of inherited arrhythmia syndromes have been linked to ankyrin dysfunction with mounting data that ankyrin defects may also play a role in common forms of acquired heart disease. The first such disorder linked to ankyrin dysfunction was inherited long-QT syndrome (LQTS), characterized by abnormalities on the electrocardiogram (ECG), and increased susceptibility to polymorphic ventricular arrhythmias and sudden death. Mutations within ion channels that are involved in the generation and termination of action potentials make up the majority of inherited LQTS (Splawski et al., 2000). However, work nearly a decade ago identified the first loss-of-function gene variant in *ANK2* in large pedigrees with LQTS (Mohler et al., 2003). Since this initial discovery, *ANK2* loss-of-function variants are now well documented to cause dominantly inherited type 4 LQTS (LQT4) and have been identified throughout the world in cohorts with phenotypes linked with LQT4 (Mohler et al., 2004b, 2007). Beyond loss-of-function mutations, analysis of large human populations has linked *ANK2* single-nucleotide polymorphisms with alterations in the cardiac QT interval (Sedlacek et al., 2008) further supporting the role of ankyrin-B in cardiac electrical regulation.

The development a mouse model heterozygous for a null mutation in ankyrin-B (ankyrin-$B^{+/-}$) was critical for understanding the pathophysiology of the human disease. In fact, ankyrin-$B^{+/-}$ mice phenocopy human LQT4. It is also imperative to highlight that mice homozygous for null mutations in ankyrin-B (ankyrin-$B^{-/-}$) also demonstrate abnormalities on ECG that mirrors those of LQT4 with abnormal QT rate adaptation resulting in greater QT prolongation with heart rate deceleration (Chauhan et al., 2000 #2). In regards to the heterozygous model, ankyrin-$B^{+/-}$ mice and human *ANK2* E1425G carriers display bradycardia, prolonged QT interval, heart rate variability, and episodes of isorhythmic atrio-ventricular dissociation (Mohler et al., 2003; Le Scouarnec et al., 2008). Furthermore, similar to their human counterparts, ankyrin-$B^{+/-}$ mice display polymorphic ventricular tachycardia, syncope, and sudden cardiac death in response to exercise and increased circulating catecholamines. In light of the complex phenotype presented by human *ANK2* variant carriers

that includes ventricular and supraventricular defects, the disease name was revised to the moniker "*Ankyrin-B Syndrome*."

Great strides have been made over the past 10 years to establish the link between ankyrin-deficiency and increased susceptibility to cardiac arrhythmia. At the level of the single myocyte, loss of ankyrin-B results in aberrant membrane targeting and regulation of the NKA, NCX, K_{ATP}, and IP3R. Loss of these proteins ultimately produces defects in intracellular Na^+ and Ca^{2+} regulation, that in the cardiomyocyte leads to increased sarcoplasmic reticulum calcium load, inappropriate calcium release, and early and delayed after depolarizations in the presence of high levels of circulating catecholamines. Subsequent computational modeling has further supported the link between ankyrin-deficiency, loss of membrane function of NCX and NKA, intracellular Na^+ and Ca^{2+} accumulation, and increased susceptibility of myocytes to develop SR Ca^{2+} overload (Wolf et al., 2010). In particular, the ankyrin-deficient cellular phenotype was illustrated to produce frequent spontaneous Ca^{2+} release events and increased the incidence of pro-arrhythmic after depolarizations during rapid pacing and/or elevated catecholamines (Wolf et al., 2010). This biophysical phenotype was independently confirmed in a recent report investigating Ca^{2+} handling in intact cardiomyocytes isolated from ankyrin-$B^{+/-}$ mice (Camors et al., 2012).

ANKYRIN-B AND SINUS NODE DISEASE

Beyond ventricular arrhythmias, loss-of-function variants in ankyrin-B have more recently been linked with sinus node dysfunction (Le Scouarnec et al., 2008). Similar to the discovery of the link between *ANK2* and human ventricular phenotypes, large human pedigrees were initially utilized to provide genetic linkage between sinus node disease and the *ANK2* locus. Related directly to sinus node disease, two large European cohorts mutant *ANK2* alleles were shown to display highly penetrant bradycardia and exaggerated heart rate variability. Immunoblotting of muscle biopsies in affected individuals confirmed loss of ankyrin-B expression associated with the phenotype (Le Scouarnec et al., 2008). The degree of penetrance and the age of onset of sinus node dysfunction were truly remarkable in affected individuals. Sinus node dysfunction is largely a disease of the elderly population (Bernstein and Parsonnet, 1992). However, carriers of the *ANK2* mutation received pacemakers (devices implanted underneath the skin of the clavicle with leads attached to the heart designed to pace the heart at a safe heart rate if the native conduction system falters) at a mean age of 30-extraordinarily rare in clinical practice. Akin to these patients with ankyrin-B-associated sinus node disease, mice lacking ankyrin-B display severe sinus node dysfunction, with reduced expression of NKA, NCX, and IP3R (Le Scouarnec et al., 2008). However, unlike ventricular cardiomyocytes, $I_{Ca,L}$ was also significantly reduced in isolated ankyrin-$B^{+/-}$ sinoatrial node cells, further altering calcium handling and sinoatrial node automaticity (Le Scouarnec et al., 2008). Together these data link ankyrin-B dysfunction to sinus node disease and provide key mechanistic insights into molecular basis for the disease at the cellular level. Subsequent studies have provided new insight into the role of ankyrin-B in cardiac pacing at the level of the whole heart. This past year, regulation of automaticity in wild-type and ankyrin-$B^{+/-}$ mouse hearts was examined using optical mapping (Glukhov et al., 2010). As expected in wild-type mice, isoproterenol accelerated the sinoatrial rate and shifted the leading pacemaker site whereas acetylcholine had the opposite effect. In contrast, following isoproterenol, ankyrin-$B^{+/-}$ mice exhibited significant beat-to-beat variability (bradycardia followed by abrupt bursts of tachycardia), a disorganized shift of the leading pacemaker, and multiple competing pacemaker foci- phenotypes consistent with the human sinus node disease. Ankyrin-$B^{+/-}$ mouse hearts also displayed reduced sensitivity to acetylcholine. Collectively these data highlight the importance of the functional anatomy of the entire sinoatrial node pacemaker complex, and clearly demonstrate the role of ankyrin-B in cardiac automaticity in humans and in mice.

ANKYRIN-B AND ATRIAL FIBRILLATION

This past year, the role of ankyrin-B in cardiovascular electrophysiology and disease has continued to expand by the linkage of ankyrin-B dysfunction in human atrial fibrillation (AF; Cunha et al., 2011). Specifically, individuals harboring *ANK2* loss-of-function alleles presented with a high incidence of early-onset AF, commonly progressing to permanent AF. In line with this finding, continuous telemetry ECG recordings from ankyrin-B-deficient mice revealed atrial arrhythmias similar to those observed in human carriers of *ANK2* variants, including bradycardia with erratic atrial activity, lack of discrete P waves, and variable ventricular response (Cunha et al., 2011). Ankyrin-$B^{+/-}$ mice also showed increased susceptibility to induction of AFin response to atrial burst pacing. Primary atrial myocytes from ankyrin-$B^{+/-}$ mice displayed shortened action potentials, a hallmark of AF, and a sharp decrease of L-type Ca^{2+} current. Subsequent molecular studies determined that loss of L-type calcium current was due to loss of $Ca_v1.3$, but not $Ca_v1.2$ in atrial cardiomyocytes also explaining previously discovered reductions in $I_{Ca,L}$ in sinoatrial node myocytes (Le Scouarnec et al., 2008). Moreover, $Ca_v1.3$ was identified as a novel ankyrin-B-interacting protein, and this direct interaction was shown to be mediated by a short motif in the C-terminus. As a final link between ankyrin-B and AF, analysis of human paroxysmal AF showed decreased ankyrin-B, as well as $Ca_v1.3$ expression. Together, these data provided a new molecular mechanism underlying congenital AF, a new binding partner for ankyrin-B in heart, and implicate ankyrin-B in the pathogenesis of common acquired forms of AF. These studies also provide insight into how defects in a single protein can result in a broad spectrum clinical disease with unique regional manifestations. In other words, the complexity of "ankyrin-B syndrome" arises from the fact that ankyrin-B is both broadly expressed in the heart and forms distinct, region-specific macromolecular complexes.

ANKYRIN-B, ISCHEMIA, AND ARRHYTHMIAS

In addition to roles in congenital arrhythmias, ankyrin-B has more recently been linked with more common forms of heart disease. Electrical and structural remodeling in the peri-infarct zone creates a substrate favorable for the initiation and maintenance of reentrant arrhythmias following myocardial infarction, in particular, ventricular fibrillation and tachycardia. An essential component of this remodeling process involves the redistribution of ion channels and transporters in surviving myocytes near the

infarct (Baba et al., 2005). Recently, ankyrin-B mRNA and protein levels, along with downstream ankyrin-B-associated channels were identified as differentially regulated following coronary artery occlusion in the canine (Hund et al., 2009). Specifically, a marked increase in ankyrin-B mRNA levels 5 days post-occlusion were seen, presumably in response to the observed decrease in protein levels of ankyrin-B at the same time. Protein levels and/or membrane expression of NCX, NKA, and IP3R were also decreased at 5 days post-occlusion, in line with their known role as ankyrin-B-binding partners (Hund et al., 2009). These changes were observed in absence of any changes to β_2-spectrin. By 14 days post-occlusion, ankyrin-B protein levels had returned to baseline, and the recovery of NCX, NKA, and IP3R expression were, also, underway, consistent with the normalization of electrical remodeling observed in this model. While these studies focus on a chronic stage of myocardial infarction, ankyrin-B likely is involved in heart's response to acute ischemia. Specifically, ankyrin-B plays a pivotal role in ATP-sensitive K^+ channel (K_{ATP}) regulation. Ankyrin-B associates with the K_{ATP} channel via the major subunit, $K_{ir}6.2$, a key component of the cellular apparatus required for intrinsic cardioprotection from ischemia (Li et al., 2010). Hearts and isolated cardiomyocytes lacking ankyrin-B display a loss of $K_{ir}6.2$ membrane expression resulting in decreased membrane $I_{K,ATP}$ (Li et al., 2010). K_{ATP} channels serve a cardioprotective role in ischemia via a K_{ATP} channel-mediated shortening of the action potential (Suzuki et al., 2001), hence decreased expression of K_{ATP} could increase the susceptibility to arrhythmias. More recently, we have observed similar changes in ankyrin and related proteins (e.g., EHD family of trafficking proteins) in a variety of animal models of ischemic and non-ischemic disease and in human heart failure (Gudmundsson et al., 2010, 2012). In summary, these data suggest potential new roles for ankyrin-B in the processes of electrical and structural remodeling in common forms of heart disease (myocardial infarction/heart failure).

Clearly, loss of membrane ion channels and transporters is an important link between ankyrin dysfunction and disease. However, recent findings suggest that ankyrin-B regulation extends beyond these integral membrane proteins to cardiac signaling in health and disease. For example, in ventricular myocytes, ankyrin-B has also been identified as a binding partner of B56α, the regulatory subunit of PP2A (Bhasin et al., 2007). PP2A is a multifunctional serine/threonine phosphatase known to regulate cardiac β-adrenergic signaling (Klein et al., 2003; De Arcangelis et al., 2008). Cardiomyocytes lacking ankyrin-B expression display a marked loss of B56α (Bhasin et al., 2007). As inhibition of B56α in myocytes results in RyR2 hyper-phosphorylation [by calcium/calmodulin-dependent kinase II (CaMKII)] and myocyte electrical instability, it is reasonable to speculate that defects in CaMKII-based signaling pathways may contribute to human ankyrin-B-linked arrhythmias (Terentyev et al., 2009). In summary, work from the past decade has illustrated complex roles for ankyrin-B in myocyte function and in disease. The multifactorial nature of ankyrin-B and its broad distribution present unique challenges and opportunities as we consider the development of therapies to treat patients with inherited or acquired ankyrin-deficiency and associated disease.

ANKYRIN-G AND BRUGADA SYNDROME

While much focus has been placed on the role of ankyrin-B in heart due in part to the identified cohorts of human patients with ankyrin-B mutations, the influence of ankyrin polypeptides on heart function extends beyond ankyrin-B. Specifically, ankyrin-G has been shown to play roles in heart health and disease. Importantly, similar to its role in neurons, ankyrin-G has been shown to target Nav1.5, for maintenance of normal cardiac cell excitability. Ankyrin-G and Na channels directly associate and are co-localized in heart at the intercalated disk, where neighboring cells are electrically and mechanically coupled. Silencing of ankyrin-G in myocytes results in loss of $Na_v1.5$ expression, membrane targeting, and membrane current (Lowe et al., 2008).

Defects in ankyrin-G-based targeting pathway have been linked to excitable cell disease. Interestingly, ankyrin-G has emerged as a candidate in multiple GWAS studies as associated with bipolar disorder (Ferreira et al., 2008 #2309; Scott et al., 2009 #5597). In heart, disruption of ankyrin-G/Nav1.5 interaction has been linked to the Brugada Syndrome, an autosomal-dominant, cardiac arrhythmia syndrome characterized by ST segment elevation in the precordial leads associated with right bundle branch block and T-wave inversion (Antzelevitch et al., 2005). *SCN5A* encodes $Na_v1.5$, and gene variants account for nearly a third of Brugada Syndrome cases. *SCN5A* variants largely lead to biophysical defects that disrupt the inward sodium current, which is a key current in cellular depolarization during the action potential. A *SCN5A* variant (E1053K) that is localized to the ankyrin-G-binding motif in Nav1.5 and disrupts ankyrin-G/$Na_v1.5$ association results in Brugada Syndrome (Mohler et al., 2004a). Mechanistically, this variant disrupts normal membrane targeting of Nav1.5 resulting in loss of membrane sodium channel current density and impaired excitability (Mohler et al., 2004a).

Beyond $Na_v1.5$ membrane targeting, ankyrin-G plays additional roles in $Na_v1.5$ membrane regulation via recruitment of $Na_v1.5$ regulatory proteins. Specifically, Hund et al. (2010) recently demonstrated that β_{IV}-spectrin is co-localized and interacts with ankyrin-G and $Na_v1.5$ at the cardiac intercalated disk. Moreover, β_{IV}-spectrin through direct binding, targets CaMKII to the intercalated disk to directly phosphorylate $Na_v1.5$ at a specific residue (571) in the DI–DII linker. This study revealed that the β_{IV}-spectrin/CaMKII complex has a direct effect on membrane excitability, and mutant β_{IV}-spectrin mice lacking CaMKII binding activity (qv^{3J}) displayed defects in Nav1.5 phosphorylation, activity, excitability, and heart function (Hund et al., 2010). Based on these initial findings, it is interesting to consider the potential role of β_{IV}-spectrin/ankyrin-G-based signaling platform in regulating excitable cell function in human disease (Hund et al., 2010).

Apart from regulating membrane excitability, ankyrin-G was recently proposed to play secondary roles in regulation of intercalated disk structural integrity. Delmar and colleagues identified ankyrin-G as member of a larger complex of $Na_v1.5$ and gap junctions at the cardiac desmosome (Sato et al., 2011). Loss of ankyrin-G caused significant changes in the subcellular distribution and abundance of desmosomal proteins. This complex could potentially play key roles in arrhythmia susceptibility as human

mutations in desmosomal genes have been linked with the development of arrhythmogenic right ventricular dysplasia (Pilichou et al., 2006), characterized by inflammation, fibrosis or adiposis, and susceptibility to cardiac arrhythmia and sudden cardiac death (Sen-Chowdhry et al., 2010). In summary, based on the ability to regulate both critical electrical and structural signaling, ankyrin-G-based pathways have been shown to play key roles in myocyte physiology and likely are a central key node for human structural and electrical heart disease.

SUMMARY

The past decade has illustrated critical roles for ankyrins in cardiac physiology and in disease. Unlike their classic roles in the erythrocyte, ankyrins play dynamic roles in protein targeting and stability, and secondary unexpected roles in protein regulation and cardiac signaling. While ankyrins are linked with congenital ventricular and atrial arrhythmias, current data suggest that these proteins serve central nodal points of regulation for common forms of acute and chronic cardiovascular disease. Moreover, based on expression, it is not unreasonable that ankyrins likely play key roles in other types of human cardiovascular disease including inflammation and metabolic and vascular disease.

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Funny current and cardiac rhythm: insights from HCN knockout and transgenic mouse models

Annalisa Bucchi, Andrea Barbuti, Dario DiFrancesco and Mirko Baruscotti*

Department of Biosciences, University of Milano, Milano, Italy

Edited by:
Carol Ann Remme, University of Amsterdam, Netherlands

Reviewed by:
Jeanne M. Nerbonne, Washington University School of Medicine, USA
Dénise Den Haan, Academic Medical Center Amsterdam, Netherlands

****Correspondence:***
Mirko Baruscotti, Department of Biosciences, University of Milano, via Celoria 26, 20133 Milano, Italy.
e-mail: mirko.baruscotti@unimi.it

In the adult animal the sinoatrial node (SAN) rhythmically generates a depolarizing wave that propagates to the rest of the heart. However, the SAN is more than a simple clock; it is a clock that adjusts its pace according to the metabolic requirements of the organism. The Hyperpolarization-activated Cyclic Nucleotide-gated channels (HCN1–4) are the structural component of the funny (I_f) channels; in the SAN the I_f current is the main driving electrical force of the diastolic depolarization and the HCN4 is the most abundant isoform. The generation of HCN KO and transgenic mouse models has advanced the understanding of the role of these channels in cardiac excitability. The HCN4 KO models that were first developed allowed either global or cardiac-specific constitutive ablation of HCN4 channels, and resulted in embryonic lethality. A further progress was made with the development of three separate inducible HCN4 KO models; in one model KO was induced globally in the entire organism, in a second, ablation occurred only in HCN4-expressing cells, and finally in a third model KO was confined to cardiac cells. Unexpectedly, the three models yielded different results; similarities and differences among these models will be presented and discussed. The functional effects of HCN2 and HCN3 knockout models and transgenic HCN4 mouse models will also be discussed. In conclusion, HCN KO/transgenic models have allowed to evaluate the functional role of the I_f currents in intact animals as well as in single SAN cells isolated from the same animals. This opportunity is therefore unique since it allows (1) to verify the contribution of specific HCN isoforms to cardiac activity in intact animals, and (2) to compare these results to those obtained in single cell experiments. These combined studies were not possible prior to the development of KO models. Finally, these models represent critical tools to improve our understanding of the molecular basis of some inheritable arrhythmic human pathologies.

Keywords: HCN KO mouse models, cardiac pacemaking, sinoatrial node

INTRODUCTION

In mammals, cardiac pacemaking originates from cells in the sinoatrial node (SAN) constitutively able to generate rhythmic action potentials (APs) due to the presence of a spontaneous diastolic depolarization (Boyett et al., 2000; Mangoni and Nargeot, 2008). SAN physiologists have long studied the molecular mechanisms responsible for the diastolic/pacemaker depolarization since it is this phase that temporally separates and electrically triggers consecutive APs (DiFrancesco et al., 1986; Biel et al., 2009; Baruscotti et al., 2010a). Several ionic currents and mechanisms that contribute to SAN cell's activity are shared with other types of cardiomyocytes; however, the functional expression of the "funny" (I_f) current in the adult is mostly restricted to the SAN and the conduction system (Baruscotti et al., 2010a).

Its tissue-specific expression, together with the evidence that I_f is an inward depolarizing current activated within the diastolic depolarization range of potentials (−40/−60 mV) and modulated by the second messenger cAMP, clearly indicate that this current provides a substantial contribution to cardiac pacemaking (Baruscotti et al., 2005; Verkerk et al., 2007a). Since its discovery and until a decade ago, the vast majority of studies on the I_f current were carried out in single SAN cells isolated from lower mammals, typically rabbit; recent evidence in humans has confirmed previous conclusions on I_f properties and function. More specifically: (1) the presence of I_f and HCN1 and HCN4 isoforms has been verified also in human SAN cells (Verkerk et al., 2007a,b; Chandler et al., 2009); (2) genetically determined alterations of the I_f channel have been associated with mild or severe forms of arrhythmias in humans (Baruscotti et al., 2010b); (3) an I_f specific blocker, ivabradine, acts as pure heart rate slowing drug and is currently used in the therapy of chronic stable angina and heart failure (DiFrancesco and Camm, 2004).

The molecular determinants of the funny current belong to the family of Hyperpolarization-activated Cyclic Nucleotide-gated (HCN) channels which comprises four isoforms (HCN1–4; Barbuti et al., 2007; Biel et al., 2009). The subunits can assemble as homotetramers and/or heterotetramers to form functional channels. The structural arrangement of each single subunit comprises six membrane-spanning domains (S1–S6), which include a putative voltage sensor (S4) and a pore (P) region between the S5 and S6 segments, and a cyclic-nucleotide-binding domain (CNBD) in the C-terminus.

Analysis of HCN distribution in the adult heart indicates that the HCN4 isoform is the major component of pacemaker channels in the SAN of all the species analyzed (mouse, rat, rabbit, dog, human). Species-dependent expression of HCN1 and HCN2 has also been reported in the SAN, but always at much lower levels than HCN4; the HCN3 is absent from the SAN; (Ishii et al., 1999; Shi et al., 1999; Moosmang et al., 2001; Marionneau et al., 2005; Tellez et al., 2006; Liu et al., 2007; Thollon et al., 2007; Stillitano et al., 2008; Brioschi et al., 2009; Chandler et al., 2009; Fenske et al., 2011; Herrmann et al., 2011b). A clear understanding of the presence of different HCN isoforms in working myocytes is complicated because of interspecies variability and of contrasting results between transcriptional and protein data. Despite this, it is reasonable to say that HCN2 is the predominant isoform both in atria and ventricles even though at a lower level of expression compared to the HCN4 level in SAN and the conduction system. HCN4, HCN3, and HCN1 proteins are only occasionally reported (Shi et al., 1999; Han et al., 2002; Dobrzynski et al., 2003; Marionneau et al., 2005; Zicha et al., 2005; Yamamoto et al., 2006; Liu et al., 2007; Stillitano et al., 2008; Chandler et al., 2009; Fenske et al., 2011; Herrmann et al., 2011b). Taken together, the observations outlined above and a whole set of experimental findings and numerical reconstruction data (Baruscotti et al., 2005; DiFrancesco, 2010) indicate clearly the involvement of the I_f current in cardiac pacemaking. What is the degree of I_f involvement in the complex set of mechanisms that underlie the whole of the cell-cycling processes active in pacemaker cells is however still a very much debated issue (Lakatta and DiFrancesco, 2009; DiFrancesco and Noble, 2012a,b). To determine this, it is also important to consider that despite the copious amount of studies carried out at single cell, any conclusion deriving from this work cannot automatically be held valid for the function of the SAN as a whole, in the absence of experimental confirmation. The availability of the knockout technology has finally allowed to explore the role of the I_f current within its complex setting of interactions in the living animal and the pathological states associated with its removal.

HCN4 KO AND TRANSGENIC MOUSE MODELS AND EMBRYOLOGICAL DEVELOPMENT

HCN KO and transgenic mice have allowed to gain insight on the role of HCN subunits during embryonic development (**Table 1**). Constitutive deletion of the HCN4 gene, either globally or specifically in the myocardium, causes a premature death of homozygous embryos between ED9.5 and ED11.5 (Stieber et al., 2003). Analysis of isolated ED9.5 hearts revealed that HCN4 deletion results in a significant bradycardia (36.7% reduction of heart rate when compared to wild type animals). The HCN4 knockout resulted in a 75–90% decrease of the I_f current, with the residual current being possibly also carried by HCN1 and HCN2 according to the authors (Stieber et al., 2003). These data confirm that the HCN4 isoform is the major component of the f-channel also in the developing SAN. Importantly, studies with specific deletion of HCN1 and HCN2 indirectly confirmed the role of HCN4 since in these HCN1 (Nolan et al., 2003) and HCN2 (Ludwig et al., 2003) knockout animals there was no evidence of cardiac alterations in developing embryos. The fundamental role of HCN4 during cardiac development is supported also by molecular data on the distribution of HCN4 mRNA in the embryonic heart. In the mouse, the *Hcn4* gene is indeed already expressed in the cardiac mesoderm at ED7.5; with the progression of development this expression remains confined to the sinus venosus/SAN and to the conduction system (Garcia-Frigola et al., 2003; Christoffels et al., 2010; Vicente-Steijn et al., 2011).

Recently, a similar pattern of HCN4 expression has been found in the chick embryo (Vicente-Steijn et al., 2011). This early expression of HCN4 is in agreement with previous evidence that in the chick embryonic heart, pacemaker activity initiates from the sinus venosus (the prospective SAN) before the first contraction takes place (DeHaan, 1965; Van Mierop, 1967). In mice, the first cardiac contraction is visible around embryonic day 8.5 (ED8.5) at the tubular heart stage, and at this stage most cardiomyocytes display rhythmic contractions and express the I_f current (Yasui et al., 2001). After this period, the proportion of cardiac embryonic cells maintaining a spontaneous activity decreases by about 60–70%, a decrease that is accompanied by a significant reduction (∼80%) of the current (Yasui et al., 2001).

Maintenance of a regular, if slower, rate until ED11.5 following deletion of HCN4 suggests that other mechanisms contribute to cardiac pacemaking in these early stages. Why HCN4 is then robustly expressed so early in the developing heart? A possible answer comes from the analysis of another transgenic HCN model. Recently Harzheim et al. (2008) have developed a mouse carrying a single amino acid substitution (R669Q) within the CNBD of HCN4 which makes the subunit completely insensitive to cAMP stimulation. Surprisingly, $HCN4^{R669Q/R669Q}$ homozygous mice die at ED111–ED12 and before that time point they show a reduced heart rate similar to that of both global and cardiac-specific constitutive HCN4 KO mice. When HCN4 protein expression and I_f current were analyzed in $HCN4^{R669Q/R669Q}$ mice, they did not show any down-regulation and the only difference was the expected lack of sensitivity to cAMP with a consequent activation at more negative potentials. These data indicate that, although HCN4 channels contribute to pacemaking as soon as the tubular heart starts to contract, their contribution becomes more important after ED9.5 when the SAN starts to develop from mesenchymal precursors (Wiese et al., 2009). At ED12.5, when the SAN is formed, $HCN4^{R669Q/R669Q}$ channels failure to respond to adrenergic stimulation becomes incompatible with life as previously indicated (Portbury et al., 2003; Chandra et al., 2006). Other evidence confirming the importance of HCN4 channels during embryonic development derives from another KO mouse with a constitutive deletion of Shox2, a transcription factor participating in the specific development of the SAN (Christoffels et al., 2010). The removal of shox2 does not cause any functional alterations in the heart up to ED10.5 when a severe bradycardia (50% compared to wild type animals) and a decrease of HCN4 expression become evident. Shox2-deleted mice die between ED11.5 and ED12.5 (Espinoza-Lewis et al., 2009) as observed with HCN4 KO mice.

ROLE OF HCN4 CHANNELS IN ADULT MICE

Previous studies using constitutive global or cardiac-specific deletion of HCN4 isoforms (Stieber et al., 2003) have revealed that the presence of HCN4 is required for the correct functional development of the embryonic heart; nonetheless the prenatal lethality

Table 1 | Phenotypic manifestations of HCN KO/transgenic mouse models.

Models	*In vivo* effects in embryos	Isolated heart	Isolated cardiomyocytes	Reference
Constitutive global HCN4 KO	Embryonic lethality (ED 9.5–11.5)			Stieber et al. (2003)
Constitutive cardiac-specific HCN4 KO	Embryonic lethality (ED 9.5–11.5)	↓ HR (−36.7%) No response to cAMP	I_f: ↓ (−75/−90%) No response to cAMP APs: absence of mature Pacemaker-like APs No response to cAMP	Stieber et al. (2003)
Constitutive global $HCN4^{R669Q/R669Q}$ transgenic	Embryonic lethality (ED 11–12)	↓ HR (−40/−60%) No response to Iso	I_f: slower activation and shift of the AC (−13 mV) No response to cAMP APs: ↓ rate (−36/−56%)	Harzheim et al. (2008)
	***In vivo* effects in adult**	**Isolated SAN cells**		
Inducible global HCN4 KO	Sinus arrhythmias(pauses) Normal response to Iso	I_f: ↓ (−75%) Faster activation; no shift of the AC Normal response to Iso APs: no spontaneous activity in 90% of the cells; when present, spontaneous rate is normal		Herrmann et al. (2007)
Inducible HCN4-expressing cells HCN4 KO (KiT-HCN4 KO)	Arrhythmia (sinus pauses) Normal response to Iso ↑ Response to Carb	I_f: ↓ (−80%) Faster activation, no shift of the AC APs: no spontaneous activity in 45% of the cells; when present, spontaneous rate is normal		Hoesl et al. (2008)
Inducible cardiac-specific HCN4 KO (Ci-HCN4 KO)	↓ Basal HR (−50%) PQ prolongation ↓ Maximal response to Iso AV block and death	I_f: ↓ (−70%) No changes in kinetics, no shift of the AC Normal response to Iso APs: ↓ Rate (−61%) ↓ Maximal response to Iso		Baruscotti et al. (2011)
Inducible cardiac-specific HCN4–573X KI	↓ Basal HR (−20%) ↓ Exercise-induced HR	I_f: no reduction Slower activation; shift of the AC (−20 mV) No response to Iso APs: irregular pacemaker activity in 88% of the cells ↓ Rate (−33%) ↓ Maximal response to Iso		Alig et al. (2009)
Constitutive global HCN2 KO	Absence epilepsy and sinus dysrhythmia	I_f: ↓ (−30%) Slower activation; no shift of the AC Normal response to cAMP		Ludwig et al. (2003)
Constitutive cardiac-specific HCN2 KO	Sinus dysrhythmia			Ludwig et al. (2003)
Constitutive global HCN3 KO	Regular sinus rhythm ↑ T-wave amplitude (+63.5%) and duration (+15%) at low heart rates ↑ QT interval (+12%)	**Isolated epicardial cells** I_f: ↓ (−30%) APs: shortening of the AP duration		Fenske et al. (2011)

associated with this model has prevented further investigation of the role of the I_f current in adult cardiac pacemaking. This aspect was therefore addressed by means of temporally controlled induction of gene knockout methodology.

INDUCIBLE KO MODELS

The first inducible HCN4 KO model was developed in 2007 by Herrmann et al. (2007) who engineered a transgenic mouse line by inserting LoxP sites in the regions flanking the exon 4 of the *Hcn4* gene (encoding the pore and the S6 transmembrane domain). This line was then crossed with the CAGGCre-ERTM mouse line (Hayashi and McMahon, 2002) to ensure the ubiquitous expression of a tamoxifen-inducible Cre construct and thus a generalized removal of the HCN4 isoforms from the entire organism. The following year the same group (Hoesl et al., 2008) produced a new transgenic mouse line with an inducible Cre inserted in-frame into the *Hcn4* start codon; thus allowing the knockout event to occur only in HCN4-expressing cells. The effective deletion of HCN4 channels was confirmed in both models by PCR, western blot, and immunoistochemical detection experiments, and by patch-clamp studies in single SAN cells isolated from KO mice; the latter showed that the I_f current decreased of about 75–80%. Despite this large current reduction, ECG recordings in freely moving HCN4 KO mice did not reveal major alterations of the cardiac

electrical activity. The only evidence of an arrhythmic behavior was the presence of sinus pauses (8–16 pauses/min with an average duration of 321 ms) between periods of normal activity characterized by mean basal rates which did not differ from that of control mice. Interestingly, the number of pauses was inversely related to the heart rate, and the highest number of pauses (up to 8.1/min) occurred at heart rates of about 300–450 bpm; the presence of sinus pauses was also confirmed in ECG recordings of isolated hearts (Herrmann et al., 2007). Studies of spontaneous activity of isolated single SAN cells did not produce similar results since the authors report that a large fraction (45–90%) of the cells lack the ability to fire spontaneously (Herrmann et al., 2007; Hoesl et al., 2008) and in those cells in which repetitive APs were present, their features did not differ from those observed in SAN cells from wild type animals. This remarkable difference between the appearance of sinus pauses in the intact animal/isolated heart and the lack of normal spontaneous activity as observed in single cells remains an issue for which a convincing explanation is still lacking.

The I_f current has previously been shown to be an important target of autonomic modulators and single cells studies have shown that the β-adrenergic/muscarinic modulation of single cell firing rate reflects the increased/reduced I_f current flowing during diastole (Bucchi et al., 2007; Mangoni and Nargeot, 2008). For this reason these mouse models were also employed to verify whether autonomic modulation of heart rate could be altered in the absence of HCN4 channels. Maximal sympathetic activation due to isoproterenol administration (0.5 mg/kg i.p.) or physical exercise (treadmill) in freely moving HCN4 knockout mice elicited a rate response that was not different from that of control mice (∼700 bpm), but upon return to basal rate the number of pauses observed in KO mice was increased up to fourfold (Herrmann et al., 2007). Perhaps a more interesting finding came from muscarinic stimulation since the heart rate reduction observed in HCN4 KO mice was significantly larger than that elicited in control mice, thus suggesting that the depolarizing contribution of HCN4 channels may be required to partially counteract the bradycardic effect due to strong activation of the parasympathetic arm of the sympathovagal balance (Herrmann et al., 2007; Hoesl et al., 2008).

Taken together, the results obtained with these inducible models suggest that ablation of HCN4 in the adult animal has limited effects on cardiac pacemaker generation and modulation. These results appear therefore to challenge the previously accepted idea that considered the I_f current/HCN4 channels a major determinant of SAN pacemaking.

However, a pitfall of the previous inducible models was the lack of cardiac-specificity. Indeed, both the generalized removal of the HCN4 isoform in the entire organism (Herrmann et al., 2007) and the selective ablation only in HCN4-expressing cells (Hoesl et al., 2008) imply that HCN4 knockout occurs not only in the heart, but also in brain areas expressing the HCN4 channels. It is therefore likely that neuronal- and cardiac-induced effects of the KO process overlap thus confounding the interpretation of data. In order to allow for a cardiac-specific HCN4 removal, our group developed a mouse knockout model by crossing a novel floxed HCN4 mouse line (Baruscotti et al., 2011) with a Cre mouse carrying the Cre-recombinase under the control of the cardiac-specific α-myosin heavy chain (αMHC) promoter (Sohal et al., 2001). This knockout protocol ensures an inducible and cardiac-restricted ablation of the HCN4 isoforms. In this model the loxP sites were inserted in the region flanking the exon 2 which encodes for the region comprising most of the S1 up to the entire S4 transmembrane segments. The recombination process led to a frame-shift generating an early stop codon but it is not known whether the putative truncated protein is made or not (**Figure 1A**). The knockout process was induced by i.p. injection of tamoxifen for five consecutive

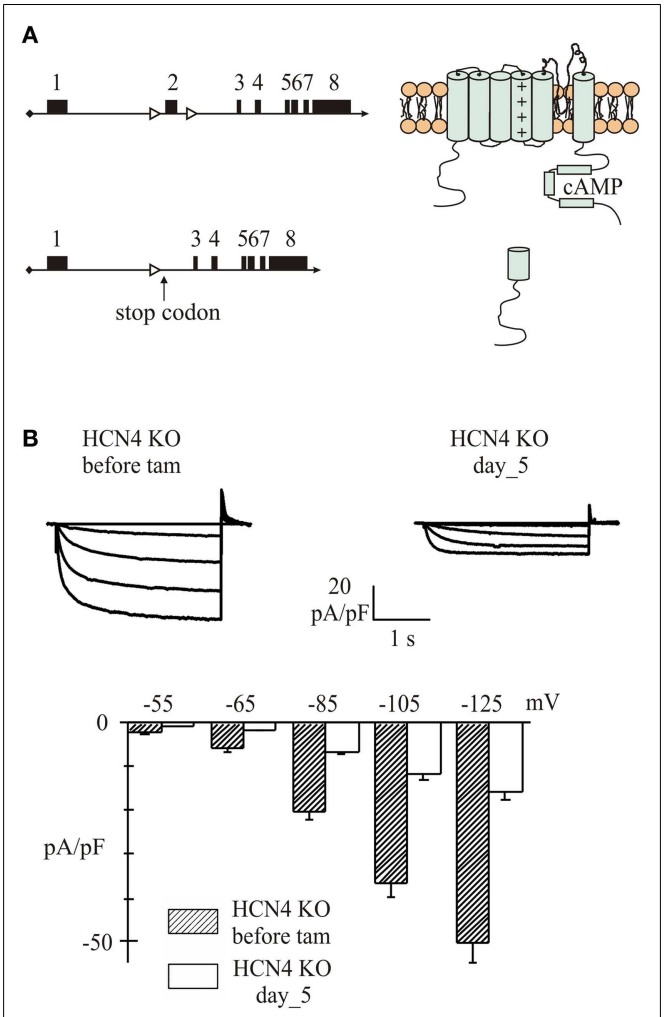

FIGURE 1 | (A) Top, left: structure of the floxed HCN4 gene carrying the loxP sites (triangles) in the introns flanking exon 2 of the inducible cardiac-specific HCN4 KO mouse model. Top, right: structure of the wild type HCN4 protein isoform. Bottom, left: HCN4 gene structure after the Cre-induced recombination; the gene is now lacking exon 2. Bottom, right: Removal of exon 2 eliminates the first four transemembrane segments and generates a frame-shift leading to an early stop-codon 3 residues downstream to the recombination site. The hypothetical resulting protein should therefore be formed by the N-terminus and by a small portion of the S1 segment. No data on the presence/absence of both transcript and protein are available **(B)** Bottom: Mean I_f current densities measured during hyperpolarization steps at the indicated potentials (holding potential −35 mV) from SAN cells isolated from cardiac-specific HCN4 KO mice before (dashed bars) and 5 days after (empty bars) KO induction (tamoxifen treatment). Top: Representative I_f traces recorded at the same potentials.

days. This model yielded completely different results than previous non-cardiac-specific models.

The KO induction in freely moving mice resulted in the development of a progressive slowing of the heart rate, without the appearance of anomalous sinus pauses. Five days after the initiation of the tamoxifen treatment, the average heart rate observed by telemetric ECG recordings was reduced by about 50% relative to control (**Figure 2**). In this model, therefore, removal of HCN4 channels was clearly associated with the appearance of severe bradycardia. The effective ablation of HCN4 proteins was confirmed by immunolabeling of SAN tissue and single cells and by western blot experiments; patch-clamp recordings quantified the KO effect in a 70% reduction of the I_f current (**Figure 1B**). The cellular origin of the bradycardia was then further investigated by comparing the spontaneous activity of single SAN cells isolated from control and KO animals. KO induction reduced but did not abolish the spontaneous firing of SAN cells (by about 61% at day 5) and the time-course of this effect nicely overlapped the bradycardic onset in intact animals (**Figure 2B**). Clearly, the similar time courses of heart rate bradycardia and slowing of spontaneous firing of single cells, proceeding in parallel with the reduction of the I_f current, indicate a strict correlation between I_f current and rate.

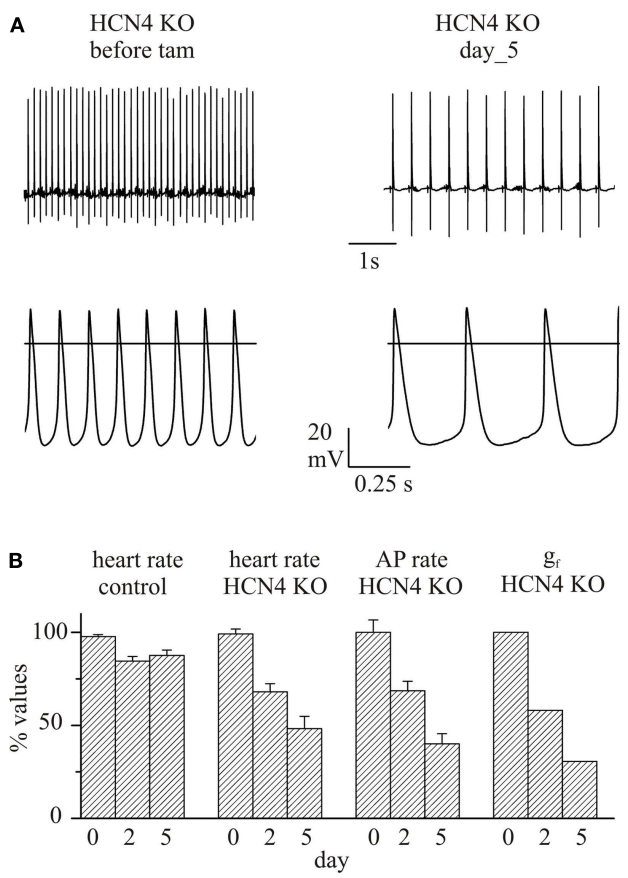

FIGURE 2 | (A) Top: Representative telemetric ECG traces recorded from freely moving mice before (left) and after (right) cardiac-specific HCN4 ablation (five tamoxifen injections). Bottom: Sample action potentials recorded from SAN cells isolated before (left) and after (right) the knockout. **(B)** Bar-graph showing normalized telemetric heart rates of control (left) and KO (middle left) mice recorded before (day 0) and during the Tam-procedure (day 2 and day 5). The normalized spontaneous activity (middle right) and the I_f current conductance (right) of SAN cells isolated from KO mice in the same days. Clearly the induction of the knockout decreases the heart rates of freely moving animals, the spontaneous activity of isolated SAN cells, and the I_f current conductance (gf) in a similar manner. Only a minor heart rate reduction was observed in control animals undergoing the same treatment.

This knockout model also allowed the evaluation of the relevance of the I_f current to the modulation of the cardiac rate induced by β-adrenergic activation. Isoproterenol stimulation (Iso, 0.1 mg/kg, i.p.) increased rate in both control and KO mice but maximal rate values were significantly different (705 vs. 485 bpm, respectively). In agreement with the results in intact animals, the average maximal rate of spontaneously beating single cells perfused with Iso (1 μM) was significantly higher in control cells (489 bpm) than in KO SAN cells (280 bpm). Despite these differences in maximal rates, the Iso-induced accelerations were similar in telemetric recordings from control and KO animals (193 vs. 185 bpm, respectively) and in single control and HCN4 KO SAN cells (120 vs. 143 bpm, respectively). The evidence that knockout of HCN4 channels reduces both basal rate and maximal β-adrenergic response in intact animals and in single cells provides a direct demonstration that the I_f current plays a prominent role in basal rhythm generation and modulation.

Bradycardia and chronotropic incompetence were not the only effects observed in the cardiac-specific HCN4 knockout model. HCN4 knockout also leads to a prolongation of the PQ interval (from 32.7 ms before induction to 45.6 ms after full tamoxifen treatment) and AV block, eventually leading to heart arrest and death of the animal. During AV block, sinus rhythm was always present albeit with a reduced rate. The unexpected observation that HCN4 KO impairs more dramatically the AVN than the SAN function (i.e., the AVN is more sensitive than the SAN to f-channel removal) remains a significant and intriguing result that awaits further confirmation and interpretation.

COMPARISON AMONG ADULT INDUCIBLE HCN4 KNOCKOUT MODELS

Ideally all adult HCN4 KO models investigated should have yielded similar or at least coherent results, which however were not observed. Herrmann and Hoesl's models (Herrmann et al., 2007; Hoesl et al., 2008) led to the conclusion that the I_f current does not substantially contribute to basal heart rate or rate acceleration induced by sympathetic stimulation. On the contrary, our cardiac-specific model revealed strong effects since a 50% reduction in rate was observed in KO mice, all mice died due to complete AV block, and chronotropic response to maximal β stimulation was about 30% smaller than in control mice. We do not have a simple explanation for the observation that complete and HCN4-expressing restricted knockout do not lead to the deep bradycardia observed with cardiac-specific knockout. However, in addition to the intrinsic difference in the models, two additional aspects should be considered. A first difference between our KO mice and Herrmann and Hoesl's (Herrmann et al., 2007; Hoesl et al., 2008) is that while in Herrmann and Hoesl's models the knockout procedure ablates exon 4, and does not produce a frame-shift of the remaining sequence, our model ablates exon 2 and generates a

stop codon three residues downstream the site of recombination (Baruscotti et al., 2011). Whether this difference may or may not produce any different results is not clear, since apparently both procedures generate non-functional channels. The second aspect relates to the genetic background of the mice. Although all the discussed mouse models have the same C57BL6 genetic background, this does not necessarily mean that they are genetically similar since recent evidence indicates the existence of multiple C57BL6 sub-strains (Kiselycznyk and Holmes, 2011), and significant phenotypic differences among sub-strains have been reported (Blum et al., 1982; Bryant et al., 2008; Matsuo et al., 2010). We therefore cannot fully rule out that some functional differences among HCN4 KO and transgenic models may arise from the combination of methodological and genetic factors.

Among different models, when single SAN cell data collected in control conditions are considered, a large variability in cell capacitance, spontaneous rate, and kinetics features of the I_f current is observed among different models (Herrmann et al., 2007; Hoesl et al., 2008; Baruscotti et al., 2011). This variability is not a specific feature of knockout model studies, since it is largely present throughout the whole SAN cell literature (Boyett et al., 2000; Baruscotti et al., 2005; Mangoni and Nargeot, 2008). Whether this difference arises from a biased selection of specific cells is difficult to establish.

Upon physical exercise, or under isoprenaline stimulation, all HCN4 KO models display heart/cell rate accelerations which are not different from those elicited in control mice. Despite the similar acceleration, the maximal rate attained by the cardiac-specific KO mice model is substantially lower than that elicited in control and in complete or HCN4-restricted mice (Herrmann et al., 2007; Hoesl et al., 2008; Baruscotti et al., 2011); this is at least in part due to the fact that cardiac-specific KO mice have a lower basal rate. While taken at face value the permanence of modulation in cardiac-specific KO mice apparently suggests that HCN4 channels are not responsible for this modulation, this conclusion is contradicted by the evidence that the remaining I_f current in cardiac HCN4 KO mice is still amply modulated, and therefore can still contribute to iso-induce acceleration.

Although the experimental data amongst models are substantially different, we believe that an organ-specific gene ablation is the correct technique to investigate functional roles of mechanisms restricted to the organ itself. We also think that the ultimate purpose of a KO mouse model is to improve the understanding of the physiological aspects and pathological consequences in humans; our model is adequate to the purpose, since results obtained with the mouse KO model correspond to results obtained in humans, where it is known that a reduction of the I_f current either resulting from genetic mutations (Baruscotti et al., 2010b) or pharmacological blockade (DiFrancesco and Camm, 2004) leads to sinus bradycardia.

INDUCIBLE TRANSGENIC MODELS

The role of HCN4 channels in the adult heart has been evaluated also by Alig et al. (2009) who developed a transgenic mouse model with an α-MHC promoter and a Tet-Off system-controlled cardiac-specific expression of an engineered human HCN4 subunit carrying a mutation previously identified in a patient with SAN dysfunction (hHCN4–573X; Schulze-Bahr et al., 2003). The patient had marked sinus bradycardia (41 bpm) and intermittent episodes of atrial fibrillation; a diagnosis of idiopathic sinus node dysfunction was made and a pacemaker was implanted. *In vitro* experiments based on patch-clamp-studies of the heterologously expressed mutant channels showed that the mutation abolished the cAMP-dependent regulation of HCN4 channels in a dominant negative manner (Schulze-Bahr et al., 2003). This model therefore did not induce an HCN4 knockout, but rather conferred to cardiac, and in particular to SAN cells, the presence of a mutated human HCN4 subunit which is fully insensitive to cAMP modulation.

Analysis of the ECG of conscious animals overexpressing the mutant hHCN4–573X channels revealed that the heart rate at rest was some 20% slower than that of control mice and no changes in PQ and QTc intervals were observed. In transgenic mice physical exercise was still able to elicit a positive modulation of heart rate, although to a significantly lesser degree than that observed in control mice exposed to a similar activity. The I_f current recorded in transgenic SAN cells exhibited slower activation kinetics and a negative shift of about 20 mV of its voltage-dependence. Spontaneous oscillations of these cells were also severely impaired since most of the cells were either quiescent (∼28%) or showed an arrhythmic behavior (∼61%) consisting in periods of sub-threshold membrane potential oscillations followed by regular firing though at a reduced rate. Regular firing was observed only in 11% of the cells investigated. Iso application resulted in pacing acceleration and restoration of regular pacemaker activity in arrhythmic mutant cells, although maximal rates were significantly slower than in controls cells.

This model (Alig et al., 2009) is substantially different from previous inducible models and therefore trying to compare the results would be partially inappropriate. Nevertheless, in these mice the functionality of sinoatrial I_f current was severely impaired due to a negative shift of its voltage-dependence and a lack of cAMP modulation; thus, the conclusion that a reduced contribution of the I_f current leads in mice to bradycardia and chronotropic incompence is compatible with the results from our KO model (see above). On the other hand, the observation that most of single isolated SAN cells were quiescent points to a similarity with the results of Herrmann et al. (2007) and Hoesl et al. (2008).

The transgenic hHCN4–573X mouse model was also used by Marger et al. (2011) to investigate the role of HCN4 channels in AVN cells. This study showed that while basal spontaneous rate of transgenic AVN cells was reduced when compared to control cells, the maximal rates attained under Iso stimulation was not different between the two groups. While cells isolated from the SAN and the AVN of hHCN4–573X mice display similarly reduced basal rates, their response to maximal stimulation is clearly different although the underlying mechanism are not clear.

MOUSE MODELS OF INDUCIBLE KNOCKOUT OF SAN CELLS

Recently Herrmann et al. (2011a) developed a mouse model where it is possible to eliminate in an inducible manner HCN4-expressing cardiomyocytes. The model was obtained by crossing a mouse line with the Cre inserted into the ATG codon of the *Hcn4* gene (Herrmann et al., 2007; Hoesl et al., 2008) with a ROSA-eGFP-DTA line able to express Diphtheria Toxin (DT) upon Cre-induced

removal of a floxed stop sequence. Tamoxifen-induced activity of DT leads to a dose-dependent elimination of SAN cells and to their substitution with collagen fibers. Despite the large differences between this model and the inducible and cardiac-specific HCN4 KO model developed by our group (Baruscotti et al., 2011), the similarities of the phenotypic manifestations are many. In freely moving conscious mice the induction of DT (five consecutive days of tamoxifen injection) caused a substantial bradycardia (−40%: from 493 to 275 bpm) comparable with that reported in our model (50% reduction). Isoprenaline administration (0.5 mg/kg i.p.) increased heart rate in both control (by 199 bpm) and DT-expressing mice (by 69 bpm) and the maximal value attained by DT-expressing mice was 447 bpm, a value which is similar to that reported in our experiments (∼486 bpm, Iso 0.1 mg/kg i.p.).

Together with bradycardia, the DT-expressing mice also showed additional arrhythmic features such as: increased PR interval, complete heart block, sino-atrial pauses and supraventricular or ventricular tachycardia. Whether this model is adequate to describe the primary features of the age-related sick sinus syndrome is still a matter of debate (Morris et al., 2011). As noted above the destruction of HCN4-expressing cells also impacts onto the AV node conduction of the impulse as verified by the increase of the PR interval, and remarkably this effect was observed in the model of Baruscotti et al. but no in other models (Herrmann et al., 2007; Hoesl et al., 2008; Alig et al., 2009).

ROLE HCN2 AND HCN1 IN ADULT MICE

The presence of the HCN2 isoform in the mouse adult heart is mostly confined to the ventricle, while in the atria and in the SAN HCN2 expression is hardly detectable. Despite the limited expression, experiments with both global and cardiac-specific constitutive HCN2-deficient mouse models (Ludwig et al., 2003) have revealed a sinus dysrhythmic pattern. In addition, global HCN2 KO mice were also affected by absence seizures. It is interesting to note that these data are in agreement with results in humans from our laboratory showing that a loss-of-function point mutation in the HCN2 C-linker is associated with a form of generalized epilepsy with recessive inheritance (DiFrancesco et al., 2011).

ECG analysis revealed that despite control and HCN2 KO mice had similar cardiac rates at rest (531 vs. 510 bpm), the HCN2-deficient animals displayed a much increased RR variability at rest and during normal activity probably due to a dysfunction of the SAN. RR variability measured as the Standard Deviation of RR (SDRR) interval was 7.1 ms in control and in the range 14–17.1 ms in mice lacking HCN2 subunits. Upon maximal stimulation (Iso or physical activity) HCN2 KO mice were able to reach maximal rates similar to those of control mice (>670 bpm) and the observed sinus dysrhythmia disappeared (SDRR in the range 3–5 ms for both groups). Experiment in isolated SAN cells revealed a decrease of about 30% of the I_f current density, significantly slower activation kinetics of the current, and a shift of about 5 mV of the maximum diastolic potential to hyperpolarized values in global KO mice myocytes. Since the remaining (non-HCN2) I_f current in KO cells was still modulated by cAMP, these data indicate the HCN2 isoform contributes to cardiac pacemaking activity of SAN cells mainly at rest and under non-stimulated conditions.

A generalize knockout HCN1 mouse model was developed by (Nolan et al., 2003), and the authors report significant deficits in neuronal process related to spatial memory and learning ability. Since no embryo lethality or anatomical and functional alterations of the adult heart are reported it can be assumed that knockout of HCN1 subunits does not have a major impact on the heart. This evidence is in agreement with a limited presence of HCN1 proteins in the heart reported by some authors (Liu et al., 2007; Brioschi et al., 2009); however it must be noted that in some other reports HCN1 signal has been strongly detected in the SAN (Herrmann et al., 2011b).

ROLE OF HCN3 IN ADULT/EMBRYONIC MICE

The HCN3 isoform has been investigated less deeply than other isoforms in the cardiac field mostly because this isoform was initially thought to be expressed in neuronal and other types of tissue, but less so in cardiac tissue. Currently very few studies of the electrical properties of homomeric HCN3 channels have been carried out (Chaplan et al., 2003; Mistrik et al., 2005; Stieber et al., 2005). The presence of HCN3 mRNA and protein in SAN cells has been investigated so far only in rodents and there is no indication of a positive signal (Moosmang et al., 2001; Huang et al., 2007; Liu et al., 2007; Fenske et al., 2011; Herrmann et al., 2011b). There is on the other hand contrasting evidence concerning the expression of HCN3 in the working myocardium. Some groups failed to detect positive signals by *in situ* hybridization and real time PCR in mice and rat hearts (Moosmang et al., 2001; Huang et al., 2007; Herrmann et al., 2011b), while other found a weak RT-PCR positive signal in mouse heart and ventricles (Marionneau et al., 2005; Mistrik et al., 2005). Despite the positive mRNA identification Mistrik et al. (2005) failed to confirm the presence of HCN3 proteins likely because the signal was below the detection level. On the other hand, Fenske et al. (2011) reported positive mRNA identification in working myocardium and positive protein identification in mouse ventricles and generated a global and constitutive HCN3 knockout mouse by ablating the exon 2 of the *Hcn3* gene. Since HCN3-deficient mice were born at the expected Mendelian ratios with normal mice, it can be concluded that this isoform is not relevant for cardiac formation and development. In agreement with the lack of HCN3 mRNA and protein in the SAN, ECGs recorded from freely moving HCN3-deficient mice did not reveal anomalies in sinus rhythm. Important evidence supporting a contribution of the HCN3 isoform to the physiology of the working myocardium comes from the finding that at low heart rates (mean of 460 bpm) significant increases in the T-wave amplitude (+63.5%) and duration (+15%) and in the QT interval (+12%) were detected. Patch-clamp analysis of epicardial cells from HCN3 KO mice showed a substantial shortening of the action potential duration (both APD75 and APD90) and a ∼30% reduction of the I_f current. Analysis of the steady-state voltage-dependence of the HCN3 channels revealed that at the cell resting voltage (−85 mV) about 25–30% of the channels are in the conducting state. This piece of information, and the extremely slow kinetic of deactivation allow to formulate the hypothesis that the HCN3 component of the ventricular epicardial I_f current is active throughout the entire action potential and exerts its functional action by counteracting the K+ current-driven late repolarization of epicardial cells.

While these data certainly highlight a novel contribution of pacemaker channel to cardiac function we believe that there are some points that need to be addressed: (1) the contrasting reports in the literature regarding the presence of the HCN3 isoform in the heart pose the question as whether the results of this model can be generalized; (2) as pointed out by the same authors it is still to be determined why the phenotypic manifestations can only be observed in epicardial cells given that endocardial cells also express HCN3 proteins; (3) last but not the least is to verify whether HCN3 isoforms are also present in human epicardial cardiomyocytes.

CONCLUSIONS

HCN channels represent an important source of depolarizing current in the heart, and the use of specific constitutive or temporally controlled deletion of different HCN isoforms has allowed to better understand the contribution of each isoform to the different functional aspects of cardiac myocytes.

Despite some variability, in the literature there is a general agreement that the HCN2 isoform is mostly confined to the ventricle; while in other cardiac district and especially in the SAN HCN2 channels are poorly expressed. The very limited contribution of HCN2 subunits to cardiac pacemaking is confirmed by the finding that the heart rates of HCN2 KO mice at rest and under maximal chronotropic modulation do not differ from those of control animals. Furthermore HCN2 are not functionally essential to heart formation during embryogenesis and maturation.

HCN3 subunits have long been regarded as little relevant to cardiac activity. Lately however ECG recordings in global and constitutive HCN3 KO mice have been reported to display alterations of the T-wave and of the QT interval. Interpretation of these data has led to the hypothesis that HCN3 channels represent a source of ventricular background current which opposes to the repolarizing process at the end of the ventricular APs.

HCN4 is by far the most important isoform in the heart, and its presence is most abundant in the SAN where HCN4 represents about 80% of the total HCN isoforms. Several HCN4 KO and transgenic mouse models have been developed and results are extremely variable (**Table 1**). Results with the cardiac-specific and inducible knockout model indicate that the HCN4 current provides a fundamental contribution to basal heart rate maintenance and modulation since its removal leads to basal bradycardia and a strongly reduced response to sympathetic stimulation. Furthermore the HCN4 current plays a critical role also in AV node conduction.

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Zebrafish: a novel research tool for cardiac (patho)electrophysiology and ion channel disorders

Arie O. Verkerk*[1] *and Carol Ann Remme*[2]*

[1] *Department of Anatomy, Embryology, and Physiology, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands*
[2] *Department of Experimental Cardiology, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands*

Edited by:
Marcel V. Heyden, University Medical Center, Netherlands

Reviewed by:
Lai-Hua Xie, UMDNJ-New Jersey Medical School, USA
Rolf Bodmer, Sanford-Burnham Medical Research Institute, USA

****Correspondence:***
Carol Ann Remme, Department of Experimental Cardiology, Academic Medical Center, Heart Failure Research Center, University of Amsterdam, Room K2-104, Meibergdreef 15, 1100 DE, PO Box 22700, Amsterdam, Netherlands.
e-mail: c.a.remme@amc.uva.nl

The zebrafish is a cold-blooded tropical freshwater teleost with two-chamber heart morphology. A major advantage of the zebrafish for heart studies is that the embryo is transparent, allowing for easy assessment of heart development, heart rate analysis and phenotypic characterization. Moreover, rapid and effective gene-specific knockdown can be achieved using morpholino oligonucleotides. Lastly, zebrafish are small in size, are easy to maintain and house, grow fast, and have large offspring size, making them a cost-efficient research model. Zebrafish embryonic and adult heart rates as well as action potential (AP) shape and duration and electrocardiogram morphology closely resemble those of humans. However, whether the zebrafish is truly an attractive alternative model for human cardiac electrophysiology depends on the presence and gating properties of the various ion channels in the zebrafish heart, but studies into the latter are as yet limited. The rapid component of the delayed rectifier K^+ current (I_{Kr}) remains the best characterized and validated ion current in zebrafish myocytes, and zebrafish may represent a valuable model to investigate human I_{Kr} channel-related disease, including long QT syndrome. Arguments against the use of zebrafish as model for human cardiac (patho)electrophysiology include its cold-bloodedness and two-chamber heart morphology, absence of t-tubuli, sarcoplamatic reticulum function, and a different profile of various depolarizing and repolarizing ion channels, including a limited Na^+ current density. Based on the currently available literature, we propose that zebrafish may constitute a relevant research model for investigating ion channel disorders associated with abnormal repolarization, but may be less suitable for studying depolarization disorders or Ca^{2+}-modulated arrhythmias.

Keywords: action potential, arrhythmia, cardiac electrophysiology, ion channel, ion channelopathy, patch-clamp, zebrafish

INTRODUCTION

To date, genetically modified mice have been predominantly used to investigate and model human cardiac diseases, including patho-electrophysiological conditions. Although mouse models have provided valuable insight into the role of many ion channels in healthy and diseased state, they also have limitations due to their intrinsic basal high heart rate and extremely fast and large phase-1 repolarization which results in a short action potential (AP) with a very negative plateau phase potential. Furthermore, *in vivo* investigation of mouse models often requires invasive imaging and monitoring techniques. Finally, generation and maintenance of mouse lines is time-consuming and expensive.

In the last decade, the zebrafish (*Danio rerio*), a tropical freshwater teleost, has been increasingly used for various human-related disease studies (Beis and Stainier, 2006; Williams, 2010). Despite its cold-bloodedness and two-chamber heart morphology, the zebrafish has been suggested as a useful model for studies of human heart development and cardiac (patho)electrophysiology. A major advantage of the zebrafish for heart studies is it that the embryo is transparent, allowing for easy assessment of heart development, heart rate analysis and phenotypic characterization by direct visual inspection (Baker et al., 1997; Bakkers, 2011). Furthermore, using optogenetics combined with transgenic expression of light-gated ion channels in zebrafish hearts, cardiac pacemaker cells can be located and quickly and reversibly activated and deactivated in various sub-compartments of the cardiac conduction system, enabling investigations into the effects of disturbed heart rhythms on cardiac performance (Arrenberg et al., 2010). An additional advantage is that an intact blood circulation is not required for proper function of fish embryos and hearts, since diffusion of nutrients is sufficient for their survival. Therefore, *in vivo* and *ex vivo* functional studies can be performed easily without complications due to nutritional deficiency or secondary deterioration (Baker et al., 1997; Peal et al., 2011). Moreover, rapid and effective gene-specific antisense knockdown using morpholino oligonucleotides allows for relatively quick *in vivo* functional characterization of the activity and function of genes of interest (Bedell et al., 2011). Lastly, zebrafish are small in size, are easy to maintain and house, grow fast, and have large offspring size, making them a cost-efficient research model.

Today, zebrafish whole heart electrical activity is routinely recorded using *in vivo* electrocardiography (ECG) (Leong et al., 2010). In addition, various non-invasive microscopic video analysis methods have been developed to determine heart rate (Chan et al., 2009; Yoshida et al., 2009), to quantify ventricular fractional shortening [a measure of systolic contractile function (Denvir et al., 2008; Fink et al., 2009)], and to analyse blood flow dynamics by tracking movement of erythrocytes or fluorescent molecules introduced into the circulation (Schwerte and Pelster, 2000; Hove et al., 2003). For assessment of cardiac conduction and excitability, Ca^{2+}-sensitive fluorescent dyes (Ebert et al., 2005; Langenbacher et al., 2005; Milan et al., 2006) or a fluorescent Ca^{2+}indicator transgene [Tg(cmlc2:gCaMP)] (Chi et al., 2008) can be used, and transmembrane APs may be evaluated using voltage-sensitive dyes (Panáková et al., 2010). Application of these voltage-sensitive dyes during so-called optical mapping may also enable detailed investigation of cardiac conduction velocity, activation patterns, and arrhythmias. These high resolution imaging techniques are powerful tools for the study of zebrafish physiology (Jou et al., 2010), but these methods require the complete absence of cardiac contraction. Jou and colleagues (Jou et al., 2010) found that the excitation-contraction uncoupler blebbistatin, but not butanedione monoxime (BDM), abolished contractility without significantly altering AP morphology or of spontaneous APs generation.

The zebrafish as model for studies into human heart development, heart regeneration, and human cardiomyopathy diseases has recently been reviewed in detail (Poss, 2007; Bakkers, 2011). The embryonic and adult zebrafish heart is proposed as an efficient platform for testing of drugs with potential electrophysiological effects on cardiomyocytes (Mittelstadt et al., 2008; Tsai et al., 2011) and for investigating human ion channel function in healthy and diseased state (Milan and Macrae, 2008). However, whether the zebrafish is truly an attractive alternative model for human cardiac electrophysiology depends on the presence and gating properties of the various ion channels in the zebrafish heart. Here we review the available data and literature addressing the suitability of the zebrafish as a research model for human cardiac electrophysiology.

ECG PARAMETERS IN ZEBRAFISH

The zebrafish heart is a tubular structure with a single atrium and ventricle. Despite its two-chambered heart morphology, the ECG of the zebrafish is very similar to that of human. Basal heart rate of adult zebrafish is close to that of humans, with a frequency of 120–130 beats/min at 28°C (Barrionuevo and Burggren, 1999; Nemtsas et al., 2010; Tsai et al., 2011), which is the optimal water temperature for this tropical freshwater fish. The heart rate decreases at 25°C and increases at 31°C (Barrionuevo and Burggren, 1999). Pacemaker activity in the zebrafish heart starts in the sinoatrial node located at the sinus venosus (Sedmera et al., 2003). The AP propagates uniformly though the atria with average atrial activation times of ≈20 ms. After a delay of ≈50 ms due to slow propagation in the atrioventricular canal, the ventricle becomes activated first in the apical region. This similarity in propagation of the AP in zebrafish and human hearts mirrors the similarity of ECG morphology (Leong et al., 2010). Like human, zebrafish show a distinct P-wave, QRS-complex, and T-wave on ECG recording (Milan et al., 2006), suggesting that depolarization and repolarization in the zebrafish heart is comparable to that in humans (Leong et al., 2010). The mean QT interval and optically mapped ventricular AP duration is ≈300 ms as shown in **Figure 1A** (adapted from Tsai et al., 2011). Thus, the QTc interval is slightly shorter than that in humans. However, it must be noted that zebrafish ECG and AP values are obtained at lower temperatures as compared to human, and that human atrial and ventricular APs significantly prolong at lower temperatures (Amos et al., 1996).

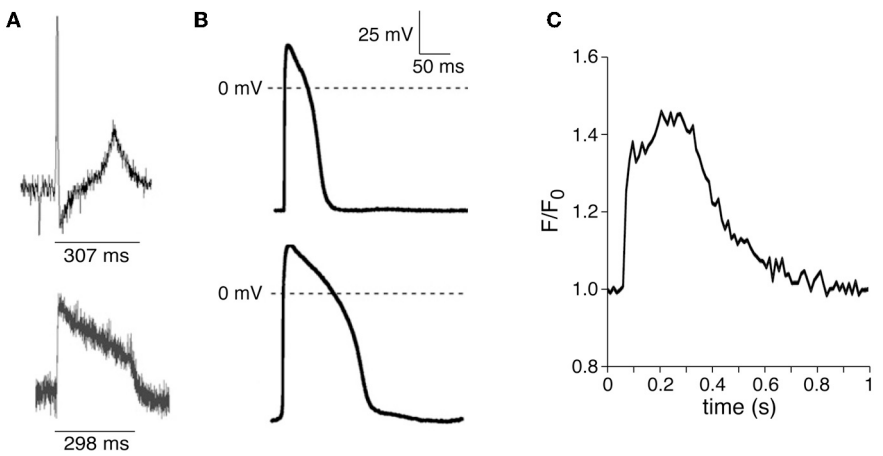

FIGURE 1 | (A) Typical ECG recording (*top*) and optically mapped action potential (*bottom*) in adult zebrafish heart. Adapted from Tsai et al. (2011), with the permission of Elsevier. **(B)** Typical atrial (*top*) and ventricular (*bottom*) action potentials recorded from spontaneously beating intact zebrafish hearts. Adapted from Nemtsas et al. (2010), with the permission of Elsevier. **(C)** Typical intracellular Ca^{2+} transient measured within a Fluo-4-loaded isolated ventricular myocyte. Adapted from Zhang et al. (2011), with the permission of the American Physiological Society.

ACTION POTENTIALS

Cardiac APs of zebrafish may be recorded from the intact heart through use of micro-electrodes (Nemtsas et al., 2010), patch-clamp technology (Jou et al., 2010), and voltage-sensitive dyes (Panáková et al., 2010; Wythe et al., 2011). Nemtsas and colleagues (Nemtsas et al., 2010) recorded both atrial and ventricular monophasic APs from intact adult zebrafish hearts that were beating spontaneously at the physiological temperature of 28°C (see **Figure 1B** for typical examples). They observed a rapid AP upstroke in zebrafish myocardium, but the maximum AP upstroke velocity was substantially lower in zebrafish atria and ventricle than in human and mouse myocardium. The resting membrane potential was similar in zebrafish and human, indicating that the differences in upstroke velocity between species were not due to differences in Na^+ channel availability. In zebrafish, the AP upstroke was followed by a long-lasting plateau phase that was shorter in atrial than in ventricular tissue, and ended with a phase of rapid terminal repolarization. APs from excised hearts from 48 hours-old zebrafish larvaes displayed similar long-lasting plateau phases, with shorter atrial APs compared to ventricular (Jou et al., 2010; Wythe et al., 2011). In human, atrial and ventricular AP also have a prominent plateau phase with a shorter AP in atria (Koumi et al., 1995; Amos et al., 1996). Nemtsas et al. (2010) concluded that the overall shape of the adult ventricular zebrafish AP is comparable to that of the human heart, and that human APs appear more similar to zebrafish APs than mouse APs. It must be kept in mind, however, that in large mammals (such as human) AP morphology is heterogeneous within both atria and ventricle, and between left and right sides of the heart (see Beuckelmann et al., 1993; Wang et al., 1993; Bénardeau et al., 1996; Näbauer et al., 1996; Gong et al., 2008; Verkerk et al., 2009b; Verkerk et al., and primary references cited therein).

For recording of cardiac APs in isolated cardiomyocytes, Ca^{2+} tolerant cells need to be isolated through enzymatic dissociation (Brette et al., 2008; Nemtsas et al., 2010; Zhang et al., 2011). Isolated ventricular (Brette et al., 2008) and atrial (**Figure 2A**) zebrafish myocytes appear rod-shaped, but it is evident from **Figure 2A** that the zebrafish myocyte is quite narrow compared to that of the human myocyte. It has previously been estimated that freshly isolated ventricular zebrafish myocytes are ≈100 by ≈5 by ≈6 μm in size (length × width × height; Brette et al., 2008). These morphological characteristics of zebrafish myocytes are in contrast with findings in human, where myocytes are much wider. The smaller size of zebrafish myocytes is also evident from their much smaller membrane capacitance. Zebrafish atrial and ventricular myocyte capacitance is ≈26 and ≈30 pF, respectively (Nemtsas et al., 2010), while that of human is ≈90–150 pF (Amos et al., 1996; Verkerk et al., 2007) and ≈165–285 pF (Amos et al., 1996; Li et al., 1998), respectively. It is likely that due to the more narrow shape of the zebrafish myocyte, the relative amount of intercalated disc area is also lower. In mammalian myocytes, intercalated discs, important for AP propagation, are not only found at the cell ends, but also along the lateral sides of the myocyte (Peters et al., 1993). The narrow shape of the zebrafish myocytes may thus influence impulse propagation importantly, but further studies are needed to address this topic in detail.

By patch-clamp analysis, it has been demonstrated that freshly isolated atrial (**Figures 2B,C**) and ventricular (Brette et al., 2008) myocytes of adult zebrafish display APs with a clear plateau phase. **Figure 2B** shows typical atrial APs of a zebrafish and human myocyte recorded at 1 Hz; average AP characteristics are summarized in **Figure 2C**. Compared to human, zebrafish myocytes display a slower AP upstroke velocity resulting in a lower AP amplitude. Zebrafish AP duration during the early phases of repolarization appear longer than in human, but the AP durations at 90% repolarization (APD_{90}) are similar (**Figure 2C**). In isolated ventricular myocytes of adult zebrafish, APD_{90} is ≈150 ms at 0.1 Hz, and it decreases at higher stimulus frequencies. While the frequency dependency is similar to findings in isolated human atrial and ventricular myocytes (Le Grand et al., 1994; Li et al., 2004), the AP duration in single ventricular zebrafish myocytes is much shorter than in isolated human ventricular myocytes (O'Hara et al., 2011).

Thus, embryonic and adult ventricular myocytes of zebrafish show APs with a clear plateau phase and an AP configuration closely resembling that of ventricular myocytes of large mammals, notably human. However, not the AP configuration itself, but the underlying membrane currents will determine whether the zebrafish is suitable as a model for human cardiac electrophysiology.

MEMBRANE CURRENTS

Using patch-clamp analysis and specific ion channel blockers, the presence and function of various inward and outwardly directed membrane currents have previously been investigated in zebrafish cardiomyocytes.

Na^+ CURRENT

Two orthologs of the cardiac Na^+ channel have been identified in zebrafish (*scn5Laa* and *scn5Lab*), which both encode and form typical voltage-gated Na^+ channels/currents (Novak et al., 2006; Chopra et al., 2010). Furthermore, a Na^+ current (I_{Na}) has been observed in both cultured embryonic and freshly isolated adult zebrafish myocytes (Baker et al., 1997; Warren et al., 2001). In single adult atrial myocytes, I_{Na} has a more negative voltage-dependency of inactivation as compared to single adult ventricular myocytes (Warren et al., 2001). Similarly, I_{Na} displays distinct biophysical properties in atrial versus ventricular myocytes in mammalians, with again a more negative voltage-dependency of inactivation (Burashnikov et al., 2007). According to studies by Warren and colleagues (Warren et al., 2001), zebrafish cardiomyocyte I_{Na} density may be up to 4-fold smaller than in mammalian cardiac myocytes, which likely explains the slower AP upstroke velocity found in zebrafish (Nemtsas et al., 2010). Consistent with the importance of I_{Na} in determining AP upstroke (Berecki et al., 2010; and primary refs cited therein), the I_{Na} blocker tetrodotoxin (100 nM) substantially reduced both atrial and ventricular AP upstroke velocity in intact adult hearts (Nemtsas et al., 2010). In contrast, AP duration was not affected by tetrodotoxin, suggesting that a sustained (non-inactivating) I_{Na} is not present under normal conditions. Thus, although similarities with human I_{Na} exists, the limited I_{Na}

density and consequent slow AP upstroke velocity in zebrafish may render it less suitable as research model for depolarization disorders.

Ca^{2+} CURRENTS

It has been demonstrated that cultured embryonic and adult freshly isolated zebrafish myocytes display both the T-type and L-type Ca^{2+} current ($I_{Ca,T}$ and $I_{Ca,L}$, respectively) (Baker et al., 1997; Nemtsas et al., 2010). In isolated atrial and ventricular myocytes of adult zebrafish, $I_{Ca,L}$ showed a typical bell-shaped current-voltage (I-V) relationship with a maximum around 0 mV (Brette et al., 2008; Nemtsas et al., 2010; Zhang et al., 2011). In adult zebrafish, the $I_{Ca,L}$ blocker nifedipine significantly shortened the plateau phase and consequently the AP duration in both atria and ventricles (Nemtsas et al., 2010). The $I_{Ca,L}$ activator BayK8644 prolonged QTc interval in a dose-dependent manner (Tsai et al., 2011). These experiments demonstrate that the requirement of $I_{Ca,L}$ for shaping AP duration is conserved between zebrafish and mammals (Nemtsas et al., 2010). The presence of $I_{Ca,T}$ in zebrafish myocytes contrasts with findings in adult human working myocardium (Ono and Iijima, 2005). The effects of $I_{Ca,T}$ blockers on zebrafish AP configuration has not yet been investigated, and the functional relevance of $I_{Ca,T}$ in zebrafish myocytes thus remains unclear.

K^+ CURRENTS

Patch-clamp experiments and drug studies have indicated the presence of various K^+ currents in both cultured and freshly isolated zebrafish myocytes.

Rapid component of the delayed rectifier K^+ current (I_{Kr})

I_{Kr} has been observed in both cultured embryonic (Baker et al., 1997) and adult freshly isolated myocytes from zebrafish (Nemtsas et al., 2010). In adult hearts, the I_{Kr} blocker E4031

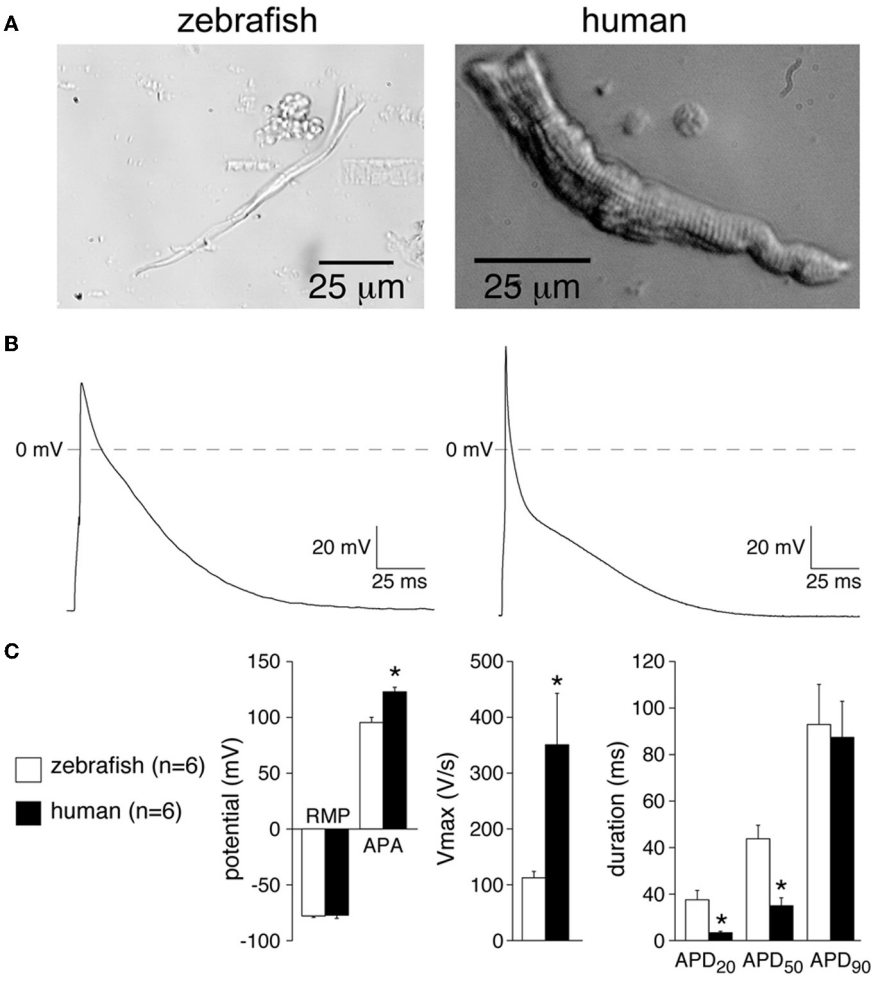

FIGURE 2 | (A) Photographs of atrial myocytes of zebrafish and human enzymatically isolated as described in detail previously (Verkerk et al., 2009a), except that the isolation temperature in the zebrafish procedure was decreased to 28°C. **(B)** Typical action potentials (APs) of a single zebrafish and human atrial myocyte measured at 1 Hz. Zebrafish and human atrial APs were recorded at 28 and 36°C, respectively [See Verkerk et al., 2009a (zebrafish) and Verkerk et al., 2007 (human) for solutions used]. **(C)** Average AP characteristics of zebrafish and human atrial myocyte recorded at 1 Hz. RMP, resting membrane potential; APA, AP amplitude; Vmax, maximal AP upstroke velocity; APD_{20}, APD_{50}, and APD_{90}, AP duration at 20, 50, and 90% repolarization, respectively. Values are mean ± SEM; $^*P < 0.05$ (*t*-test).

prolonged zebrafish atrial and ventricular APs (Nemtsas et al., 2010) and QTc interval in a dose-dependent manner (Tsai et al., 2011). These observations are in agreement with findings in humans (Jost et al., 2005). In addition, E4031 decreased heart rate, suggesting a role for I_{Kr} in zebrafish pacemaker formation (Tsai et al., 2011), consistent with findings in mammalian studies (Ono and Ito, 1995).

Scholz et al. (2009) analyzed in Xenopus oocytes the biophysical properties of heterologously expressed cloned zebrafish orthologue (*zERG*) of the human ether-à-go-go-related gene *hERG*, encoding the pore-forming subunit of I_{Kr}. *zERG* conduct rapidly activating and inactivating K^+ currents. However, compared to *hERG*, the half-maximal activation voltage of *zERG* is shifted toward more positive potentials and the half-maximal steady-state inactivation voltage is shifted toward more negative potentials. *zERG* activation is slowed while deactivation is accelerated significantly, but the time course of *zERG* during AP clamp experiments is highly similar to that of *hERG*. Therefore, the authors concluded that zebrafish represent a valuable model to investigate human I_{Kr} channel-related diseases (Scholz et al., 2009). Indeed, a number of studies have applied zebrafish for investigations into human syndromes associated with both loss and gain of I_{Kr} (see below).

Slow component of the delayed rectifier K^+ current (I_{Ks})

In their voltage clamp experiments on isolated adult myocytes, Nemtsas et al. (2010) observed no effect of the I_{Ks} blocker HMR1556 on membrane currents, suggesting that I_{Ks} is absent in zebrafish myocytes. During AP measurements in intact zebrafish hearts, they observed an unexpected prolongation of AP duration by HMR1556 due to a reduction in $I_{Ca,T}$ (Nemtsas et al., 2010). In contrast, Tsai et al. (2011) mentioned expression of the *KCNQ1* transcript (which underlies part of I_{Ks}) in zebrafish myocardium, and found that the I_{Ks} blocker chromanol 293B prolonged both QTc interval and AP duration in a dose-dependent manner in isolated adult zebrafish hearts. In addition, chromanol 293B was also able to decrease heart rate in this study (Tsai et al., 2011). In human ventricular myocytes, I_{Ks} is present but I_{Ks} blockade only results in significant AP prolongation when the "repolarization reserve" is attenuated or under conditions of sympathetic activation (Jost et al., 2005). Clearly, further studies are needed to elucidate the contrasting findings of I_{Ks} blockade on the zebrafish AP.

Ultrarapid component of the delayed rectifier K^+ current (I_{Kur})

Baker et al. (1997) mentioned as "unpublished work" the presence of a K^+ current with the properties of I_{Kur} in cultured embryonic zebrafish myocytes. However, no other studies have as yet provided evidence of I_{Kur} in zebrafish myocytes. I_{Kur} and I_{Kur}-related channel proteins are absent/small in human ventricular myocytes, but is the major repolarizing current in atrial myocytes (Amos et al., 1996; Wettwer et al., 2004; Ordög et al., 2006). Additional studies are thus required to address the presence and function of I_{Kur} in zebrafish myocytes.

Inward rectifier K^+ current (I_{K1})

Nemtsas et al. (2010) measured I_{K1} during depolarizing ramp pulses as Ba^{2+} sensitive current. I_{K1} was observed in both isolated adult atrial and ventricular myocytes, but I_{K1} was ≈5 times larger in ventricular myocytes. These observations are consistent with findings in mammals (Koumi et al., 1995; Amos et al., 1996; Panama et al., 2007). I_{K1} regulates the late phase of AP repolarization and stabilizes the resting membrane potential, thus atria of zebrafish hearts may be more susceptible to diastolic depolarization compared to ventricle. Indeed, diastolic depolarization is observed in atria, but not in the ventricle, of intact zebrafish embryonic hearts (Jou et al., 2010).

Transient outward K^+ current (I_{to1})

Again, the presence of a K^+ current with the properties of I_{to1} in cultured embryonic zebrafish myocytes has so far only been mentioned as "unpublished work" by Baker et al. (1997), and no other studies have provided evidence of I_{to1} in zebrafish myocytes. Since I_{to1} is a prominent current determining human atrial and ventricular AP morphology (Shibata et al., 1989; Amos et al., 1996), further studies are essential to investigate the presence and function of this current in zebrafish heart.

Acetylcholine-activated K^+ current ($I_{K,ACh}$)

In intact adult zebrafish hearts, atrial but not ventricular APs are abbreviated upon exposure to carbachol, an agonist for $I_{K,ACh}$ (Nemtsas et al., 2010). Thus, atrial myocytes of zebrafish display functional $I_{K,ACh}$ in agreement with findings in human (Dobrev et al., 2001). In human, acetylcholine activates $I_{K,ACh}$in both atrial and ventricular myocytes, with however a three-times smaller current and a greater half-maximal stimulation concentration in atrial myocytes (Koumi and Wasserstrom, 1994). Additional detailed studies in zebrafish are required to excluded the presence of $I_{K,ACh}$ in zebrafish ventricular myocytes.

HYPERPOLARIZATION-ACTIVATED "FUNNY" CURRENT

Patch-clamp analysis of cultured myocytes from zebrafish embryos (Baker et al., 1997) and isolated adult myocytes (Warren et al., 2001) reveals the prominent presence of the hyperpolarization-activated "funny" current (I_f), also known as the pacemaker current (I_h). In adult zebrafish myocytes, I_f has chamber-specific properties, i.e., the atrial I_f density is larger than the ventricular I_f density (Warren et al., 2001). The clear presence of I_f in atrial and ventricular zebrafish myocytes contrasts with findings in human, where I_f is mainly found in cells of the conduction system (Han et al., 2002; Verkerk et al., 2007), is small in atrial myocytes (Hoppe and Beuckelmann, 1998), and only observed in ventricular myocytes during pathophysiological conditions such as heart failure (Cerbai et al., 2001). Nevertheless, a functional role for I_f in sinoatrial pacemaking and heart rate regulation has also been described in zebrafish (see below; Baker et al., 1997; Warren et al., 2001).

Na^+-Ca^{2+} EXCHANGE CURRENT (I_{NCX})

Patch-clamp analysis of the Na^+-Ca^{2+} exchange current (I_{NCX}) has not yet been performed in zebrafish myocytes. However, Langenbacher et al. (2005) have reported the presence of a *NCX1* zebrafish homologue (i.e., *NCX1h*) in the heart of zebrafish, and knockdown studies indicate a functional role for NCX1 in maintaining normal Ca^{2+} homeostasis in the zebrafish heart, as discussed below.

EXCITATION-CONTRACTION COUPLING

The zebrafish heart displays clearly visible contractions and these are frequently used to assess the beating rate of the whole heart. Isolated myocytes of adult zebrafish show clear cross-striations, indicating the presence of sarcomeres (Brette et al., 2008; Nemtsas et al., 2010), but zebrafish ventricular myocytes lack t-tubules (Brette et al., 2008). The latter observation contrasts with ventricular myocytes from mammals. It is thought that t-tubules allow the excitation wave to spread from the cell surface deep into the muscle fibers for efficient release of Ca^{2+} from the sarcoplasmic reticulum (SR) (for review, see Brette and Orchard, 2003). The lack of t-tubules thus may have consequences for Ca^{2+} transients, but it is also possible that due to the small diameter of the zebrafish myocytes, t-tubules are not required.

Experimentally, Ca^{2+} transients can be visualized and measured in both single adult myocytes (Zhang et al., 2011) and whole embryonic hearts (Jou et al., 2010). **Figure 1C** shows a typical example of a Ca^{2+} transient recorded in an isolated ventricular myocyte (Figure adapted from (Zhang et al., 2011)). In the intact embryonic heart, Ca^{2+} transients are shorter in atria then in ventricle, and this correlates with the time course of atrial and ventricular APs (Jou et al., 2010; Nemtsas et al., 2010; Wythe et al., 2011). The exact pattern and mechanisms of excitation-contraction coupling in zebrafish myocytes has not yet been studied in detail. In the mammalian myocardium, Ca^{2+} released from the SR is the main source for generating Ca^{2+} transients. Compared to mammals, however, the SR in lower vertebrates is underdeveloped, has a lower ability to store and release Ca^{2+}, and has less importance in excitation-contraction coupling [see Xie et al. (2008), and primary refs cited therein]. To assess the role of SR function in generating Ca^{2+} transients in zebrafish myocytes, Xie and colleagues (2008) recorded Ca^{2+} transients before and after the addition of caffeine in embryonic hearts. The caffeine-induced depletion of SR Ca^{2+} increased diastolic Ca^{2+} levels, as well as Ca^{2+} transient amplitudes. However, caffeine did not halt the repetitive Ca^{2+} transients, implying that Ca^{2+} entry across the sarcolemmal membrane is sufficient for a relatively synchronous and uniform rise in whole cell intracellular Ca^{2+} concentration. These data thus indicate the presence of a functional SR in zebrafish myocytes, although the effects of caffeine were relatively modest. More recently, Zhang and co-workers (Zhang et al., 2011) performed simultaneous recordings of $I_{Ca,L}$, intracellular Ca^{2+}, and/or measurements of cell shortening in adult zebrafish myocytes. Their findings suggest that $I_{Ca,L}$ is the major contributor to the activation of contraction at membrane voltages below 10 mV, whereas the contribution of reversed Na^+-Ca^{2+} exchange becomes increasingly more important at membrane potentials above 10 mV. Crucially, the apparent lesser importance of the SR for excitation-contraction coupling may also make zebrafish myocytes less susceptible to the occurrence of Ca^{2+} aftertransients and subsequent delayed afterdepolarizations. Indeed, morpholino-mediated knockdown of *Serca2*-activity caused embryonic lethality in zebrafish embryos due to defects in cardiac contractility and morphology, but no arrhythmias were observed (Ebert et al., 2005). Thus, zebrafish may not be ideal for investigations into Ca^{2+}-modulated arrhythmias, including catecholaminergic polymorphic ventricular tachycardias.

ZEBRAFISH MODELS OF CARDIAC (PATHO)ELECTROPHYSIOLOGY

Several studies have investigated the functional effects of altered gene expression of ion channel genes in zebrafish hearts. These mutations were either spontaneously occurring or were identified from large mutagenesis screens or generated through targeted specific knockdown of the gene in question.

Na^+ CHANNELS AND CARDIAC DEVELOPMENT

Antisense morpholino knockdown of either of the two cardiac Na^+ channel orthologs identified in zebrafish (*scn5Laa* and *scn5Lab*) has been shown to severely disrupt early cardiac development in zebrafish (Chopra et al., 2010). Interestingly, pharmacological Na^+ current blockade did not affect cardiac morphology, suggesting a possible structural role for Na^+ channels in heart development (Chopra et al., 2010). Similarly, knockdown of brain-type Na^+ channel ∝- and ß-subunits affects nervous system and motoneuron development in embryonic zebrafish (Pineda et al., 2006; Fein et al., 2008).

ERG OR KCNH2 RELATED MODELS

Langheinrich and co-workers studied *zERG* encoding the pore-forming subunit of I_{Kr} in zebrafish (Langheinrich et al., 2003). Morpholino antisense oligonucleotides targeting *zERG* as well as pharmacological inhibition of *zERG* both elicited dose-dependent bradycardia and arrhythmia in zebrafish embryos, including atrioventricular 2:1 block. Moreover, they identified a mutation in a regulatory domain of the *zERG* channel in the previously identified *breakdance* mutant (*bre*), which is also characterized by a 2:1 atrioventricular block (Chen et al., 1996; Langheinrich et al., 2003). The authors concluded that zebrafish are useful for studying *ERG* function and modulation, and may also be suitable for testing potential QT prolongating effect of drugs. Similary, Arnaout et al. (2007) investigated two recessive *Kcnh2* zebrafish mutants identified with ventricular asystole. Both *Kcnh2* mutations encoded non-functional I_{Kr} channels, and *Kcnh2* mutant zebrafish embryos displayed ventricular AP prolongation, QT interval prolongation, and increased sensitivity to QT prolonging drugs, thus constituting a potential research model for human long QT syndrome (Arnaout et al., 2007). In contrast, the *reggae* mutation (*reg*) was found to reside within the voltage sensor of *zERG* and caused a gain-of-function of I_{Kr} due to defective channel inactivation (Hassel et al., 2008). Accordingly, *reg* mutant adult zebrafish displayed shortened QT intervals, and this mutation has been proposed as a relevant research model for human short QT syndrome type 1 (SQT1; Hassel et al., 2008).

L-TYPE Ca^{2+} CHANNELS AND CARDIAC PROLIFERATION

One of the first zebrafish mutants displaying a clear cardiac phenotype was *island beat* (*isl*), which presented with a silent and non-contractile ventricle and an asynchronously beating atrium resembling atrial fibrillation (Rottbauer et al., 2001). The *Isl* locus was subsequently found to encode the cardiac L-type Ca^{2+} channel. Interestingly, the atrium of *Isl* mutants was structurally

normal, but the ventricle was small and contained relatively few cardiomyocytes. These findings indicate a possible uncharacterized role for L-type Ca^{2+} channels in cardiac proliferation (Rottbauer et al., 2001).

THE *SLOW MO* GENE

A spontaneous mutation in this gene was identified in a particular strain of zebrafish, and it was found that adult zebrafish with a homozygous mutation in the *slow mo* gene were bradycardic (Baker et al., 1997). Through patch-clamp analysis, it was revealed that most cardiac ion currents, including Na^+, K^+, and Ca^{2+} currents were unaffected by the recessive *slow mo* mutation. In contrast, it became apparent that the I_f was defective (Baker et al., 1997; Warren et al., 2001). Although the exact underlying genetic defect remains unknown, these studies in *slow mo* mutants clearly indicate a functional role for I_f in zebrafish sinoatrial pacemaker formation.

Na^+-Ca^{2+} EXCHANGER AND RHYTHMICITY

Langenbacher et al. (2005) reported the presence of a *NCX1* homologue (*NCX1h*) in the atrial and ventricular myocardium of zebrafish. Using morpholino knockdown assay and positional cloning of the zebrafish *tremblor* (*tre*) locus, the authors demonstrated that defective NCX1h activity results in chaotic cardiac movements and dys-synchronized cardiac contractions due to abnormal Ca^{2+} transients (Langenbacher et al., 2005). Another study on *tre* mutants demonstrated dysregulation of atrial rhythmicity (including fibrillation), a silent ventricle, and severe disruptions in sarcomere assembly (Ebert et al., 2005). Results from these studies thus indicate that NCX1h is required for normal development and rhythmicity in the zebrafish heart.

CONCLUSIONS

Zebrafish embryonic and adult heart rates as well as AP and ECG morphology closely resemble those of humans. However, whether the zebrafish is truly an attractive alternative model for human cardiac electrophysiology depends on the presence and gating properties of the various ion channels in the zebrafish heart. The rapid component of the delayed rectifier potassium current (I_{Kr}) remains the best characterized and validated ion current in zebrafish myocytes, and zebrafish may represent a valuable model to investigate human I_{Kr} channel-related disease, including long and short QT syndromes. Arguments against the use of zebrafish as model for human cardiac (patho)electrophysiology include its cold-bloodedness and two-chamber heart morphology, absence of t-tubuli, limited SR function, presence of $I_{Ca,T}$ and I_f, absence of I_{to1} and I_{Kur}, and lowI_{Na} density. Based on the currently available literature, we propose that the zebrafish may constitute a relevant research model for investigating ion channel disorders associated with abnormal repolarization, but may be less suitable for studying depolarization disorders or Ca^{2+}-modulated arrhythmias.

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Mouse models in arrhythmogenic right ventricular cardiomyopathy

Elisabeth M. Lodder*[1] *and* ***Stefania Rizzo*[2]**

[1] *Department of Experimental Cardiology, Heart Failure Research Center, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands*
[2] *Department of Cardiac, Thoracic and Vascular Sciences, University of Padua, Padua, Italy*

Edited by:
Carol Ann Remme, University of Amsterdam, Netherlands

Reviewed by:
Sebastian Pieperhoff, University of Edinburgh, Scotland
Weinian Shou, Indiana University, USA

****Correspondence:***
Elisabeth M. Lodder, Department of Experimental Cardiology, Heart Failure Research Center, Academic Medical Center, University of Amsterdam, Meibergdreef 15, Room L2-107, PO Box 22660, 1100DD Amsterdam, Netherlands.
e-mail: e.m.lodder@amc.uva.nl

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is a heart muscle disorder characterized by fibro-fatty replacement of cardiomyocytes. The cardinal manifestations are arrhythmias, sudden cardiac death, and seldom heart failure. Mutations in genes encoding desmosomal proteins and their interaction partners have been implicated in the pathogenesis of ARVC and it is now widely accepted that ARVC is a disease caused by abnormal cell–cell adhesion. The mechanism(s) by which mutations in desmosomal proteins lead to fibro-fatty replacement remains to be fully elucidated. To this aim over the last 10 years different transgenic and targeted mouse models have been developed, these models and what they have taught us will be discussed in this review.

Keywords: ARVC, animal models, sudden death, desmosomes

INTRODUCTION

Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an important cause of ventricular arrhythmias and sudden cardiac death, especially in the young and in athletes (Marcus et al., 1982; Thiene et al., 1988; Basso et al., 2009). Mutations in one or more genes encoding desmosomal proteins are found in ∼50% of patients (McKoy et al., 2000; Rampazzo et al., 2002; Gerull et al., 2004; Pilichou et al., 2006; Syrris et al., 2006). Desmosomes are highly conserved structures that, together with adherens junctions and gap junctions, connect cardiac myocytes end to end at the level of the intercalated disks and thereby play a crucial role in maintaining proper myocardial function (Delmar and McKenna, 2010). Recent findings indicating the presence of mixed type junctions (the area composite; Franke et al., 2006) and crosstalk between protein complexes pertaining to the different types of junctions (Delmar, 2004; Meadows and Isom, 2005; Saffitz, 2005; Tavora et al., 2009; Li and Radice, 2010; Sato et al., 2011) have markedly changed the perception of the intercalated disk. Altered junctional organization, as the result of mutations, is thought to lead to myocardial damage and replacement fibrosis, the classical histopathologic pattern of ARVC (Basso et al., 2009). In advanced stages of the disease, focal scars cause electrical isolation of cardiomyocytes within non-conducting fibrous tissue, resulting in slow conduction and delayed activation, thus forming the substrate for re-entrant circuits and ventricular electrical instability. Over the years several mouse models have been developed to investigate the mechanisms of disease development in ARVC (Pilichou et al., 2011), focusing on four of the desmosomal proteins: Plakophilin-2, Plakoglobin (γ-catenin), Desmoplakin, and Desmoglein 2. All four will be discussed in detail below; an overview of all the models is given in **Table 1**.

PLAKOPHILIN-2 TARGETED DELETION

The first mouse model described that addresses disruption of one of the desmosomal proteins is the study by Grossmann et al. (2004) that describes the targeted deletion of plakophilin-2. The heterozygous mice carrying one wild type copy of plakophilin-2 were completely viable without any cardiac phenotype. Mouse homozygous for the deletion ($Pkp2^{-/-}$) died during embryonic development around day 11 post fertilization. Embryos at day 10.5 appeared pale with blood aggregates in the interperitoneal cavity indicating the presence of small holes in the endothelial layers lining the heart and vessels. It would be interesting to see if the $Pkp2^{\pm}$ mice develop an ARVC like phenotype upon exercise.

PLAKOGLOBIN TARGETED DELETION AND OVEREXPRESSION MODELS

The first papers on mouse models involving a desmosomal protein described the targeted deletion of *Plakoglobin* (*Pg*) by two independent groups in 1996 (models Pg1 and Pg2; Bierkamp et al., 1996; Ruiz et al., 1996). Homozygous targeted deletion of *Plakoglobin* leads to embryonic lethality between embryonic day 9.5 and 16 due to cardiac malformations: thin cardiac walls and less trabeculation and frequent burst of the epicardial wall; in addition mice showed a blistering skin phenotype. Heterozygous animals appeared healthy and fertile. However, closer inspection of these mice (model Pg2) after the link between ARVC and desmosomal proteins had become clear showed that $Pg^{\pm}$ mice at 10 months after birth had enlarged right ventricles, increased spontaneous ventricular arrhythmias and right ventricular conduction slowing. No replacement fibrosis and remodeling of the junctions was observed, Cx43 localization and distribution were normal on immunofluorescence microscopy. All observed changes were exacerbated and expedited when mice were subjected to exercise

Table 1 | Arrhythmogenic right ventricular cardiomyopathy mouse models.

Nr	Protein	Gene	Mutation/targeted exons	Model type	Reference
Pk1	Plakophilin-2	*Pkp2*	Exon 1-intron 1	Targeted deletion	Grossmann et al. (2004)
Pk2	Plakophilin-2	*Pkp2*	Exon 1-intron 1	Targeted deletion	Grossmann et al. (2004)
Pg1	Plakoglobin	*Jup*	Exon 2–5	Targeted deletion	Bierkamp et al. (1996)
Pg2–Pg4	Plakoglobin	*Jup*	Exon 34	Targeted deletion	Ruiz et al. (1996), Kirchhof et al. (2006), Fabritz et al. (2011)
Pg5	Plakoglobin	*Jup*	Exon 2–3	Inducible deletion; αMHCcre induced	Li et al. (2011)
Pg6	Plakoglobin β-catenin	*Jup* *Ctnnb1*	Exon 2–3 Exon 2–5	αMHC/MerCreMer tamoxifen induced double deletion	Swope et al. (2012)
Pg7	Plakoglobin	*Jup*	Wild type	Flag tagged transgene	Lombardi et al. (2011)
Pg8	Plakoglobin	*Jup*	2365Δ2 Truncated	Transgene	Lombardi et al. (2011)
Dp1	Desmoplakin	*Dsp*	Δ281–473	Targeted deletion with extra-embryonic rescue	Gallicano et al. (2001)
Dp2	Desmoplakin	*Dsp*	Exon 2	Inducible deletion; αMHCcre induced	Garcia-Gras et al. (2006), Gomes et al. (2012)
Dp3	Desmoplakin	*Dsp*	Wild type	Flag tagged transgene, αMHC promoter	Yang et al. (2006)
Dp4	Desmoplakin	*Dsp*	R2834H	Flag tagged transgene, αMHC promoter	Yang et al. (2006)
Dp5	Desmoplakin	*Dsp*	Q90R	Flag tagged transgene, αMHC promoter	Yang et al. (2006)
Dp6	Desmoplakin	*Dsp*	V30M	Flag tagged transgene, αMHC promoter	Yang et al. (2006)
Dg1	Desmoglein 2	*Dsg2*	Wild type	Flag tagged transgene, αMHC promoter	Pilichou et al. (2009)
Dg2	Desmoglein 2	*Dsg2*	N271S	Flag tagged transgene, αMHC promoter	Pilichou et al. (2009)
Dg3	Desmoglein 3	*Dsg2*	Exon 4–6	Targeted deletion	Krusche et al. (2011), Kant et al. (2012)

training (model Pg3; Kirchhof et al., 2006). Load reducing therapy is able to prevent these symptoms of ARVC in $Pg^{\pm}$ mice (model Pg4; Fabritz et al., 2011).

To circumvent the problem of neonatal lethality, a cardiac specific targeted deletion of Pg was developed under the control of αMHCcre. $Pg^{f/f}$ αMHCcre (model Pg5) mice have ∼30% of the WT protein as measured by Western blot, no Pg was detectable by immunofluorescence on cardiac sections. Phenotypically these mice largely recapitulate the human ARVC phenotype: cardiac sudden death, progressive dilation, and fibrosis in the cardiac walls (both in the left and the right ventricle) from 2 month onward. No cardiac fat deposition was observed. With transmission electron microscopy the structure of the desmosomes seemed to be disrupted: other desmosomal proteins (e.g., Dsg2) appeared to be absent from the intercalated disk (Li et al., 2011). Cell death in the $Pg^{f/f}$ αMHCcre mice was at least partially through myocyte apoptosis in addition to myocyte necrosis in contrast to mice overexpressing N271S-Dsg2, which mainly showed myocyte necrosis (see below; Pilichou et al., 2006; Li et al., 2011). Interestingly, increased β-catenin staining was observed at the intercalated disk suggesting partial rescue by this close relative of Pg (γ-catenin). To test whether the lack of fast spontaneous death in these mice (model Pg5) was due to a partial rescue by β-catenin, double-targeted mice were created, carrying both a floxed Pg gene and a floxed β-catenin locus ($Pg^{f/f}$; $\beta\text{-}catenin^{f/f}$). Crossing with αMHC/MerCreMer mice and subsequent tamoxifen injections effected specific targeted deletion. Double-targeted mice (model Pg6) showed a strong arrhythmogenic phenotype, with 100% of the double-targeted animals dying of sudden cardiac death between 3 and 5 months after tamoxifen injections. In contrast to either single targeted deletion and wild type littermates of which 4–9% died within 6 months of tamoxifen injection (Swope et al., 2012).

Two lines overexpressing wild type (model Pg7) and mutant (model Pg8) Pg were generated (Lombardi et al., 2011); both showed similar levels of increased incidence of sudden cardiac death, an indication that even moderate levels of overexpression of *Pg* disturb the balance of the mechanical interaction and signaling functions of *Pg* independent of the introduced truncating mutation.

DESMOPLAKIN TARGETED DELETION AND OVEREXPRESSION MODELS

Desmoplakin (*Dsp*) targeted deletion mice $Dsp^{-/-}$ die at embryonic day 6.5 of malformations in the extra-embryonic tissue before assessment of a cardiac phenotype is possible (Gallicano et al., 1998). To overcome this problem the extra-embryonic phenotype was rescued by tetraploid aggregation (model Dp1). The resulting embryos die around embryonic day E11. At E10 they show severe cardiac malformation although desmosomal-like structures appear to be present by transmission electron microscopy. As these mice die well before birth they are obviously unsuitable as a proper model for ARVC (Gallicano et al., 2001). The embryonic lethality of the $Dsp^{-/-}$ mice is partially circumvented in the cardiac specific, αMHCcre induced, targeted deletion of *Dsp* (model Dp2; Garcia-Gras et al., 2006). The homozygous cardiac specific deletion of *Dsp* leads to embryonic growth arrest at day E10–E12 with embryos that appeared very pale, with no circulating red blood cells in organs. The heart was poorly formed with no chamber specification, 80% of $Dsp^{-/-}$ mice did not survive until delivery. Mice that did survive the embryonic period died within 6 weeks

after birth. Heterozygous *Dsp*$^{\pm}$ mice developed normally, however adult *Dsp*$^{\pm}$ mice had thin ventricular walls, increased left ventricular diameters and reduced left ventricular ejection fraction. Spontaneous arrhythmias were observed on surface ECG and 4/5 mice developed ventricular arrhythmias after a single ventricular extra stimulus.

A more detailed study of these *Dsp*$^{\pm}$ mice at earlier stages of disease development, when no abnormalities at the surface ECGs could be detected and no evidence of replacement fibrosis could yet be found, showed a significant increase in the activation time and inducible arrhythmias in Langendorff perfused hearts. These results indicate the presence of electrophysiological abnormalities before the onset of overt structural changes (Gomes et al., 2012). However, no electron microscopy was done to exclude the presence of disrupted desmosomes at the ultrastructural level.

Transgenic mice with cardiac overexpression of flag tagged human *Dsp* cDNA both wild type (WT-Tg, model Dp3) and with a C-terminal mutation (R2834H-Tg, model Dp4) were generated by Yang et al. (2006). Overexpression of *Dsp* with N-terminal mutations, e.g., V30M (model Dp5) and Q90R (model Dp6) led to embryonic lethality after embryonic day 13.5 due to reduced ventricular wall thickness and ventricular dilatation. R2834H-Tg mice had an increased heart weight/body weight ratio compared to both wild type littermates and Wt-Tg mice. R2834H-Tg hearts showed increased apoptosis and fibrosis along with reduced ventricular function and dilatation of both right and left ventricles. Co-immunoprecipitation indicated the disruption of the interaction between Dsp and Pg. at the ultrastructural level widening of the intercalated disk was observed in Dp4 mice.

DESMOGLEIN OVEREXPRESSION MODELS

Transgenic mice with cardiac overexpression of flag tagged Dsg2 both wild type (Tg-WT, model Dg1) and N271S-Dsg2 mutant (Tg-NS, model Dg2) were generated; the murine N271S mutation is the mouse homolog of the human ARVC mutation DSG2–N266S. While mice overexpressing of wild type *Dsg2* were indistinguishable from their wild type littermates at 2 months of age, Tg-Ns mice developed spontaneous ventricular arrhythmias, conduction slowing, ventricular dilatation and aneurysms, and replacement fibrosis, leading to sudden cardiac death from a less than 2 weeks of age. The disease process was triggered by myocyte necrosis followed by calcification and fibrous tissue replacement (Pilichou et al., 2009). These findings are consistent with the results obtained in mice carrying a targeted deletion in the extracellular adhesion domain of Dsg2 (model Dg3). Approximately 30% of the mice homozygous for the mutation survived embryonic development. These mice develop left and right ventricular dilatation, fibrosis, calcification, and spontaneous death similar to the Tg-NS mice (Krusche et al., 2011). Detailed investigation of this model by transmission electron microscopy revealed a widening of the intercellular space at the intercalated disk and loss of desmosomal structure close to macroscopically visible lesions of the heart (Kant et al., 2012).

DISCUSSION AND CONCLUSION

The diverse mouse models discussed here recapitulate ARVC disease phenotype and have led to several new insights in the etiology of the cardiac phenotype in ARVC patients. The initial targeted deletion of single desmosomal genes, although embryonically lethal, gave fundamental insights in effects of desmosomal gene dysfunction on cardiac function. All the different models surviving embryonic development, recapitulate the human phenotype in the sense of the arrhythmogenicity, replacement fibrosis, and calcification. None of the models show the clear fatty infiltrations seen in human, this is most likely a reflection of the different composition of the extracellular matrix and non-cardiomyocytes interstitial cells in the wild type murine heart, which lacks a significant adipocyte population (Pilichou et al., 2009). In all described mouse models that survive the embryonic period, the disease process is triggered by cardiac cell death upon cardiac stress after birth. However, they are inconsistent considering the type of cell death occurring, with necrosis being the prominent feature in the Desmoglein 2 models (Dg2 and Dg3) and apoptosis (in addition to necrosis) in the plakoglobin models (Pg5 and Pg6). As a consequence of the myocyte death, calcification, and replacement fibrosis occurs leading to reduced cardiac function and ventricular dilatation. This scar formation also provides a substrate for the arrhythmogenic phenotype seen both in human patients and the ARVC mouse models. The study by Fabritz et al. (2011) shows the positive effect of load reducing therapy on disease progression, clearly underwriting the current disease management strategy of discouraging strenuous physical exercise in patients.

Knowledge of the early disease stages would help in designing more effective treatments (Basso et al., 2011). However, the earliest phase of the disease remains thus far elusive due to the early postnatal severe pathogenicity of the phenotype, precluding the effective dissection of the first pathological changes in the affected hearts. The recent study of Gomes et al. suggests the presence of an electrophysiological disturbance before the development of overt cardiomyopathic changes (Kaplan et al., 2004; Gomes et al., 2012). The proposal of electrical disturbances at this early stage of the disease is an attractive one in the light of the recent data showing cross talk between the mechanical junctions, the gap junctions and the Na^+ channel complex (Sato et al., 2009, 2011) and the presence of arrhythmias early in the disease process in humans (Bauce et al., 2005). Unfortunately, this study fails to examine the desmosomal structure at this specific time point; it would be interesting to see whether there are ultrastructural changes present that cause the observed electrical changes. Furthermore, the electrophysiological data presented lacks sufficient detail (e.g., Na^+-current and action potential measurements) to be able to draw definitive conclusions on the nature of the observed early electrical disturbances.

In conclusion, since the discovery a little over a decade ago that the cause of ARVC lies within the cardiac desmosomal complex and its associated proteins, a wealth of knowledge has been build up on the etiology of the disease. The use of the murine transgenic and targeted models has played a pivotal role in this process. The quest to answer the many remaining questions on the first stages of the disease and the search for a good treatment will continue during the next decade, the developed mouse models will undoubtedly play an important role in this process.

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An introduction to murine models of atrial fibrillation

Genna Riley*[1], *Fahima Syeda*[1], *Paulus Kirchhof*[1,2] *and Larissa Fabritz*[1,2]

[1] *Centre for Cardiovascular Sciences, School of Clinical and Experimental Medicine, University of Birmingham, Birmingham, UK*
[2] *Department of Cardiology and Angiology, University Hospital Münster, Münster, Germany*

Edited by:
Carol Ann Remme, University of Amsterdam, Netherlands

Reviewed by:
Sander Verheule, Maastricht University, Netherlands
Diego Franco, Universidad de Jaen, Spain

****Correspondence:***
Larissa Fabritz, Centre for Cardiovascular Sciences, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.
e-mail: l.fabritz@bham.ac.uk

Understanding the mechanism of re-entrant arrhythmias in the past 30 years has allowed the development of almost curative therapies for many rhythm disturbances. The complex, polymorphic arrhythmias of atrial fibrillation (AF) and sudden death are, unfortunately, not yet well understood, and hence still in need of adequate therapy. AF contributes markedly to morbidity and mortality in aging Western populations. In the past decade, many genetically altered murine models have been described and characterized. Here, we review genetically altered murine models of AF; powerful tools that will enable a better understanding of the mechanisms of AF and the assessment of novel therapeutic interventions.

Keywords: atrial fibrillation, atrial tachycardia, cardiac function, electrocardiogram, electrophysiology, mouse heart, murine model, sick sinus syndrome

INTRODUCTION

Atrial fibrillation (AF) affects 1–2% of the population in Europe, and this number is expected to increase twofold to threefold in the next decade, due to both an age-dependent increase in AF and an increased incidence of the arrhythmia (Camm et al., 2010). Even when treated according to best current knowledge, AF remains associated with high residual morbidity and an excess mortality (Camm et al., 2010) that calls for better understanding, diagnosis, and therapy of the arrhythmia rather than the management of symptoms alone. The considered use of animal models has facilitated the characterization of at least four positive feedback loops that contribute to the development of AF (Schotten et al., 2011). Two of these loops augment Ca^{2+} loading and alter ion channel dynamics, which result in the shortening of atrial action potential duration (APD) and focal ectopic activity. Both can be treated with anti-arrhythmic drugs and catheter ablation (Kirchhof et al., 2009) and these techniques can help prevent AF recurrence in selected patients, but the overall recurrence rate of AF remains high.

Unfortunately, some patients with AF are predisposed to recurrent arrhythmias even on such therapy. The changes that predispose the atria toward recurrence of AF have been broadly summarized as "structural remodeling". Better characterization of these processes are required to enable the identification of patients at risk of recurrence and to develop new therapies for the arrhythmia (Schotten et al., 2011; Wakili et al., 2011).

OF MICE AND MEN

There is no doubt that the best model in which to study human disease mechanisms is the patient. However, many of the molecular changes that confer AF are more pronounced in left atrial tissue (Kahr et al., 2011), which is not readily accessible in patients. Furthermore, the polygenic and multi-factorial processes that govern AF render genetically modified models attractive tools in which to dissect the molecular mechanisms of arrhythmia. Models facilitate the control of confounding factors, enable demonstration of causation rather than association and offer opportunities to validate new therapies in an experimental setting. In recent years, owing to its small size, short gestation period, rapid maturation, and the relative ease with which genetic alterations can be achieved, the mouse has become an attractive mammalian model in which to investigate a number of human conditions. Inbred murine models reduce genetic and environmental variability, enabling disease progression and the effect of genetic modifiers including gender, diet, and physical activity on pathology, to be studied in a closed, complex physiological system. Historically, cardiac arrhythmias were studied in larger mammals such as the goat, pig, or dog, as it was believed that arrhythmia did not occur in mice due to their lack of critical cardiac mass (Janse and Rosen, 2006). Furthermore, the hearts of larger mammals such as non-human primates and dogs are more akin to the human heart than rabbits or smaller rodent species (Russell and Proctor, 2006). However, the seminal and relatively recent demonstrations, disproving the theory of critical cardiac mass (Vaidya et al., 1999), have promoted the use of mice in the study of cardiac arrhythmias. There is considerable genetic homology between humans and mice and this is reflected in the conservation of cardiac developmental pathways, morphological structure, and signaling pathways. There are however, inevitable differences between the murine and human heart: the murine heart rate is up to 10 times faster than that of the human heart and the murine APD is shorter and lacks the typical plateau phase (Fabritz et al., 2003). Yet the relationship between electrical diastole and APD in the mouse is comparable with that of humans. In summary, whilst the mouse provides an excellent model system for primary investigation, molecular underpinnings, and proof of principle strategies, all findings need to be confirmed in other animal models and *in vitro* systems prior to considering clinical application.

MURINE MODELS OF ATRIAL FIBRILLATION

In terms of cardiac assessment, the pipelines of large-scale phenotyping consortia are usually limited to the analysis of blood chemistry, the assessment of heart weight relative to tibial length and the assessment of left ventricular function which, whilst good indicators of cardiac dysfunction, will no doubt often fail to identify an arrhythmic phenotype. Electrocardiograms (ECGs) in lightly sedated and freely roaming mice can be used to diagnose atrial arrhythmias. To study electrophysiological mechanisms of atrial arrhythmias, the use of isolated, beating, perfused hearts have been invaluable. These can be subjected to catheter-based recording of electrograms and action potentials; to electrical stimulation and other arrhythmia provocation techniques, and can be used in conjunction with optical mapping to visualize membrane potential and calcium transients (Eloff et al., 2001; Mathur et al., 2009). These techniques, first developed to study ventricular arrhythmias in mice, have more recently been adapted to study atrial arrhythmias as well (de Diego et al., 2008; Lang et al., 2011). There has been a recent increase in the number of murine models reported to harbor atrial arrhythmias, some of which have been reviewed before (Schotten et al., 2011; Wakili et al., 2011). In this review, murine models were grouped according to the molecular signaling pathway with which they have been most associated as follows: alterations in: G-protein coupled receptor (GPCR) signaling; ion channel dynamics, anchoring, and junctional complexes, calcium homeostasis; transcriptional, post transcriptional, and epigenetic regulation; cytokines and growth factors. In this review we have provided a simplified schematic depicting the position of these murine models both within the context of previously established molecular signaling pathways of atrial arrhythmia and subcellular compartment (**Figure 1**). We have also generated a tabulated list of murine models in which AF or a surrogate parameter was reported (**Table 1**). The table indicates whether structural atrial changes were found and gives an idea of whether AF occurred spontaneously under free roaming conditions, under anesthesia, or was provoked during programmed stimulation.

In the following text we will highlight several of these models to exemplify the complex and divergent changes that can result in

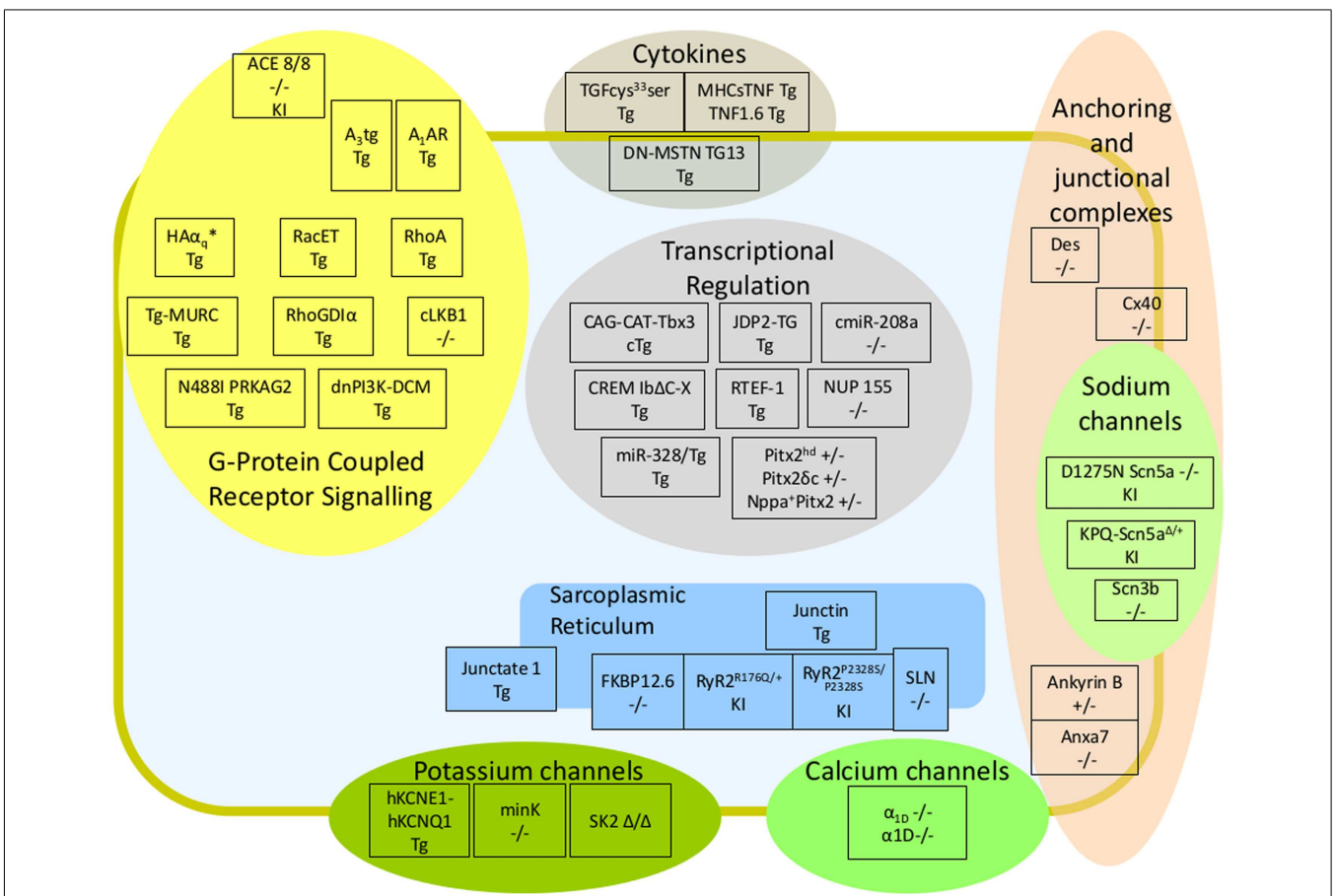

FIGURE 1 | Murine Models of Atrial Fibrillation. Schematic diagram depicting murine models of atrial arrhythmia within the context of arrhythmic signaling pathway and subcellular localization. Models were subdivided into altered G-Protein Coupled Receptor signaling; altered ion channels dynamics, anchoring, and junctional complexes; altered calcium homeostasis; altered transcriptional, post transcriptional, and epigenetic regulators; and cytokines and growth factors. The genotype of knockout models associated with atrial fibrillation is provided; knockin models are followed by the suffix (KI), and transgenic models by the suffix (Tg). A brief description of each model, the experimental circumstances used to elicit AF and references can be found within **Table 1**.

Table 1 | Murine Models of Atrial Fibrillation.

Mouse mutant	Description of transgenic (promoter)	Increased AT/AF telemetric ECG	Increased AT/AF in sedated mice	Increased AT/AF in EP study	AVB	Conduction	A APD	A ERP	Contractile function	Atrial dilation	Atrial thrombi	Atrial fibrosis	Reference
ALTERED G-PROTEIN COUPLED RECEPTOR SIGNALING													
ACE 8/8$^{-/-}$ (KI)	Knockin mutant of α*Mhc* promoter at angiotensin I converting enzyme locus (α*Mhc*)	✓			✓				V=	✓		✓	Xiao et al. (2004)
A_1AR (Tg)	Transgenic mutant overexpressing adenosine A1 receptor (α*Mhc*)			✓	✓		=		=	×			Matherne et al. (1997), Kirchhof et al. (2003)
A_3tg (Tg)	Transgenic mutant overexpressing adenosine A3 receptor (α*Mhc*)			✓	✓				↓	✓		✓	Black et al. (2002), Fabritz et al. (2004)
HAα_q*(Tg)	Transgenic mutant overexpressing constitutively active Gαq (α*Mhc*)	✓	✓	✓	✓	✓	↑		↓	✓	✓	✓	Mende et al. (1998), Hirose et al. (2009), Laakmann et al. (2010)
RhoA (Tg)	Transgenic mutant overexpressing constitutively active Rho A (α*Mhc*)		✓		✓				↓	✓		✓	Sah et al. (1999)
RhoGDIα (Tg)	Transgenic mutant overexpressing bovine Rho GDP dissociation inhibitor α (α*Mhc*)		✓		✓			↑	**V**=	✓		×	Wei et al. (2004)
RacET (Tg)	Transgenic mutant overexpressing constitutively active Rac1 (α*Mhc*)		✓	✓	✓	✓	=	=	↓	✓		✓	Sussman et al. (2000), Adam et al. (2007), Reil et al. (2010)
cLKB1$^{-/-}$	Conditional knockout of serine/threonine kinase 11 using α*Mhc-cre*.		✓						↓	✓	✓	✓	Bardeesy et al. (2002), Ikeda et al. (2009)
Tg-MURC (Tg)	Transgenic mutant overexpressing muscle-related coiled-coil protein (α*Mhc*)		✓		✓				↓	✓	✓	✓	Ogata et al. (2008)
N488I PRKAG2 (Tg)	Transgenic mutant overexpressing human protein kinase AMP-activated gamma 2 subunit carrying N488I mutation (α*Mhc*)	✓			×	×			V↓	✓			Arad et al. (2003)
dnPI3K-DCM (Tg)	Double transgenic mutant overexpressing dominant negative phosphatidyl inositol 3 kinase lacking kinase activity and overexpression of macrophage stimulating 1 (both α*Mhc*)	✓	✓		✓				↓	✓	✓	✓	Pretorius et al. (2009)
ALTERED ION CHANNEL DYNAMICS, ANCHORING, AND JUNCTIONAL COMPLEXES AND CALCIUM HOMEOSTASIS													
Potassium channels													
minK$^{-/-}$	Systemic knockout of the KCNE1 (minK) potassium channel	✓		✓		✓	↓		=				Kupershmidt et al. (1999), Temple et al. (2005)
SK2 $^{\Delta/\Delta}$	Systemic knockout of the SK2 potassium channel			✓	✓		↑	=	=				Bond et al. (2004), Li et al. (2009)
hKCNE1-hKCNQ1 (Tg)	Transgenic mutant overexpressing hKCNE1-hKCNQ1 fusion protein (α*Mhc*)			✓		=	↓	=	=				Marx et al. (2002), Chiello Tracy et al. (2003), Sampson et al. (2008)

(Continued)

Table 1 | Continued

Mouse mutant	Description of transgenic (promoter)	Increased AT/AF telemetric ECG	Increased AT/AF in sedated mice	Increased AT/AF in EP study	AVB	Conduction	A APD	A ERP	Contractile function	Atrial dilation	Atrial thrombi	Atrial fibrosis	Reference
Sodium channels													
$Scn5a^{\Delta/+}$ (KI)	Knockin mutant expressing Scn5a sodium channel subunit with 1505-KPQ-1507 deletion			✓	✓	✓	↑	↑	=	✓	×	×	Nuyens et al. (2001), Dautova et al. (2009), Guzadhur et al. (2010), Blana et al. (2010)
$Scn3b^{-/-}$	Systemic knockout of Scn3b sodium channel subunit			✓	✓		↓						Hakim et al. (2008), Hakim et al. (2010)
D1275N $Scn5a^{-/-}$ (KI)	Knockin mutant expressing Scn5a sodium channel subunit with D1275N mutation		✓		✓	✓	↑		V↓				Watanabe et al. (2011)
Anchoring and junctional complexes													
$Des^{-/-}$	Systemic knockout of desmin			✓	=	✓		↓				✓	Li et al. (1996), Milner et al. (1996), Schrickel et al. (2010)
$Cx40^{-/-}$	Systemic knockout of connexin40			✓	✓	↓		↑					Kirchhoff et al. (1998), Hagendorff et al. (1999), Verheule (1999)
$Ankyrin_B\pm$	Systemic knockout of ankyrin2			✓			↓						Scotland et al. (1998), Cunha et al. (2011)
Altered calcium homeostasis													
$\alpha1D^{-/-}$	Systemic knockout of the α1D subunit of the L-type Ca^{2+} channel		✓	✓	✓	↓	=			×			Platzer et al. (2000), Mancarella et al. (2008)
$\alpha_{1D}{}^{-/-}$	Systemic knockout of the α1D subunit of the L-type Ca^{2+} channel			✓	✓		↑	=					Namkung et al. (2001), Zhang et al. (2002a), Zhang et al. (2005)
$SLN^{-/-}$	Systemic knockout of sarcolipin		✓				↑		A/V↑			✓	Babu et al. (2007a), Xie et al. (2012)
$RyR2^{R176Q/+}$ (KI)	Knockin mutant expressing ryanodine receptor 2 carrying R176Q mutation			✓		=	=	=		×		×	Chelu et al. (2009)
$RyR2^{P2328S/P2328S}$ (KI)	Knockin mutant expressing Ryanodine Receptor 2 carrying P2328S mutation			✓		=	=	=					Goddard et al. (2008), Zhang et al. (2011)
$FKBP12.6^{-/-}$	Systemic knockout of FK506 binding protein 12.6			✓		=		=		×		×	Wehrens et al. (2003), Sood et al. (2008), Li et al. (2012)
Junctate 1 (Tg)	Transgenic mutant overexpressing Junctate 1 (α*Mhc*)		✓						↓	✓	✓	✓	Hong et al. (2008)
Junctin (Tg)	Transgenic mutant overexpressing canine junctin (α*Mhc*)		✓				↑		↓	✓	✓	✓	Hong et al. (2002), Kirchhefer et al. (2004)
$Anxa7^{-/-}$	Systemic knockout of annexin Aa7			✓	✓			=	=				Herr et al. (2001), Schrickel et al. (2007)

(Continued)

Table 1 | Continued

Mouse mutant	Description of transgenic (promoter)	Increased AT/AF telemetric ECG	Increased AT/AF in sedated mice	Increased AT/AF in EP study	AVB	Conduction	A APD	A ERP	Contractile function	Atrial dilation	Atrial thrombi	Atrial fibrosis	Reference
ALTERED TRANSCRIPTIONAL, POST TRANSCRIPTIONAL, AND EPIGENETIC REGULATORS													
CREM-IbΔC-X (Tg)	Transgenic mutant expressing human cardiac CREM isoform (α*Mhc*)	✓	✓	✓	✓		↑		V ↑ A ↓	✓	✓		Müller et al. (2005), Kirchhof et al. (2011a)
miR-328/Tg (Tg)	Transgenic mutant overexpressing premiR-328 (α*Mhc*)		✓				↓				✓		Lu et al. (2010)
cmiR-208a$^{-/-}$	Conditional knockout of miR-208a using β*Actn-cre*		✓						↓				Callis et al. (2009)
Pitx2 δc$^{+/-}$	Systemic knockout of Pitx2c isoform			✓	=		↓		=	×			Liu et al. (2002), Kirchhof et al. (2011b)
Pitx2$^{hd+/-}$	Systemic knockout of Pitx2			✓									Lu et al. (1999), Wang et al. (2010)
Nppa^{+} Pitx2$^{-/-}$	Conditional knockout of Pitx2 using *Nppa-cre*									✓			Gage et al. (1999), Chinchilla et al. (2011)
cTg-CAG-CAT-Tbx3 (Tg)	Conditional transgenic overexpressing human T-box 3 crossed to *Nppa-cre*		✓		✓							✓	Hoogaars et al. (2007)
NUP155$^{+/-}$	Systemic knockout of nucleoporin 155	✓								×			Zhang et al. (2008)
JDP-Tg (Tet-JDP2/α-MHC-tTA)$^{+/-}$	Tetracycline-regulated overexpression of Jun dimerization protein 2 using α*Mhc-tTA*		✓		✓	↓			=	✓		×	Kehat et al. (2006)
RTEF-1 (Tg)	Transgenic mutant overexpressing human TEA domain family member 4 (α*Mhc*)	✓	✓	✓	✓	↓				✓	✓		Chen et al. (2004)
Cytokines/Growth Factors													
MHC-TGFcys^{33}ser (Tg)	Transgenic mutant overexpressing human TGF-beta 1 carrying C33S mutation (α*Mhc*)			✓	✓		=	=		×		✓	Nakajima et al. (2000), Verheule et al. (2004), Choi et al. (2012)
MHCsTNF (Tg)	Transgenic mutant overexpressing tumor necrosis factor (α*Mhc*)		✓	✓	✓			↑		✓			Li et al. (2000), Sivasubramanian et al. (2001), Sawaya et al. (2007)
TNF1.6 (Tg)	Transgenic mutant overexpressing tumor necrosis factor (α*Mhc*)	✓		✓	✓		=		A ↓	✓	✓	✓	Kubota et al. (1997), Saba et al. (2005)
DN-MSTN TG13 (Tg)	Transgenic mutant overexpressing inhibitory N-terminal pro-peptide (α*Mhc*)		✓	✓	✓				=/↑	✓		✓	Rosenberg et al. (2012)

Murine models of AF were divided into five categories: Altered G-Protein Coupled signaling; altered ion channel dynamics, anchoring, and junctional complexes, altered calcium homeostasis; altered transcriptional, post transcriptional, and epigenetic regulators; cytokines and growth factors. Models were identified via a publication search on Pubmed using the terms "mouse atrial arrhythmia." This returned a total of 351 hits all which were then individually screened for relevance in terms of: spontaneous atrial tachycardia and/or fibrillation (AT/AF) during telemetric or sedated analysis; increased induced occurrence of atrial tachycardia and/or fibrillation (AT/AF) in electrophysiology (EP) studies; atrial ventricular block (AVB) including PQ/PR prolongation as AVBI; effect on atrial conduction (direct and indirect); effect on atrial action potential duration (APD); effect on atrial effective refractory period (ERP); effect on contractile function refractory period, ERP; contractile function (V-ventricular, A-atrial); presence of atrial dilation; atrial thrombi, and atrial fibrosis. ✓ indicates presence of that condition, × indicates absence of the condition, unticked boxes do not necessarily mean that a condition has been completely ruled out for a given model, only that there is no evidence as yet. =, unaltered. References are shown in the right hand column. The first reference for each model describes the generation of that model.

murine AF. We hope that understanding mechanisms of arrhythmia in such models will further our understanding and treatment of AF in humans.

MODELS OF AF WITH ALTERED G-PROTEIN COUPLED RECEPTOR SIGNALING

Systemically, GPCRs are involved in extracellular-intracellular signaling and underpin a wide variety of biological responses (Neer, 1994; Park et al., 2004). The most commonly studied GPCRs in the heart include the adrenergic, angiotensin, endothelin, and adenosine receptors (Salazar et al., 2007).

ANGIOTENSIN II

The role of renin-angiotensin signaling within cardiac pathological states, particularly in regard to blood pressure, is well documented. However, there is increasing evidence that angiotensin II exerts a more local stimulatory effect on heart function (Sancho et al., 1976; Lindpaintner et al., 1988; De Mello and Frohlich, 2011). Furthermore, atrial angiotensin II expression levels are increased in patients with AF (Boldt et al., 2003; Cong et al., 2010; De Jong et al., 2011). *In vitro*, rapid pacing of atrial myocytes increased the paracrine secretion of angiotensin II (Tsai et al., 2011), suggesting that fibrillation promotes angiotensin II production and therefore signaling. Cardiac-specific overexpression of angiotensin I converting enzyme (ACE), a peptidase that converts angiotensin I to its biologically active counterpart, angiotensin II, in mice, resulted in atrial dilation, fibrosis, and spontaneous AF under free roaming conditions (Xiao et al., 2004), suggesting that increased angiotensin II signaling promotes the development of AF. Yet, cardiac-specific overexpression of the angiotensin receptor AT_{1a} caused marked atrial enlargement, bradycardia, and abnormal atrio-ventricular (AV) conduction, but failed to increase AF susceptibility in neonatal mice (Hein et al., 1997).

ADENOSINE RECEPTORS

Adenosine receptors are a type of purinergic G-Protein coupled receptor, activated by adenosine, that have an inhibitory effect on adenlyl cyclase signaling (and therefore cAMP levels) and voltage-gated ion channels, whilst concomitantly activating potassium channels (Priori et al., 1993; Headrick et al., 2011). Short-term adenosine A1 receptor (A_1AR) stimulation induces bradycardia and AV block in humans (DiMarco et al., 1983). Transgenic models of adenosine A1 and A3 receptors were developed primarily to evaluate the protective effect of receptor overexpression on myocardial ischemia (Matherne et al., 1997). However, enhanced expression of either A1 or A3 adenosine receptors also provoked atrial bradycardia and AV block and increased susceptibility to AF dependent on the degree of bradycardia ("tachycardia-bradycardia syndrome"; Kirchhof et al., 2003; Fabritz et al., 2004), that could potentially lead to bradycardiomyopathy (Fabritz et al., 2004). Therefore, increased cAMP levels (as shown by this model and that of ACE overexpression) appear to contribute to the development of AF.

GALPHA Q

Heterotrimeric G-proteins are membrane-associated complexes that comprise an alpha, beta, and gamma subunit and are the intracellular effectors of GPCRs (Neer, 1994; Park et al., 2004; Salazar et al., 2007). In the heart, Gαq associates with alpha1-adrenergic, endothelin (ET_A), and angiotensin II type I (AT_1) receptors. Following ligand activation, Gαq is phosphorylated which results in the activation of numerous downstream effectors including phospholipase C (PLC), protein kinase C (PKC), thymoma viral protein-oncogene (Akt), and Ras homologous (Rho; Salazar et al., 2007; Filtz et al., 2009; Ben-Ami et al., 2011; Pfreimer et al., 2012). To investigate the putative role of Gαq in cardiac hypertrophy, two transgenic mouse lines overexpressing endogenous and a constitutively active *Gαq* subunit under the control of the α*Mhc* promoter were independently generated (D'Angelo et al., 1997; Mende et al., 1998). Both models developed cardiac hypertrophy in the absence of myocyte disarray or necrosis, followed by diffuse atrial and ventricular fibrosis and heart failure (D'Angelo et al., 1997; Mende et al., 1998; Hirose et al., 2009). A number of these mice developed left atrial thrombi, another pathological characteristic associated with AF (Mende et al., 1998; Laakmann et al., 2010), and indeed, AF was recorded as occurring spontaneously under roaming conditions (Hirose et al., 2009). Similar to the Ras homolog gene family member A (*RhoA*) overexpression model discussed below, there is recent evidence to suggest that in this model, AF may be a primary pathology within its own right that does not occur secondary to ventricular remodeling (Sah et al., 1999; Laakmann et al., 2010).

RAS HOMOLOG GENE FAMILY MEMBER A

Initially identified for its role within hypertrophic signaling pathways (Sah et al., 1996), RHOA is a small membrane-associated GTPase involved in actin cytoskeleton organization (Ridley and Hall, 1992). Mice overexpressing either wild type RHOA or an activated form of RHOA under the control of the cardiac-specific α*Mhc* promoter died prematurely and developed cardiac enlargement, cellular hypertrophy, interstitial fibrosis, and heart failure (Sah et al., 1999). Atrial enlargement was more pronounced than ventricular enlargement, suggesting atrial dilation preceded ventricular dilation, which is consistent with the reported temporal cardiac expression profile of α*Mhc* (Chizzonite et al., 1982; Sweeney et al., 1985; Bouvagnet et al., 1987; Colbert et al., 1997). Electrocardiography (ECG) performed under anesthesia was indicative of AF and AV block (Sah et al., 1999).

More recently overexpression of RhoGDIα, an endogenous specific GDP dissociation inhibitor for all Rho family proteins, led to atrial arrhythmias and mild ventricular hypertrophy (Wei et al., 2004). ECG and intracardiac electrophysiological analysis showed these mice developed bradycardia, AV block, and atrial arrhythmias concomitant with reduced expression of the gap junction protein, connexin 40, before the onset of cardiac hypertrophy and heart failure (Wei et al., 2004). Taken together these murine models suggest that altered expression levels of Rho family proteins can propagate a pro-arrhythmic environment.

Rac1

Rat sarcoma (RAS)-related C3 botulinum toxin substrate 1 (Rac1) has long been recognized for its role in actin cytoskeleton organization, and is a plasma membrane-associated GTPase belonging to the RAS superfamily (Ridley and Hall, 1992; Sussman et al., 2000). Homozygous mice overexpressing constitutively active *Rac1* under the control of the α*Mhc* promoter were found to be viable

(Chizzonite et al., 1982). However, a proportion of progeny died within 2 weeks of birth owing to increased postnatal α*Mhc* promoter activity as a result of increased circulating levels of thyroid hormone (Chizzonite et al., 1982; Sussman et al., 2000). Mice surviving this initial vulnerable period achieved survival comparable with that of wild type (Sussman et al., 2000), but by 2 months of age, mutants developed atrial enlargement and atrial wall thinning juxtaposed to ventricular wall thickening and reduced ventricular chamber size (Sussman et al., 2000). Interstitial fibrosis and systolic heart failure progressively developed and 75% of the transgenic mice developed spontaneous AF under anesthesia (Adam et al., 2007).

OTHER MODELS OF ALTERED GPCR SIGNALING

Several other murine models of AF have been generated by the genetic manipulation of other proteins within GPCR signaling cascades using α*Mhc* promoter-driven expression. Briefly, models reported to develop spontaneous AF under anesthesia include a knockout of serine/threonine kinase 11 (Stk11/Lkb1; Bardeesy et al., 2002; Ikeda et al., 2009; Pretorius et al., 2009); a transgenic mouse overexpressing Muscle-Related Coiled-Coil protein (MURC1; Ogata et al., 2008) and a transgenic mouse overexpressing human protein kinase AMP-activated gamma 2 subunit (PRKAG2) carrying the N488I mutation (Arad et al., 2003). A double transgenic mutant overexpressing dominant negative phosphatidyl inositol 3 kinase and macrophage stimulating 1 (Mst1) was also reported to develop spontaneous AF during telemetry (Pretorius et al., 2009).

MODELS OF AF WITH ALTERED ION CHANNEL DYNAMICS, ANCHORING, AND JUNCTIONAL COMPLEXES AND CALCIUM HOMEOSTASIS

ION CHANNEL DYNAMICS

Potassium channels

Potassium ion channels remove K^+ from the cell defining the repolarization phase of the action potential. Therefore, any genetic modification that alters the expression levels or gating of potassium channels has the potential to alter the cardiac action potential and thereby act as an arrhythmic substrate. Models include: the systemic knockout of the small conductance calcium-activated potassium channel (SK2) which increased susceptibility to AF induction (Bond et al., 2004; Li et al., 2009), and the systemic knockout of the voltage-gated subfamily E member 1 potassium channel (KCNE1) which resulted in spontaneous AF under telemetry (Kupershmidt et al., 1999; Temple et al., 2005).

Sodium channels

SCN5A. Sodium currents are responsible for the rapid depolarization of myocytes and are important for conduction, repolarization, and refractoriness. Similar to potassium channel disruption, several animal models in which sodium channel dynamics have been altered develop AF. *SCN5A* encodes the alpha subunit of the cardiac voltage-gated sodium channel (Nav1.5; Gellens et al., 1992). Mutations within *SCN5A* underlie a number of clinically defined arrhythmias including long QT syndrome type 3 (LQT3), Brugada syndrome, progressive conduction disease, and AF (Ruan et al., 2010; Wilde and Brugada, 2011) and many of these diseases harbor a propensity for atrial arrhythmias (Eckardt et al., 2001; Zellerhoff et al., 2009).

Systemic targeted disruption of *Scn5a* results in homozygous lethality whilst heterozygotes display a 50% reduction in sodium conductance (Papadatos et al., 2002). A knockin murine model, Scn5a Δ1505-1507 KPQ (ΔKPQ-Scn5a), of a human mutation in which three amino acids within the inactivation domain of Nav1.5 were deleted, resulted in a persistent inward Na^+ current thus delaying the repolarization of the action potential and causing longer QT intervals (Wang et al., 1996; Nuyens et al., 2001; Head et al., 2005). ECG recorded by telemetry and under anesthesia revealed that heterozygous mice mimic the long QT syndrome 3 phenotype found in patients (Nuyens et al., 2001). More recently, atrial electrophysiology was assessed in ΔKPQ-Scn5a mutant mice. Concurrent with ventricular action potentials, atrial action potentials were also prolonged (Dautova et al., 2009; Blana et al., 2010). ΔKPQ-Scn5a mice were more susceptible to atrial arrhythmias induced by maneuvers known to provoke torsades de pointes, namely short-long-short stimulation sequences (Blana et al., 2010). Aged ΔKPQ-Scn5a mice were reported to be more susceptible to atrial arrhythmias induced by programmed stimulation (Guzadhur et al., 2010), and have altered sinoatrial node function and intra-atrial conduction (Head et al., 2005; Wu et al., 2012).

Analysis of another knockin murine model, homozygous for the human D1275N mutation (Groenewegen et al., 2003; Remme et al., 2006), reported slowed conduction, atrial arrhythmias, sinus node dysfunction, and progressive AV block, that culminated in sudden death at around 12 weeks of age (Watanabe et al., 2011). Mutations within other accessory subunits of the cardiac sodium channel also affect channel dynamics; homozygous deletion of the beta subunit, *SCN3B*, led to conduction disturbances, bradycardia, and an increased susceptibility to induced atrial arrhythmias (Hakim et al., 2008, 2010; Olesen et al., 2011).

ANCHORING AND JUNCTIONAL COMPLEXES

Connexins

The cardiac voltage sodium channel, Nav1.5, was shown to colocalize with Connexin 43 within the intercalated disk of ventricular cardiomyocytes (Maier et al., 2002). The gap junction proteins Connexin 40 (Cx40, GJA5) and Connexin 43 (Cx43, GJA1) function to electrically couple cardiomyocytes, facilitating syncytial contraction. Cx40 and Cx43 are expressed at equal levels in atria however, only Cx43 is expressed in the ventricles (Lin et al., 2010). Both mutations in *CX40* and *CX43* have been reported in patients with idiopathic AF (Gollob et al., 2006; Thibodeau et al., 2010). Cardiac-specific loss of Cx43 and overexpression of a dominant negative mutant of Cx43 led to the development of spontaneous ventricular arrhythmias (Hong et al., 2008; Sood et al., 2008; Li et al., 2012), consistent with a predominating ventricular role in the mouse. However, systemic loss of Cx40 (or GJA5) in the mouse resulted in an increased propensity to induced atrial arrhythmias and reduced conduction velocity (Kirchhoff et al., 1998; Hagendorff et al., 1999; Verheule, 1999). Furthermore, loss/redistribution of Cx40 appears to be a common feature of other murine models of AF including overexpression of RHOA, overexpression of constitutively active RAC1, cardiac-specific overexpression of ACE,

overexpression of Tumor necrosis factor (TNF), and cardiac overexpression of cAMP Response element modulator (CREM; Sah et al., 1999; Kasi et al., 2007; Sawaya et al., 2007; Adam et al., 2010; Kirchhof et al., 2011a; see **Table 1** and **Figure 1** for further details).

Recent evidence suggests that in the heart of higher vertebrates, the protein complexes that traditionally comprise the cell–cell junctions of the adherens junctions and desmosomes of epithelial cells, actually coordinate to form a single heterogeneous junction referred to as the area composita (Borrmann et al., 2006; Franke et al., 2006). Loss of desmin, an intermediate filament that selectively associates with desmosomal complexes, has been reported to lead to atrial fibrosis and increased susceptibility to AF induction (Li et al., 1996; Milner et al., 1996; Schrickel et al., 2010). Hence, disrupted cell–cell mechanical coupling may also act as a substrate for AF.

Ankyrin B

The ankyrin family are membrane-associated adaptor proteins that serve to link membrane-associated proteins with the cytoskeleton (Bennett and Gilligan, 1993; Li et al., 1993). Loss of function of Ankyrin B (ANK2) is associated with long QT syndrome and sudden cardiac death in humans and mice (Mohler et al., 2003). Previously, ankyrin B was shown to regulate cardiac sodium channels dynamics (Chauhan et al., 2000), Na/K ATPase, Na/Ca exchanger 1, and inositol triphosphate (InsP3; Mohler et al., 2005). Loss of ankyrin B was reported to increase cardiac Na^+ channel opening times, increasing APD (Chauhan et al., 2000). Recent evidence suggests that disrupted ankyrin B function is a predisposing factor for the development of atrial arrhythmias in humans (Cunha et al., 2011). Mice heterozygous for ankyrin B were reported to develop spontaneous atrial arrhythmias under telemetry and ECG studies showed increased susceptibility to AF induction by pacing (Cunha et al., 2011). In the same work, ankyrin B was shown to bind to the alpha 1C subunit of the L-type Ca^{2+} channel (Cav1.2) and loss of ankyrin B resulted in reduced inward Ca^{2+} current and a shortened APD (Cunha et al., 2011). Hence, it would appear that ankyrin B regulates both Na^+ and Ca^{2+} currents and that disruption of ankyrin B perturbed these currents and lead to spontaneous AF.

CALCIUM HOMEOSTASIS

The voltage-dependent L-type Ca^{2+} channel contributes to the late depolarizing currents of the cardiac action potential. Comprising five subunits in total, the alpha subunit of the L-type Ca^{2+} channel is the transmembrane spanning subunit. In the heart, the alpha 1d subunit (Cav1.3) is exclusively expressed in the atria (Zhang et al., 2005), where it plays an important role in the spontaneous diastolic depolarization of pacemaker cells (Zhang et al., 2002a). Systemic deletion of Cav1.3 rendered mice susceptible to AF induction (Zhang et al., 2005; Mancarella et al., 2008).

Calcium homeostasis is important in the maintenance of normal sinus rhythm and contraction: Ca^{2+} induced Ca^{2+} release is the mainstay of mechanoelectrical coupling, clearance of Ca^{2+} from the cytosol significantly contributes to the late depolarizing currents of the cardiac action potential and Ca^{2+} acts as a secondary messenger within many intracellular signaling pathways. Therefore, any genetic modification that alters the expression levels or gating of calcium channels; or modifies signaling cascades in which Ca^{2+} features as a secondary messenger, has the potential to act as an arrhythmogenic substrate. Previous reviews of AF models have focused on calcium homeostasis therefore, we will only mention them briefly (Schotten et al., 2011; Wakili et al., 2011).

Murine models with mutations in the ryanodine receptor and associated proteins are reported to be susceptible to AF (see **Table 1**). Models include: mice carrying the human mutation, R176Q, within the outward Ca^{2+} channel of the sarcoplasmic reticulum, ryanodine receptor 2 (RyR2; Chelu et al., 2009); mice carrying the human mutation, P2328S, within RyR2 (Goddard et al., 2008; Zhang et al., 2011); systemic deletion of the RyR2 binding protein, FKBP12.6 (Wehrens et al., 2003; Sood et al., 2008; Li et al., 2012); cardiac-specific overexpression of the Ca^{2+} binding protein, junctate 1 (Hong et al., 2002, 2008); cardiac-specific overexpression of the sarcoplasmic transmembrane protein, junctin (Hong et al., 2002; Kirchhof et al., 2007), and systemic deletion of the Ca^{2+}- and GTP-dependent membrane-associated protein, annexin Aa7 (Anxa7; Herr et al., 2001; Schrickel et al., 2007). Of note, spontaneous AF was observed in mice with a 29-fold overexpression of junctin, whereas mice with 10-fold overexpression showed only reduced adaption of heart rate to stress (Hong et al., 2002; Kirchhof et al., 2007).

Sarcolipin (SLN) is a transmembrane protein located within the sarcoplasmic reticulum that like phospholamban, is a key regulator of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) function (Odermatt et al., 1998; Asahi et al., 2003; Minamisawa et al., 2003; Babu et al., 2007a; Bhupathy et al., 2009). However, unlike phospholamban, SLN activity is mediated by calcium-calmodulin dependent protein kinase II (CaMKII), not protein kinase A (PKA; Bhupathy et al., 2009). At low Ca^{2+} concentrations, SLN inhibits SERCA, reducing its affinity for Ca^{2+} which has the effect of slowing Ca^{2+} uptake into the sarcoplasmic reticulum, reducing contractility (reduced force generation and speed of relaxation; Tupling et al., 2002). Reduced levels of SLN mRNA and protein expression are associated with chronic AF in humans (Uemura et al., 2004; Shanmugam et al., 2011). Aged homozygous SLN knockout mice (Babu et al., 2007b) were reported to develop atrial fibrosis and spontaneous AF under anesthesia (Xie et al., 2012), suggesting that increased SERCA function promotes AF.

MODELS OF AF WITH ALTERED TRANSCRIPTIONAL, POST TRANSCRIPTIONAL, AND EPIGENETIC REGULATION

Dysregulation of GPCR signaling, ion channel dynamics, and calcium homeostasis have long been associated with the development and maintenance of AF however, the net effect of these pathways upon transcription and the contribution of transcriptional, post transcriptional, and epigenetic dysregulation to AF are less well understood. This represents a rapidly expanding area of AF research and it is here, within this category, the majority of the more recently described animal models of AF reside.

cAMP RESPONSE ELEMENT MODULATOR

cAMP Response element modulator belongs to the cAMP response element binding (CREB)/activating transcription factors (ATF) family of transcription factors and is a phosphorylation target of PKA. Cardiac-directed expression of CREM-IbΔC-X, a human

cardiac CREM repressor isoform, resulted in both atrial and ventricular enlargement, atrial wall thinning, atrial thrombus formation, disturbed myocyte architecture, and increased basal left ventricular function (Müller et al., 2005). Echocardiography analysis revealed that atrial chamber dilation preceded the development of AF (Kirchhof et al., 2011a). In addition to reduced wall thickness, atrial myocytes were elongated and atrial weight increased, while neither cellular hypertrophy nor fibrosis were found (Kirchhof et al., 2011a). Telemetric ECG indicated that mutants suffered from atrial ectopies, the occurrence of which increased with age until mice entered an almost constant state of atrial tachycardia (Kirchhof et al., 2011a). Reduced phosphorylation of CREM (due to overexpression of CREM-IbΔC-X) was associated with altered Ca^{2+} homeostasis concurrent with spontaneous Ca^{2+} release and reduced gap junction protein expression (CX40; Kirchhof et al., 2011a). CREM transcription factors bind to the cAMP response element found within several promoters (Foulkes et al., 1991). Whilst many of the genetic targets of CREM involved in spermatogenesis have been identified (Kosir et al., 2012), cardiac-specific gene targets remain largely unknown and are subject to current studies.

PITX2c

The paired-like homeodomain transcription factor 2 (Pitx2) is a downstream target of Nodal within the transforming growth factor beta (TGFβ) signaling pathway and was primarily recognized as having a role in establishing left-right asymmetry during embryonic development (Meno et al., 1998; Ryan et al., 1998). The highest Pitx2 isoform expressed in the murine and human heart is Pitx2c (Kitamura et al., 1999; Schweickert et al., 2000). Systemic loss of Pitx2c in mice lead to embryonic lethality and resulted in a number of phenotypes including abnormal cardiac morphogenesis (Gage et al., 1999). During embryogenesis, Pitx2c was shown to synergistically drive expression of natriuretic peptide A (*Nppa)* in the presence of the homeobox transcription factor, *Nkx2.5* (Ganga et al., 2003) which in turn, suppressed the expression of the pacemaker channel gene, *Hcn4*, and the T-box transcription factor (*Tbx3*), gene, thus delineating contractile atrial myocardium from the sinoatrial node (Hoogaars et al., 2007; Mommersteeg et al., 2007).

Genome-wide association studies (GWAS) identified sequence variation within 4q25 as conferring increased susceptibility to AF (Schott et al., 1995; Gudbjartsson et al., 2007; Kääb et al., 2009). Hence, owing to its genomic location within this candidate region, *PITX2* was considered a prime candidate gene for the development of atrial arrhythmias in man. Indeed, expression analysis confirmed that *PITX2C* was also expressed in adult left atrial tissue, further supporting a biological function beyond that of embryonic left-right patterning (Wang et al., 2010; Kirchhof et al., 2011b). In mice carrying a hypomorphic allele of *Pitx2c*, sedation and pacing revealed an increased tendency of atrial flutter and tachycardia in heterozygous mutants (Wang et al., 2010). Isolated, beating hearts carrying a heterozygous *Pitx2c*-deletion had a reduced APD and were also susceptible to AF (Kirchhof et al., 2011b). Echocardiography suggested there were no apparent morphological defects (Kirchhof et al., 2011b). In contrast, homozygous deletion of *Pitx2c* in the heart, appeared to provoke structural changes later in life as well as changes in ion channel expression (Chinchilla et al., 2011), which could also offer mechanistic insight into the observed arrhythmic phenotype.

Given its role in suppressing "pacemaker" genes and defining the contractile myocardium, it has been proposed that loss of Pitx2c results in a "less left" atrial phenotype promoting ectopic left atrial activity however, how this is achieved in fully differentiated, adult myocardium is unclear at present.

T-BOX TRANSCRIPTION FACTOR 3

Similar to loss of Pitx2c, mice over expressing Tbx3, another gene implicated in the specification of pacemaker cells, in a cardiac-specific manner, developed atrial ectopic beats (Hoogaars et al., 2007).

RTEF-1

Transcription enhancer factor-1-related factor (RTEF-1) is a transcriptional target of alpha1-adrenergic signaling (Stewart et al., 1998; Ueyama et al., 2000). Cardiac-specific overexpression of human RTEF-1 in mice led to the development of spontaneous AF under telemetry, an increased tendency to AF induction by burst pacing in younger mice and the development of sustained AF in older (12 months) transgenic founder mice (Chen et al., 2004). Atrial dilation and thrombi were reported in this transgenic model however, there was no evidence of fibrosis. Owing to reduced atrial conduction velocity, the expression levels of phosphorylated compared to dephosphorylated Cx40 were assessed: The levels of phosphorylated Cx40 were found to be reduced concomitant with an increase in dephosphorylated Cx40 suggesting that gap junction complexes were compromised in this model of AF (Chen et al., 2004).

JUN DIMERIZATION PROTEIN 2

c-Jun dimerization protein 2 (JDP2) is a transcriptional repressor belonging to the basic leucine zipper (bZIP) family of transcription factors (Aronheim et al., 1997; Jin et al., 2001). Tetracycline-regulated cardiac-specific overexpression of JDP2 resulted in atrial dilatation and atrial myocyte hypertrophy and reduced conduction velocity (Kehat et al., 2006). Furthermore, spontaneous AF under anesthesia was reported in a "small number" of mice (Kehat et al., 2006). Cx40 protein levels were also perturbed in this model (reduced expression level of total Cx40), which was indicative of the inhibitory effect of JDP2 on Cx40 promoter activity (Kehat et al., 2006). Hence loss of Cx40 may also be an attributing factor to the observed conduction defects and the development of AF in this model.

NUCLEOPORIN 155

Nucleoporin 155 (NUP155) is a major component of the nuclear pore complex within the nuclear envelope that facilitates the transport of DNA and mRNA from the nucleus to the cytoplasm (Zhang et al., 2002b). Systemic heterozygous deletion of NUP155 in mice, reportedly lead to spontaneous AF under roaming conditions (Zhang et al., 2008). More importantly, a single nucleotide polymorphism that evokes a missense mutation within NUP155 has been identified as contributing to AF in patients (Zhang et al., 2008). *In vitro* analysis showed that this mutation impaired

nuclear permeability to *Nup155* gene products (Zhang et al., 2008). NUP155 was recently shown to interact with histone deacetylase 4 (HDAC4), hence this arrhythmic phenotype may also be the consequence of altered transcriptional activity (Kehat et al., 2011).

MICRORNAs

In recent years a number of microRNAs (miRNA), endogenous non-coding ∼22mer RNA molecules that regulate gene expression by gene silencing, have been increasingly implicated in cardiac pathology (Wakili et al., 2011; Wang et al., 2011). This highlights the potential role of post transcriptional regulation alongside other such control mechanisms as transcriptional regulation, translational regulation, and post-translational modification.

miR-328

miR-223, miR-328, and miR-664 were found to be upregulated in a canine model of AF (Yue et al., 1997; Lu et al., 2010). The generation of a transgenic mouse overexpressing miR-328 under the control of the α*Mhc* promoter, resulted in the development of spontaneous AF under anesthesia (Lu et al., 2010). Furthermore loss of miR-328 protected against AF induction via pacing following muscarinic acetylcholine receptor stimulation (Lu et al., 2010). The alpha 1c and beta 1 subunits of the L-type Ca^{2+} channel were identified as targets of miR-328, and the expression levels of the L-type Ca^{2+} channel protein subunits encoded by these genes, Cav1.2 and Cavβ1, were also reduced, suggesting that the observed shorter APD in this model is the result of reduced L-type Ca^{2+} currents (Lu et al., 2010).

miR-208

Transgenic mice overexpressing miR-208a displayed reduced cardiac function by 3 months of age and went on to develop first and second degree AV block (Callis et al., 2009). Conversely, loss of miR-208a in a murine knockout model had a protective effect on cardiac remodeling following thoracic aortic banding (van Rooij et al., 2007). Despite the seemingly positive effect of reducing miR-208a on cardiac pathology, miR-208a knockout mice also developed spontaneous AF under anesthesia (Callis et al., 2009). The effect of miR-208b on cardiac conduction has yet to be investigated but it differed from miR-208a by only 3 bp and shared identical seed regions (Callis et al., 2009), hence it seems likely that miR-208b regulates the same targets as miR-208a (Callis et al., 2009) and that both feature within arrhythmogenic signaling pathways. miR-208a and miR-208b are encoded within intron 27 of the α*Mhc* and β*Mhc* genes respectively (van Rooij et al., 2007) and regulatory targets of miR-208a include thyroid hormone associated protein 1 and myostatin (see next paragraph; Callis et al., 2009).

CYTOKINES AND GROWTH FACTORS

MYOSTATIN

Myostatin was among the first proteins to be identified as a negative regulator of hypertrophy (McPherron et al., 1997). Very recently, cardiac-specific expression of the inhibitory N-terminus of myostatin pro-peptide was shown to increase susceptibility to AF induction in mice (Rosenberg et al., 2012). Taken together, these results suggest that loss of myostatin expression can act as a substrate for AF. Preliminary data has shown the voltage-gated potassium channel Kv1.4 (KCNA4) to be upregulated in this model, although the impact of this on APD has yet to be determined (Callis et al., 2009; Rosenberg et al., 2012).

TRANSFORMING GROWTH FACTOR BETA 1

Transforming growth factor beta 1 (TGFß1) is a cytokine that regulates numerous cellular processes including growth, differentiation, adhesion, migration, and apoptosis. However, in the adult heart, TGFß1 is a key regulator of fibrosis. Enhanced fibrosis reduces the electrical coupling of cardiomyocytes (Shaw and Rudy, 1997; Allessie et al., 2005). The atria have been shown to be more susceptible to fibrotic remodeling (Burstein et al., 2008) and pacing of cultured atrial-derived myocytes was shown to increase expression of TGFβ1 (Yeh et al., 2011). In patients, increased expression levels of TGFß1 are associated with chronic AF, valvular heart disease, atherosclerosis, and pressure overload (Lamirault et al., 2006; Xu et al., 2010; Creemers and Pinto, 2011). In mice, cardiac-specific overexpression of constitutively active TGFß1 caused atrial fibrosis, atrial conduction disturbances, and AF (Verheule et al., 2004). Recent data suggested that triggered activity arising due to reduced APD and spontaneous Ca^{2+} release, contribute to the development of AF in this murine model (Choi et al., 2012). Increased levels of angiotensin II and reactive oxygen species were shown to increase the promoter activity of *Tgfβ1*, connective tissue growth factor (*Ctgf*), and collagen in fibroblasts thus perpetuating a profibrotic environment that could promote conduction defects (Hao et al., 2011; Tsai et al., 2011).

TUMOR NECROSIS FACTOR

Tumor necrosis factor (formerly referred to as TNFα) is another inflammatory cytokine implicated in the development and pathology of AF in humans (Cao et al., 2011; Deng et al., 2011). Cardiac-specific overexpression of TNFα in mice resulted in atrial dilation, fibrosis, thrombi development, and spontaneous AF under roaming conditions (Kubota et al., 1997; Saba et al., 2005). In a second TNF overexpression model, downregulation of the gap junction protein Connexin 40 was also reported (Sawaya et al., 2007).

CONCLUSION

A long list of murine models that confer increased susceptibility to atrial arrhythmias, further substantiates the value of genetically altered murine models in advancing our understanding of the processes that initiate and propagate AF. In this review, models were grouped into signaling pathways already associated with AF. However, this is not to say that the proteins they encode are restricted to any single pathway. Each "pathway" most likely represents a small component of a much larger, more complex, pro-arrhythmic pathway.

In the vast majority of models, atrial arrhythmia was reported to be induced in a non-physiological setting such as anesthesia or pacing (more than three quarters of mouse mutants reported in **Table 1**). The relationship between anesthesia and AF is still of clinical relevance (Laakmann et al., 2010). However pacing induced arrhythmia suggests that whilst there is a substrate for atrial arrhythmia, this can be well tolerated and undetected in the absence of a triggering factor. Reported cases of spontaneous atrial arrhythmias in freely roaming mice were relatively rare (less than a quarter of the mouse mutants reported in **Table 1**) and almost

always accompanied by atrial enlargement and/or fibrosis. We were intrigued to find that the majority of models reported to develop spontaneous AF without anesthesia or interventions were transgenic overexpression mutants. High levels of overexpression may have a toxic effect on the cell that also contributes to atrial phenotype, whereby overexpression leads to structural changes beyond those observed during "normal" pathophysiology. It is also possible that such expression levels may actually trigger a common final pathological pathway that mimics human pathology. Indeed the majority of the mutant models discussed in this review displayed dose dependent phenotypes that arose due to the heterozygous or homozygous expression and/or the positional effect of transgene integration (D'Angelo et al., 1997; Mende et al., 1998; Sussman et al., 2000; Fabritz et al., 2004; Kasi et al., 2007). The differential onset of α*Mhc* promoter expression during embryogenesis in the atria and at birth in the ventricles (Chizzonite et al., 1982; Sweeney et al., 1985; Bouvagnet et al., 1987; Colbert et al., 1997; Hein et al., 1997; Wei et al., 2004) might also mitigate a gene dosage effect across the heart.

Atrial fibrillation is a complex arrhythmia, and is likely to depend on multiple intertwined mechanisms. While the principal dysregulations in ion channel function and intracellular calcium homeostasis have been characterized, more recent genetic findings suggest that dysregulation of gene transcription and an imbalance in major regulatory pathways of cell function may contribute to the complex genesis of AF. Future challenges include the identification and investigation of the downstream components of these pathways and henceforth, the identification of therapeutic targets. The use of GWAS will lead to the development of new models of AF that better mimic human pathology from a different perspective. The differences between man and mice notwithstanding, murine models offer unique opportunities to investigate the functional and biochemical consequences of such complex regulatory changes, and to integrate these domains into our understanding of the genesis of AF.

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Induced pluripotent stem cell derived cardiomyocytes as models for cardiac arrhythmias

Maaike Hoekstra[1]*, Christine L. Mummery[2], Arthur A. M. Wilde[1], Connie R. Bezzina[1] and Arie O. Verkerk[3]

[1] *Department of Clinical and Experimental Cardiology, Heart Failure Research Center, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands*
[2] *Department of Anatomy and Embryology, Leiden University Medical Center, Leiden, Netherlands*
[3] *Department of Anatomy, Embryology, and Physiology, Heart Failure Research Center, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands*

Edited by:
Carol Ann Remme, University of Amsterdam, Netherlands

Reviewed by:
Teun P. De Boer, University Medical Center Utrecht, Netherlands
Isabelle Baro, CNRS, France

****Correspondence:***
Maaike Hoekstra, Department of Clinical and Experimental Cardiology, Heart Failure Research Center, Academic Medical Center, University of Amsterdam, room K2-113, Meibergdreef 15, PO Box 22700, 1100 DE Amsterdam, Netherlands.
e-mail: m.hoekstra@amc.uva.nl

Cardiac arrhythmias are a major cause of morbidity and mortality. In younger patients, the majority of sudden cardiac deaths have an underlying Mendelian genetic cause. Over the last 15 years, enormous progress has been made in identifying the distinct clinical phenotypes and in studying the basic cellular and genetic mechanisms associated with the primary Mendelian (monogenic) arrhythmia syndromes. Investigation of the electrophysiological consequences of an ion channel mutation is ideally done in the native cardiomyocyte (CM) environment. However, the majority of such studies so far have relied on heterologous expression systems in which single ion channel genes are expressed in non-cardiac cells. In some cases, transgenic mouse models have been generated, but these also have significant shortcomings, primarily related to species differences. The discovery that somatic cells can be reprogrammed to pluripotency as induced pluripotent stem cells (iPSC) has generated much interest since it presents an opportunity to generate patient- and disease-specific cell lines from which normal and diseased human CMs can be obtained These genetically diverse human model systems can be studied *in vitro* and used to decipher mechanisms of disease and identify strategies and reagents for new therapies. Here, we review the present state of the art with respect to cardiac disease models already generated using IPSC technology and which have been (partially) characterized. Human iPSC (hiPSC) models have been described for the cardiac arrhythmia syndromes, including LQT1, LQT2, LQT3-Brugada Syndrome, LQT8/Timothy syndrome and catecholaminergic polymorphic ventricular tachycardia (CPVT). In most cases, the hiPSC-derived cardiomyoctes recapitulate the disease phenotype and have already provided opportunities for novel insight into cardiac pathophysiology. It is expected that the lines will be useful in the development of pharmacological agents for the management of these disorders.

Keywords: induced pluripotent stem cells, cardiac arrhythmia syndromes, electrophysiology, cardiomyocytes, human, iPS, heart

INTRODUCTION

Cardiac arrhythmias can be life threatening and are a major cause of morbidity and mortality in developed nations (Wolf and Berul, 2008). In older patients, most arrhythmic sudden deaths occur in the setting of acute ischemia or coronary artery diseases (Zipes and Wellens, 1998). In younger patients, the great majority of sudden arrhythmic deaths have an underlying genetic cause (Wilde and Bezzina, 2005). These can be broadly subdivided into those associated with structural heart disease (such as hypertrophic cardiomyopathy) and those associated with electrical disease in the structurally normal heart (Wolf and Berul, 2008).

Over the last 15 years, much progress has been made in identifying the clinical phenotypes and cellular and genetic mechanisms underlying the various primary Mendelian arrhythmia syndromes, including the Long QT syndrome (LQTS), Brugada Syndrome (BrS), and catecholaminergic polymorphic ventricular tachycardia (CPVT) (Wilde and Bezzina, 2005). This has provided important insights into these disorders and as a consequence improved the management of affected patients. The availability of genetic tests has added an important diagnostic tool permitting early (presymptomatic) identification of patients at risk and allowing for the timely implementation of preventive strategies (Hofman et al., 2010). Studies into genotype–phenotype relationships have uncovered important gene-specific aspects of disease and indicated that patient management must take the nature of the gene affected into consideration (Priori, 2004). However, there is considerable variation in phenotypic expression of arrhythmia syndromes even within families carrying the same mutation (Scicluna et al., 2008).

Studying the electrophysiological and molecular consequences of a mutation associated with cardiac arrhythmia is ideally

done in the native cardiomyocyte (CM) environment. However, obtaining ventricular cardiac biopsies from patients is a highly invasive procedure and not without significant risk. Consequently the majority of functional studies on specific mutations associated with the Mendelian rhythm disorders have relied on heterologous expression systems, primarily *Xenopus oocytes*, human embryonic kidney (HEK) cells, and Chinese Hamster Ovary (CHO) cells (Watanabe et al., 2008), in which the mutated ion channel of interest is expressed. Such cellular models have significant shortcomings since they lack important constituents of cardiac ion channel macromolecular complexes that might be required to reproduce the exact molecular and electrophysiological phenotype of the mutation. For example, the behavior of the Na^+ channel in cell expression systems seems to be different from that in CMs (Remme et al., 2008). One way of overcoming this has been to generate transgenic mice carrying specific mutations (Sabir et al., 2008). However, the generation of such mouse models is costly and time-consuming and not practical for high-throughput screening of rare inherited arrhythmia mutations. Moreover, there remain crucial differences between mouse and human cardiac electrophysiological characteristics, such as the high basal heart rate (>500 bpm), the very negative action potential (AP) plateau phase, and the short AP compared of the mouse compared to human (Watanabe et al., 2011). These differences are amongst others due to the different biophysical properties in the transient outward currents (I_{to}) of human and mouse (for review, see Nerbonne and Kass, 2005).

The discovery of somatic cell reprogramming to generate induced pluripotent stem cells (iPSC) (Takahashi and Yamanaka, 2006) has created much excitement because of the possibility of producing unique patient- and disease-specific human iPSC (hiPSC) lines (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Yu et al., 2007). With this technique, somatic cells can be turned into embryonic stem cell-like cells which can differentiate into all cells of the human body and be propagated indefinitely in culture. Thus, hiPSC can provide investigators with genetically diverse human model systems to study mechanisms of disease and identify strategies for potential new therapies. Zhang et al. (2009) were the first to show that hiPSC can differentiate to functional CMs, making it possible to generate patient-specific human CMs which are by definition on different genetic backgrounds. hiPSC-derived CMs (hiPSC-CMs) therefore represent a new model system for studying Mendelian arrhythmia syndromes.

Here we provide a short overview of hiPSC generation, culturing, and differentiation methods. Further we will discuss in detail the electrophysiological characteristics of hiPSC-CMs and review hiPSC models for cardiovascular diseases, including LQT1, LQT2, LQT3/BrS, LQT8/Timothy syndrome, and CPVT.

DERIVATION OF hiPSC MODELS

CELL ORIGIN

Although the first hiPSC lines were derived from dermal fibroblasts (Takahashi et al., 2007) hiPSC can now be generated from a wide variety of somatic cells. It is important to consider easily accessible sources, which are efficient to reprogram and give minimal burden to the patient. Easily accessible sources used successfully for reprogramming include keratinocytes from skin or plucked hair (Aasen et al., 2008), peripheral blood (Loh et al., 2009), mesenchymal cells in fat (Sun et al., 2009), dental pulp (Tamaoki et al., 2010), and oral mucosa (Miyoshi et al., 2010).

CELL REPROGRAMMING

Somatic cells can be reprogrammed to a pluripotent state by introducing pluripotency-associated genes. The first iPSC reported were generated by transducing mouse fibroblasts with four retroviral vectors OCT4, SOX2, KLF4, and C-MYC (Takahashi and Yamanaka, 2006). The first hiPSC were generated using the same four retroviral vectors (Takahashi et al., 2007) or OCT4, SOX2, LIN28, and NANOG (Yu et al., 2007). Later studies reported reprogramming with other combinations and numbers of pluripotency factors. The hiPSC thus generated can be kept in culture indefinitely using a variety of undefined fibroblast feeder cells and fetal calf serum-based methods (Takahashi et al., 2007; Yu et al., 2007) or defined mTESr/Matrigel-based protocols. Transplantation of hiPSC into immune-compromised mice leads to the formation of teratomas with derivatives of the three embryonic germ layers, demonstrating the pluripotent potential of these cells (Takahashi et al., 2007; Yu et al., 2007). In addition, differentiation of hiPSC *in vitro* also results in derivatives of the three germ layers. The review by Narsinh et al. (2011) provides a detailed overview of the methods to reprogram somatic cells to iPSC and discusses the advantages and disadvantages of the different techniques.

GENERATION OF iPSC-DERIVED CARDIOMYOCYTES

When iPSC are removed from differentiation suppression conditions and/or when grown in suspension aggregates [called embryoid bodies (EBs)] spontaneous differentiation to cells of the three germ layers occurs. CMs originate from the mesodermal germ layer, so that CM differentiation first requires efficient differentiation toward the mesodermal lineage. Directed differentiation toward the cardiac lineage is mainly achieved by one of the following strategies: (1) The first involves the formation of EBs in the presence of growth factors and repressors known to influence heart development (Kehat et al., 2001); (2) The second relies on the influence of endoderm on cardiac differentiation during embryogenesis (Mummery et al., 2003). Here, co-culture of iPSC with mouse END-2 is used to produce CMs; (3) The third involves monolayer culture at high density of iPSC seeded on Matrigel with sequential treatment with activin A and BMP4 (Laflamme et al., 2007). This method was developed using human embryonic stem cells (hESC) but has been transferred to hiPSC. Beating areas from differentiated EBs usually appear in 7–10 days. These EBs can be microscopically dissected and dissociated in single cells. For electrophysiological and immunofluorescence analysis, the dissociated cells can be seeded onto glass coverslips.

CHARACTERISTICS OF hiPSC-DERIVED CARDIOMYOCYTES

MOLECULAR AND STRUCTURAL CHARACTERISTICS

The first hiPS-CMs were generated by Zhang et al. (2009). In these cells the investigators examined the gene expression of the transcription factor Nkx2.5, the myofilament proteins cardiac

troponin T, α-myosin heavy chain, α-actinin, the atrial and ventricular isoforms of myosin light chain 2, atrial natriuretic factor, and phospholamban (PLN). Low levels of cardiac troponin T and the atrial isoform of myosin light chain 2 were found in undifferentiated hiPSC and high expression of all the cardiac genes were found in the hiPSC-CMs, which was comparable to the expression of these genes in adult ventricular myocardium. Immunohistochemistry showed a typically striated pattern for α-actinin and myosin light chain. However, these cells had multiangular morphologies and relatively disorganized sacromeres (Dick et al., 2010). Novak and co-workers demonstrated by transmission electron microscopy analysis that hiPSC-CM had an immature ultra structure without t-tubuli (Novak et al., 2012).

ACTION POTENTIALS

Using the patch clamp technique, Zhang et al. (2009) were the first to measure the APs in spontaneously contracting cells isolated from hiPSC-EBs. The majority of the cells showed ventricular-like APs (70–74% of cells for two distinct hiPSC lines), but atrial-like and nodal-like APs were also observed. The distinction was made on AP phenotype, with a negative diastolic membrane potential, a rapid AP upstroke and a long plateau phase for ventricular-like APs. The absence of a prominent plateau phase was a characteristic of atrial-like APs, resulting in shorter AP duration compared to ventricular-like APs. Nodal-like APs showed a more positive maximum diastolic potential (MDP), a slower AP upstroke and a prominent phase 4 depolarization. Other studies also described ventricular-like, atrial-like, and sometimes nodal-like APs (Moretti et al., 2010; Fatima et al., 2011; Itzhaki et al., 2011a; Ma et al., 2011; Matsa et al., 2011; Jung et al., 2012; Lahti et al., 2012), with the ventricular-like phenotype being the most prominent AP form (76–48%) (Zhang et al., 2009; Moretti et al., 2010; Itzhaki et al., 2011a; Ma et al., 2011; Lahti et al., 2012). Comparison of hiPSC-CMs to hESC-CMs seems valuable, since hESC-CMs are more established. In hESC-CM, ventricular-like APs are also observed more frequently [50–60% (Zhang et al., 2009, 2011)]. Moretti et al. (2010) employed single-cell reverse-transcriptase-PCR in combination with patch-clamp in the same cell to show that the designation as ventricular-like, atrial-like, and nodal-like APs based on cellular electrophysiological features correlated with gene-expression of specific myocyte-lineage markers.

Table 1 summarizes the reported AP characteristics of hiPSC-CMs, hESC-CMs, and native ventricular CMs. The APs measured in hiPSC-CM differ from APs measured in freshly isolated native CMs (**Table 1**). The first remarkable difference is that most of the hiPSC-CMs studied, including the ventricular-like and atrial-like cells, are spontaneously active, with beating rates between 28 and 108 bpm (**Table 1**). Whether, in these studies, the spontaneous activity was used as a tool for CM selection and that non-beating CMs were also present, or whether it is a typical feature of hiPSC-CMs is unknown. We recently (Davis et al., 2012) performed experiments on non-spontaneously beating hiPSC-CM. For this we selected quiescent cells which were able to contract upon field stimulation. In these non-spontaneous beating hiPSC-CM, the resting membrane potential (RMP) was more negative then the MDP in most studies reporting on spontaneously active hiPSC-CMs (**Table 1**). Compared with native human ventricular CMs, where the reported RMP varies from –81.8 to –87 mV (**Table 1**), the MDP of ventricular-like hiPSC-CM APs is less negative with values ranging from –57 to –75 mV (**Table 1**). In spontaneously beating hiPSC-CM, the ventricular-like AP has a maximal upstroke velocity (dV/dt_{max}) ranging from 9 to 40 V/s which is slow compared to those non-spontaneously beating hiPSC-CM with a dV/dt_{max} of 115 V/s, and native ventricular CMs with a dV/dt_{max} of 215–234 V/s (Magyar et al., 2000).

Table 1 | Action potential characteristics in hiPSC-CM and human ventricular cardiomyocytes.

Cell type	BPM	APD_{90} (ms)	dv/dt_{max} (V/s)	APA (mV)	MDP/RMP (mV)	Exp. conditions	References
iPSC-CM V IMR90C4	43.8 ± 2.7	320.1 ± 17	40.5 ± 4.6	87.7 ± 2.6	−63.5 ± 1.7	37°C, me LJP: corr.	Zhang et al., 2009
iPSC-CM V ForeskinC1	44.2 ± 3.5	312.5 ± 11.2	27.2 ± 3.7	87.9 ± 2.4	−63.3 ± 1.5	37°C, me LJP: corr.	Zhang et al., 2009
iPSC-CM V	68.2 ± 2.7	381.3 ± 35.3	9 ± 0.2	107.8 ± 2.1	−63.5 ± 2.1	35°C, wc LJP:nk	Moretti et al., 2010
iPSC-CM V	72 ± 1.2	314.4 ± 17.6	26.8 ± 6.3	113.2 ± 2.4	−63.4 ± 1.3	36°C, pp LJP: nk	Lahti et al., 2012
iPSC-CM V	35.5 ± 2.1	414.7 ± 21.8	27.8 ± 4.8	101.5 ± 2.5	−75.6 ± 1.2	35–37°C pp, LJP: nk	Ma et al., 2011
iPSC-CM V	28 ± 5	495 ± 36	9.5 ± 1.8	109 ± 3	−57 ± 1	32°C, wc LJP: nk	Itzhaki et al., 2011a
iPSC-CM* ns	60	173.5 ± 12.2	115.7 ± 18.4	106 ± 3.2	−72.4 ± 0.9	37°C, pp LJP: corr	Davis et al., 2012
hESC-CM V	47.1 ± 23.3	247.2 ± 66.7	13.2 ± 6.2	85.4 ± 9.3	−53.9 ± 8.6	37°C, me LJP: nk	He et al., 2003
hESC-CM V		285.8 ± 52.6	11.4 ± 2.8	86.8 ± 52.6	−62.3 ± 8.6	rt, wc LJP: nk	Zhang et al., 2011
VM*	50	213 ± 7	215 ± 33	106.7 ± 1.4	−81.8 ± 3.3	37°C, wc LJP: nk	Magyar et al., 2000
VM endo*	60	330 ± 16	234 ± 28	105 ± 2	−87.1 ± 1	37°C, wc LJP: nk	Drouin et al., 1998
VM epi*	60	351 ± 14	228 ± 11	104 ± 2	−86 ± 1	37°C, wc LJP: nk	Drouin et al., 1998

*iPS-CM V, iPS-CM of ventricular-like phenotype; iPS-CM ns, not specified to action potential type; VM, native human ventricular myocyte; endo, endocardial; epi, epicardial; BPM, beats per minute; APD_{90}, action potential duration at 90% of repolarization; dV/dt_{max}, maximal upstroke velocity; APA, action potential amplitude; MDP, maximum diastolic potential; RMP, resting membrane potential; *, non spontaneous beating cells; hESC-CM V, hESC-CM of ventricular-like phenotype; me, micro electrode; wc, whole cell patch-clamp configuration; ljp: corr, corrected for liquid junction potential; ljp:nk, not known if corrected for liquid junction potential; rt, room temperature.*

The duration of ventricular-like hiPSC-CM APs, for example at 90% of repolarization (APD_{90}), is longer in spontaneous active cells (313–495 ms) compared to non-spontaneously active hiPSC-CMs (173 ms) and native freshly isolated CMs (213–351 ms) (**Table 1**). The AP amplitude (APA) for most ventricular-like hiPSC-CM APs (87–113 mV) is comparable to native ventricular CMs (104–106 mV), which due to the depolarized MDP in hiPSC-CM results in a higher overshoot of the hiPSC-CM AP. The direct comparison between the APs is complicated by the differences in experimental techniques used. Most hiPSC-CM studies used a temperature between 35 and 37°C; only in the study of Itzhaki was a temperature of 32°C used. This could be a possible explanation of the depolarized MDP, slow dV/dt_{max} and long APD_{90} in this latter study (Itzhaki et al., 2011a). The majority of the studies used the ruptured whole-cell patch-clamp technique, and three studies (Ma et al., 2011; Davis et al., 2012; Lahti et al., 2012) used the perforated patch-clamp technique. In the latter technique it is possible to perform experiments more close to physiological conditions since cell dialysis is minimal and EGTA, a buffer for Ca^{2+} ions, is absent.

MEMBRANE CURRENTS

The shape of the AP is the result of the various inwardly and outwardly directed ion currents present in the CM. A schematic overview of the different ionic membrane currents underlying the ventricular AP and their course is depicted in **Figure 1**. Because of the clear differences in AP shape between native CMs and hiPSC-CMs, one can assume that differences exist in the content and function of the various cardiac ion channels between the two cell types. Thus, before hiPSC-CMs can be used as a cell model in the study of cardiac arrhythmia syndrome, it is important to carry out a detailed comparison between the cardiac ion currents in hiPSC-CMs with those in native CMs. In the description of the cardiac ion currents below, a comparison between hiPSC-CMs displaying ventricular-like APs and healthy native human ventricular CMs is made, unless stated otherwise.

Sodium current

The cardiac Na^+ current (I_{Na}) is responsible for the AP upstroke in ventricular CMs [see (Berecki et al., 2010), and primary references cited therein]. Mutations in the genes encoding the α- and β-subunits of the cardiac Na^+ channel can alter the kinetics and availability of the cardiac Na^+ current (Remme et al., 2008). As stated before, the upstroke velocity in hiPSC-CM APs is extremely low compared to the AP upstroke of freshly isolated human ventricular CMs. In hiPSC-CM, I_{Na} was studied in detail in two reports (Ma et al., 2011; Davis et al., 2012). Ma et al. (2011) report a half-maximal potential ($V_{1/2}$) of activation and inactivation of –34.1 and –96.1 mV, respectively. Davis et al. (2012) report a $V_{1/2}$ of activation of ∼42 mV. The findings of these studies are consistent with values reported for native human ventricular CMs (Sakakibara et al., 1993) (**Table 2**). The low temperature and

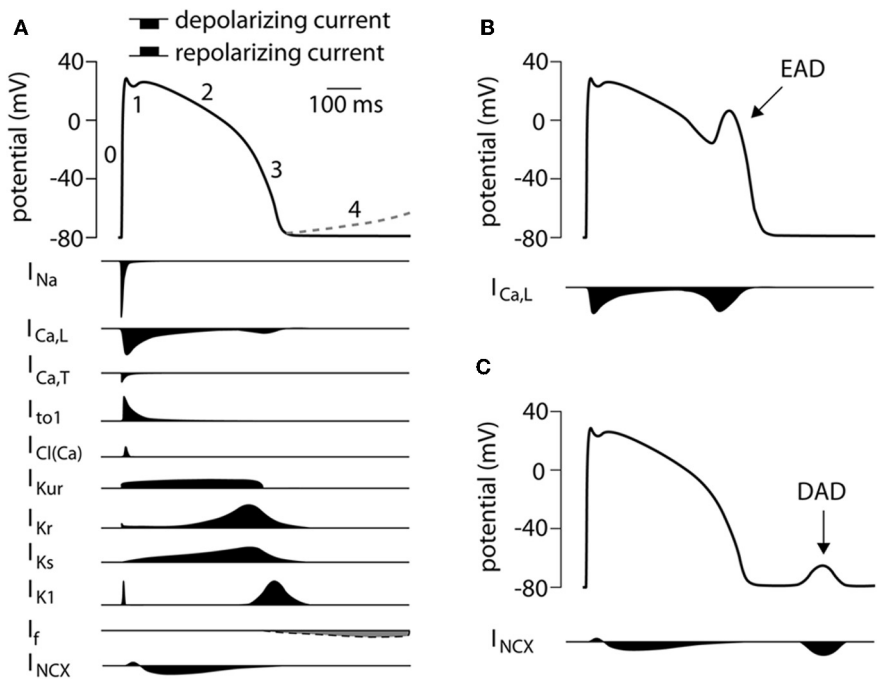

FIGURE 1 | (A) Schematic representation of a human ventricular action potential (top panel). Numbers denote the different phases of the ventricular action potential. The dashed line represents phase 4 depolarization normally present in cells from the conduction system and not in ventricular CMs. Underlying ionic membrane currents and their schematic time course are depicted below. **(B)** Schematic representation of an early afterdepolarization (EAD) and its underlying mechanism. **(C)** Schematic representation of a delayed afterdepolarization (DAD) and its underlying mechanism. I_{Na}, Na^+ current; $I_{Ca,L}$, L-type Ca^{2+} current; $I_{Ca,T}$, T-type Ca^{2+} current; I_{to1}, transient outward current type 1; $I_{Cl(Ca)}$, Ca^{2+} activated Cl^- current, also called I_{to2}; I_{Kur}, ultra rapid component of the delayed rectifier K^+ current, I_{Kr}, rapid component of the delayed rectifier K^+ current; I_{Ks}, slow component of the delayed rectifier K^+ current; I_{K1}, inward rectifier K^+ current; I_f, funny current; I_{NCX}, Na^+/Ca^{2+} exchange current.

Table 2 | Ion channel current and gating properties in hiPS-CM and human cardiomyocytes.

	Cell type	Peak current (pA/pF)	$V_{1/2}$ act (mV)	k act	$V_{1/2}$ inact (mV)	k inact	Exp. conditions	Reference
I_{Na}	iPS-CM	−216.7 ± 18.7	−34.1	5.9	−72.1	−5.7	35–37°C wc, LJP: nk	Ma et al., 2011
	iPS-CM	∼280 (Figure 8C)	∼ −42 (Figure 8D)				37°C, pp LJP: nc	Davis et al., 2012
	hESC-CM	−72 ± 21	−34	5.7	−78	−4.6	20°C, wc LJP: nk	Jonsson et al., 2012
	VM	−20.2 ± 2.2	−42.4 ± 3.0	6.3 ± 0.5	−100 ± 2.1	−6.3 ± 0.5	17°C, wc LJP: nk	Sakakibara et al., 1993
$I_{Ca,L}$	iPS-CM	−17.1 ± 1.7	−14.9	6.6	−29.1	−4.9	35–37°C wc, LJP: nk	Ma et al., 2011
	iPS-CM	−3.3	∼ −45 (Figure 2E)				25°C, wc LJP: nk	Yazawa et al., 2011
	hESC-CM	−4.3 ± 0.6	−12	5.5			37°C, wc LJP: nk	Jonsson et al., 2012
	VM	−10.2 ± 0.6	−4.7 ± 0.7	3.7 ± 0.3	−19.3 ± 1.2	−3.45 ± 0.6	37°C, wc LJP: nk	Magyar et al., 2000
	VM	−3.8 ± 0.5	−4.2 ± 0.8	7 ± 0.3	−23.5 ± 1.4	−5.5 ± 1.4	21–23°C wc, LJP: nk	Mewes and Ravens, 1994
	AM	−2.2 ± 0.3	−12.1 ± 1.3	5.8 ± 0.4	−26.8 ± 1.3	−5.7 ± 0.2	21–23°C wc, LJP: nk	Mewes and Ravens, 1994
I_{to1}	iPS-CM	∼2.4 (Figure 6D)					35–37°C wc, LJP: nk	Ma et al., 2011
	iPS-CM	∼30 (Suppl. Figure 10A)					35°C, wc LJP: nk	Moretti et al., 2010
	hESC-CM	6.0 ± 0.9					37°C, wc LJP: nk	Sartiani et al., 2007
	VM endo	∼5 (Figure 2B)	29.1 ± 1.2	12.9 ± 1.1	−17.6 ± 1.0	−8.9 ± 0.9	35°C, wc LJP: nk	Nabäuer et al., 1996
	VM epi	∼16 (Figure 2A)	32.0 ± 1.11	14.9 ± 0.8	−9.5 ± 0.38	−5.1 ± 0.4	35°C, wc LJP: nk	Nabäuer et al., 1996
	VM endo	2.3 ± 0.3	23.1 ± 4.2	12.9 ± 0.8	−25.3 ± 3.0	−4.7 ± 0.8	20–22°C wc, LJP: nk	Wettwer et al., 1994
	VM epi	7.9 ± 0.7	15.4 ± 0.7	9.7 ± 1.6	−31.9 ± 1.5	−4.6 ± 0.2	20–22°C wc, LJP: nk	Wettwer et al., 1994
I_{Kr}	iPS-CM	0.95 ± 0.02	−22.7	4.9			35–37°C wc, LJP: nk	Ma et al., 2011
	iPS-CM	∼1.9 (Figure 4A)					35°C, wc LJP: nk	Moretti et al., 2010
	iPS-CM	∼1.9 (Figure 5C)					36°C, pp LJP: nk	Lahti et al., 2012
	iPS-CM	∼0.55 (Figure 3D)					32°C, wc LJP: nk	Itzhaki et al., 2011a
	hESC-CM	−11.5 ± 1.8					37°C, wc LJP: nk	Jonsson et al., 2012
	VM	∼0.25 (Figure 1C)	−5.74	5.63			37°C, wc LJP: nk	Iost et al., 1998
	VM	0.31 ± 0.02					37°C, wc LJP: nk	Magyar et al., 2000
I_{Ks}	iPS-CM	0.31 ± 0.09					35–37°C wc, LJP: nk	Ma et al., 2011
	iPS-CM	∼2.5 (Figure 4A)					35°C, wc LJP: nk	Moretti et al., 2010
	hESC-CM	0.65 ± 0.12					37°C, wc LJP: nk	Jonsson et al., 2012
	VM	0.18					37°C, wc LJP: nk	Virag et al., 2001
I_{K1}	iPS-CM	∼ −3.8 (Figure 6C)					35–37°C wc, LJP: nk	Ma et al., 2011
	hESC-CM	−2.67 ± 0.3					37°C, wc LJP: nk	Jonsson et al., 2012
	VM	∼ −10 (Figure 3B)					37°C, wc LJP: nk	Magyar et al., 2000
I_f	iPS-CM	−4.1 ± 0.3	−84.6	8.8			35–37°C wc, LJP: nk	Ma et al., 2011
	hESC-CM	−10 ± 1.1	−74	4.5			37°C, wc LJP: nk	Jonsson et al., 2012
	VM	−1.18 ± 0.21	−111 ± 0.06				22°C, wc LJP: nk	Hoppe et al., 1998
	SAN	∼ −8.0 (Figure 3B)	−96.9 ± 2.7	8.8 ± 0.5			36°C, wc LJP: corr	Verkerk et al., 2007

VM, native human ventricular myocyte; AM, native human atrial myocyte; endo, endocardial; epi, epicardial; SAN; native sinoatrial node myocyte; ∼, estimated from figure. I_{Na}, Na^+ current; $I_{Ca,L}$, L-type Ca^{2+} current; I_{to1}, Transient outward current type 1; I_{Kr}, rapid compontent of the delayed rectifier K^+ current; I_{Ks}, slow component of the delayed rectifier K^+ current; I_{K1}, inward rectifier K^+ current; I_f, funny current; $V_{1/2}$ act, potential of half maximal activation; k act, slope factor of activation curve; $V_{1/2}$ inact, potential of half maximal inactivation; k inact, slope factor of inactivation curve hESC-CM; V, hESC-CM of ventricular-like phenotype; wc, whole cell patch-clamp configuration; ljp: corr, corrected for liquid junction potential; ljp: nk, not known if corrected for liquid junction potential, ljp: nc, not corrected for liquid junction potential.

reduced Na^+ concentration used to study the maximal peak I_{Na} in native human ventricular CM (Sakakibara et al., 1993) prevents comparison with the maximal peak I_{Na} measured in hiPSC-CM (Ma et al., 2011; Davis et al., 2012). Other I_{Na} characteristics, such as recovery from inactivation and slow inactivation have not been reported to date. In the presence of the Na^+ channel blocker tetrodotoxin (TTX) the upstroke of the AP in hiPSC-CMs is delayed and the dV/dt_{max} is reduced (Ma et al., 2011). In hESC-derived CMs (hESC-CM) the Na^+ channel blocker lidocaine also reduced the spontaneous beating rate (Kuzmenkin et al., 2009). Whether I_{Na} plays a role in spontaneous activity in hiPSC-CM is unknown. However, hiPSC-CMs have prominent Na^+ currents with characteristics close to that of native human ventricular CMs, despite any information to compare maximal peak I_{Na}, it seems that the low dV/dt_{max} of spontaneously active ventricular-like hiPSC-CM APs seems thus due to lower functional availability of Na^+ channels (related to the relative positive value of the RMP) rather than differences in I_{Na} density.

Calcium current

Two types of Ca^{2+} current exist in the mammalian heart, i.e., the L-type ($I_{Ca,L}$) and T-type ($I_{Ca,T}$) Ca^{2+} current [for review, see (Nerbonne and Kass, 2005)]. Patch clamp studies have demonstrated the presence of the $I_{Ca,L}$ in hiPSC-CM with a $V_{1/2}$ of activation and inactivation of –15 and –29 mV, respectively (Ma et al., 2011). These values are more comparable to those found in native atrial CMs (–12 and –27 mV, for $V_{1/2}$ of activation and inactivation, respectively) (Mewes and Ravens, 1994) than native ventricular myocytes (–4.2 and –4.7 mV and –23.5 and –19.3 mV) (Mewes and Ravens, 1994; Magyar et al., 2000). The maximal peak $I_{Ca,L}$ in hiPSC-CM reported by Ma et al. (2011) is 16.4 pA/pF, which is much higher than the 3.3 pA/pF reported in hiPSC-CM by Yazawa et al. (2011). In native ventricular CMs the amplitudes of the maximal current density varies between 2.2 and 10.2 pA/pF (Mewes and Ravens, 1994; Magyar et al., 2000) The higher maximal peak $I_{Ca,L}$ in the study of Ma et al. (2011) may be explained by the higher extracellular Ca^{2+} and higher temperatures used in their experiments. Blocking of the $I_{Ca,L}$ with nifedipine results in shortening of the AP duration and field potential duration (FDP) with minimal effects on dV/dt_{max} (Itzhaki et al., 2011a; Ma et al., 2011). Long-term application of nifedipine resulted in cessation of beating in some EBs (Itzhaki et al., 2011a).

Functional presence of the $I_{Ca,T}$ has not been reported in hiPSC-CM. Ma et al. (2011) did not find clear evidence for its presence. $I_{Ca,T}$ is present in the human heart conduction system, where it plays a role in facilitation of pacemaker depolarization, but is not functionally present in healthy human native ventricular CMs (Ono and Iijima, 2010). The T-type Ca^{2+} channels are re-expressed in atrial and ventricular CMs under pathological conditions such as cardiac hypertrophy and heart failure (Ono and Iijima, 2010).

Transient outward current

Two transient outward current components are found in native mammalian cardiac cells, one carried by K^+(I_{to1}), the other by Cl^- ions (I_{to2}) [for review, see (Nerbonne and Kass, 2005)]. While it is not yet known whether I_{to2} is present in hiPSC-CMs, native human ventricular CMs are known to lack I_{to2} (Verkerk et al., 2001). On the otherhand, I_{to1} has been found in hiPSC-CMs (Moretti et al., 2010; Ma et al., 2011), but data about gating properties are not known. Reported peak current densities of I_{to1} in hiPSC-CM display large variation, namely between 2.4 (Ma et al., 2011) and 30 pA/pF (Moretti et al., 2010), both at 60 mV. Values reported for native ventricular CMs vary between 2.3 and 16 pA/pF (Wettwer et al., 1994; Nabäuer et al., 1996). Although an exact comparison of I_{to1} density between hiPSC-CM and native human CMs is hampered by differences in experimental conditions, I_{to1} current density also depends on the site from which the native ventricular CMs are isolated, with larger current densities reported in human subepicardial ventricular myocytes compared to endocardial ventricular CMs (Beuckelmann et al., 1993; Wettwer et al., 1994).

Studies of I_{to1} block on hiPSC-CM APs have not yet been performed. However, due to the depolarized MDP values in hiPSC-CM, I_{to1} function may be limited because most channels will be inactivated (Varro and Papp, 1992). Further studies are required to address the function of I_{to1} in hiPSC-CM in detail.

The delayed rectifier potassium current

In the mammalian heart, the delayed rectifier K^+ current (I_K) is composed of three different components: the ultrarapid (I_{Kur}), the rapid (I_{Kr}), and the slow (I_{Ks}) components [for review, see (Nerbonne and Kass, 2005)]. To our knowledge, studies to elucidate the presence and function of I_{Kur} in hiPSC-CMs are lacking.

I_{Kr} has been reported in hiPSC-CM with a maximal I_{Kr} density varying between 0.55 and 1.9 pA/pF (Moretti et al., 2010; Itzhaki et al., 2011a; Ma et al., 2011; Lahti et al., 2012), values comparable to those in native human CMs where I_{Kr} densities are between 0.25 and 0.6 pA/pF (Iost et al., 1998; Magyar et al., 2000; Jost et al., 2009). $V_{1/2}$ of activation is –22.7 mV (Ma et al., 2011) in hiPSC-CM and –5.74 mV in native ventricular CMs (Iost et al., 1998) and –14 mV in atrial CMs (Wang et al., 1994). In hiPSC-CMs, blockade of I_{Kr} by E4031 resulted in a significant AP prolongation In addition, E4031 induced early afterdepolarizations (EADs) (Itzhaki et al., 2011a; Ma et al., 2011; Matsa et al., 2011; Lahti et al., 2012), that is afterdepolarization during the AP (**Figure 1B**) due to the reactivation of $I_{Ca,L}$ (Verkerk et al., 2000b). Thus, I_{Kr} plays a prominent role in the repolarization phase of hiPSC-CM APs. Interestingly, in hESC-CM E4031 reduced the beating rate (Kuzmenkin et al., 2009) suggesting that I_{Kr} also plays a role in spontaneous activity.

The presence of I_{Ks} in hiPSC-CMs has been reported in two studies. Ma et al. (2011) found I_{Ks} in 5 out of 16 cells studied, and when present the average I_{Ks} density was 0.31 pA/pF. In contrast, Moretti et al. (2010) measured I_{Ks} in all studied cells and the average density was around 2.5 pA/pF [estimated from Figure 4A (Moretti et al., 2010)]. In native left ventricular human CMs, Virag et al. (2001) identified I_{Ks} in 31 out of 58 cells and the maximal current density was approximately 0.18 pA/pF. In hiPSC-CMs, blockade of I_{Ks} by chromanol 293B results only in minimal prolongation of the AP (Ma et al., 2011). This is consistent with the relative small number of cells exhibiting I_{Ks} and

the small I_{Ks} densities found in their study, but contrast with the effects of a loss-of-function I_{Ks} mutation which results in a prominent AP prolongation (see paragraph "LQT1"). A study by Wang et al. (2011) in hESC-CMs suggests altered expression of the β-subunit mink encoded by the *KCNE1* gene, as a mechanism for variable I_{Ks} function in the developing heart and in disease.

Inward rectifier current

In atrial and ventricular CMs, the inward rectifier K^+ current (I_{K1}) is an important contributor to the maintenance of the RMP and contributes to the terminal phase of repolarization (Dhamoon and Jalife, 2005). I_{K1} was found to be present in hiPSC-CM (Ma et al., 2011). I_{K1} density in hiPSC-CM is four times smaller than that reported in native ventricular CMs, 0.9 (Ma et al., 2011) and 3.6 pA/pF (Magyar et al., 2000), respectively. In hESC-CMs I_{K1} is significantly increased in longer cultured hiPSC-CMs, these cells also displayed a flattened diastolic depolarization rate and decreased spontaneous activity (Sartiani et al., 2007).

The small I_{K1} densities in hiPSC-CMs may explain the frequently observed spontaneous activity in these cells. However, whether all hiPSC-CMs have a low I_{K1} density or whether a bias is introduced by the selection of spontaneously active cells for patch-clamp needs to be elucidated.

The acetylcholine-activated K^+ current

The acetylcholine-activated K^+ current ($I_{K,ACh}$) is involved in parasympathetic regulation of heart rate (Tamargo et al., 2004). $I_{K,ACh}$ is to our knowledge not yet studied in hiPSC-CM. Studies addressing the presence of $I_{K,ACh}$ in hiPSC-CM might be particularly important in modeling atrial arrhythmias, as blockers of $I_{K,Ach}$, which leave ventricular repolarization intact, are effective in the treatment of atrial fibrillation (Hashimoto et al., 2006).

The ATP-sensitive K^+ current

The ATP-sensitive K^+ current ($I_{K,ATP}$) has not been studied in detail in hiPSC-CMs. However, the $I_{K,ATP}$ channel openers nicorandil and pinacidil shorten the AP in hiPSC-CMs (Itzhaki et al., 2011a; Matsa et al., 2011), suggesting that $I_{K,ATP}$ channels are present in these cells. Further studies are required to address the presence and function of $I_{K,ATP}$ in hiPSC-CM in detail.

The hyperpolarization-activated "funny" current

The funny current (I_f) is an inward current activating at hyperpolarized membrane potentials [for review, see (Verkerk et al., 2007)]. In human sinoatrial node cells, the current density of I_f at a membrane potential of –130 mV is reported to be 8 pA/pF (Verkerk et al., 2007). I_f is also described in human atrial CMs (El Chemaly et al., 2007) and in human ventricular CMs during heart failure [(Hoppe et al., 1998)]. The current densities reported, however, are much smaller, compared to human sinoatrial node cells. hiPSC-CMs also exhibit I_f (Ma et al., 2011) and the reported current density is 4.1 pA/pF (Ma et al., 2011). The relatively high I_f density in hiPSC-CMs compared to human ventricular CMs might be attributed to the fact that these cells express higher levels of the HCN isoforms (HCN1, 2, 4) as compared to adult human CMs. (Synnergren et al., 2012). In hiPSC-CMs, I_f starts to activate at potentials negative of –60 mV and has a $V_{1/2}$ of activation of –84 mV (Ma et al., 2011) and may therefore have a role in spontaneous activity in these cells. hESC-CMs have comparable characteristics of I_f, and in these cells blockade of I_f with zatebradine resulted in slowing of spontaneous activity due to a reduced diastolic depolarization rate (Sartiani et al., 2007).

Na^+-Ca^{2+} exchange current

The Na^+-Ca^{2+} exchange current (I_{NCX}) is crucial for Ca^{2+} extrusion from the cell and plays a role in the electric activity of mammalian CMs (Sipido et al., 2007). While the functional properties of I_{NCX} have not been studied in hiPSC-CM, the presence of the Na^+-Ca^{2+} exchanger in hiPSC-CM has been demonstrated at the level of the protein (Lee et al., 2011). I_{NCX} is present in hESC-CM and its density increases with maturation (Fu et al., 2010).

In human CMs, Ca^{2+} extrusion by the Na^+/Ca^{2+} exchanger is the major mechanism to balance the Ca^{2+} influx through the $I_{Ca,L}$ (Sipido et al., 2007). In addition, the Na^+/Ca^{2+} exchanger has a function during depolarization where it contributes in its reverse mode (Ca^{2+} influx) to the total amount of Ca^{2+} influx. The amplitude of the I_{NCX} depends on the membrane potential and the intracellular levels of Na^+ ($[Na^+]_i$) and Ca^+ ($[Ca^{2+}]_i$) (Sipido et al., 2007). Altered $[Na^+]_i$ and/or $[Ca^{2+}]_i$ will lead to altered I_{NCX} and can cause cardiac arrhythmias due to spontaneous Ca^{2+} releases from the SR. Studying the I_{NCX} in hiPSC-CM might be of particular interest in cardiac arrhythmia models of CPVT and LQT3. CPVT is associated with mutations in the RyR2 which can lead to increased $[Ca^{2+}]_i$ due to altered gating properties of the RyR2 receptor. In LQT3 syndrome there is an increased persistent I_{Na} (Remme et al., 2006), which might lead to elevated levels of $[Na^+]_i$.

EXCITATION-CONTRACTION COUPLING

hiPSC-CMs display clearly visible contractions. In native adult CMs, a small influx of Ca^{2+} through the L-type Ca^{2+} channels triggers a several-fold multiplied Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyRs). This phenomenon is referred to as "$Ca^{2+}-$induced Ca^{2+} release" (CICR) (Lee et al., 2011). CICR is the key mechanism underlying excitation-contraction coupling. The key Ca^{2+} handling proteins, RyR2, SR Ca^{2+}-ATPase (SERCA), junctin (Jun), triadin (TRDN), Na^+/Ca^{2+} exchanger (NCX), calsequestrin (CASQ2), L-type Ca^{2+} channel ($Ca_v1.2$), inositol-1,4,5-trisphosphate receptor (IP3R2) and PLN are expressed in hiPSC-CM (Itzhaki et al., 2011b; Lee et al., 2011). Spontaneous rhythmic Ca^{2+} transients are present in hiPSC-CM, and blocking of the $I_{Ca,L}$ by nifedipine, abolishes Ca^{2+} transients (Itzhaki et al., 2011b). The presence of functional SR and RyRs was proven by application of caffeine, which induced a large Ca^{2+} transient (Itzhaki et al., 2011b; Lee et al., 2011), consistent with findings in human ventricular CMs (Piacentino et al., 2003). In addition, ryanodine caused a reduction in the amplitude of the Ca^{2+} transient (Itzhaki et al., 2011b; Lee et al., 2011). The pattern of Ca^{2+} transient in hiPSC-CM was studied by transverse line-scan images and revealed a U-shape Ca^{2+} wavefront (the rise of Ca^{2+} in the periphery is faster than in the center of the cell), which is typical for t-tubule deficient cells (Lee et al., 2011). This suggests that hiPSC-CMs lack t-tubuli, an

observation which is in line with that of Novak and co-workers (Novak et al., 2012) who did not find t-tubuli with transmission electron microscopy. This would mean that hiPSC-CMs likely have poor coupling between Ca^{2+} influx through L-type Ca^{2+} channels and Ca^{2+} release from the SR through RyRs.

HUMAN iPSC-CM MODELS FOR INHERITED CARDIAC ARRHYTHMIAS

hiPSC-CM MODELS FOR LQTS

LQT1

Moretti and co-workers (Moretti et al., 2010) were the first to publish on a hiPSC-CM model for a primarily electrical disease, namely LQT1. LQT1 is a repolarization disorder identified by a prolongation of the QT interval on the ECG due to mutations in the *KCNQ1* gene, encoding the α subunit of the K^+ channel responsible of I_{Ks} (Wilde and Bezzina, 2005). They investigators obtained fibroblasts from two, so far, asymptomatic patients, with the *KCNQ*1-G569A mutation and two healthy controls (Moretti et al., 2010). These fibroblasts were infected with retroviruses encoding OCT3/4, SOX2, KLF4, and c-MYC; hiPSC-CMs were differentiated as EBs. In this study AP characteristics and K^+ currents were investigated in spontaneously beating cells. Three different types of AP s were distinguished, that were designated as ventricular-, atrial-, and nodal-like. These investigators also correlated these characteristics with gene-expression analysis of specific myocyte-lineage markers (MLC2v, MLC2a, and HCN4 for ventricular-, atrial- and nodal-like cells, respectively). The delayed rectifier currents were studied in ventricular-like myocytes. In hiPSC-CMs derived from the LQT1 patient (LQT1-iPSC-CM), I_{Ks} peak and tail current densities were reduced by approximately 75%, and I_{Kr} conductance was unaffected. APs of atrial-like and ventricular-like hiPSC-CMs were significantly prolonged in LQT1-iPSC-CMs compared to control (WT-iPSC-CMs). Adaptation of the AP duration to higher pacing frequencies and the response to isoproterenol were impaired in LQT- iPSC-CM. EADs were elicited in response to isoproterenol (a β-adrenergic agonist) in 6 out of 9 LQT1-iPSC-CM and never in WT-iPSC-CM. When propranolol (a non-selective β-blocker) was applied the effect of isoproterenol was blunted. These data are in line with observations in LQT1 patients as these patients suffer from arrhythmias during increased heart rates caused by emotional stress or exercise. The data is also in line with the beneficial effects of β-blockers in suppressing arrythmias in these patients (Ruan et al., 2008). Immunocytochemistry revealed that the *KCNQ*1-G569A mutation leads to impaired trafficking and localization of the mutant channels.

LQT2

Three groups have published on hiPSC-CM models of LQT2 (Itzhaki et al., 2011a,b; Matsa et al., 2011; Lahti et al., 2012). LQT2 is a repolarization disorder caused by mutations in *KCNH2*, encoding I_{Kr} channels (Wilde and Bezzina, 2005).

Itzhaki et al. (2011a) reported on a hiPSC-CMs model generated from dermal fibroblasts obtained from a 28-year-old woman with a diagnosis of familial LQT2 due to the *KCNH2*-A614V mutation. Clinical data of the patient were not shown. Fibroblasts were reprogrammed by retroviral infection with vectors encoding for SOX-2, KLF4, and OCT4. In this study EB formation was used for differentiation of the hiPSC into CMs. In this study APs were measured from spontaneously contracting clusters and the hiPSC-CMs were classified as nodal-, atrial-, and ventricular-like. Prolongation of repolarization and predisposition to the development of EADs was shown in cells with atrial- and ventricular-like APs. For voltage clamp experiments the spontaneously beating clusters were dissociated to single cells. Peak amplitudes of I_{Kr} were found to be significantly smaller in LQT2-iPSC-CM compared to WT-iPSC-CM. FDP corrected for variations in beating frequency were longer in LQT2-iPSC-CM compared to WT-iPSC-CM. When I_{Kr} was blocked by the hERG blocker E4021, the AP prolonged and EADs were seen in 66% of the cells studied. Furthermore, the effects of agents that may have a therapeutic effect in preventing arrhythmias were also studied. These agents include nifedipine, pinacidil, and ryanodine. Because Ca^{2+} influx through L-type Ca^{2+} channels contributes to AP duration and has a role in EAD formation, inhibition of $I_{Ca,L}$ by nifedipine was proposed to be anti-arrhythmic. Another anti-arrhythmic strategy proposed was to augment the repolarization currents, by the $I_{K,ATP}$ channel opener pinacidil. Both interventions resulted in AP shortening and abolished propensity to EADs. This study also demonstrated that ranolazine, a blocker of the persistent I_{Na}, did not shorten the AP duration, but prevented EADs.

Matsa et al. (2011) generated hiPSC-CMs of a symptomatic and an asymptomatic carrier of the G1681A mutation in *KCNH2*. The symptomatic patient, female with a QTc interval of up to 571 ms, experienced 11 episodes of syncope in 12 months. As is typical for LQT2, episodes occurred at arousal from sleep and not during competitive sports. Her mother was the asymptomatic individual studied; although her QTc-interval was prolonged she had not experienced any symptoms. The hiPSC-CMs were derived from punch biopsies of skin, which were reprogrammed by lentiviral delivery of OCT4, SOX2 NANOG, and LIN28; EB formation was used for differentiation of cells into CMs. I_{Kr} current characteristics were not studied. The derived hiPSC-CMs showed APs which were categorized as ventricular-, atrial- and pacemaker-like. Ventricular- and atrial-like APs from the symotomatic patient and her mother showed increased durations compared to APs of the genetically unrelated control; AP duration was less in the maternal hiPSC-CMs compared to those of the patient. Application of isoprenaline resulted in 25% of LQT2-iPSC-CMs in electrophysiological abnormalities, including EADs. Isoprenaline-induced arrhythmias were ameliorated by nadolol or propanolol, non-selective β-blockers. This is in line with the clinical picture in LQT2 as these patients experience arrhythmias due to increased heart rates caused by emotional and similar stress, mainly auditory stimulation and arousal from sleep. As for LQT1, LQT2 patients are often treated with β-blockers for prevention of cardiac events (Ruan et al., 2008). I_{Kr} blockade by E4031 resulted in prolongation of the AP duration and EADs in 30% of the LQT2-iPSC-CMs, but never in control cells. Nicorandil, an $I_{K,ATP}$ channel opener, and PD-118057, an I_{Kr} channel enhancer, shortened the AP in LQT2-iPSC-CM, showing that potassium channel activators can normalize the prolonged repolarization in LQT2.

Lahti et al. (2012) derived hiPSC-CMs from an asymptomatic carrier of the R176W mutation in the *KCNH2*. This mutation is one of the four founder mutations of LQTS cases in Finland, and present in one in 400 Finns (Marjamaa et al., 2009). The QTc intervals of patients carrying the R176W mutation range from 386 to 569 ms, with a mean of 448 ms (Fodstad et al., 2006). In the study of Lahti et al. (2012), fibroblasts were infected with lentivirus followed by retroviruses encoding for OCT4, SOX2, KLF4, and MYC to generate iPSC-CMs. CM differentiation was achieved by co-culturing hiPSC with END-2 cells. APs were divided into two types, atrial- and ventricular-like APs. Only the ventricular-like APs showed significantly increased APD_{90}. The AP frequency had a tendency toward slower frequencies in LQT2-iPSC-CM. EADs were present in 1 of 20 LQT2 iPSC-CMs and were never observed in WT-iPSC-CMs. I_{Kr} step and tail current densities were reduced by 40–46%. The APs of LQT2-iPSC-CMs had a significantly prolonged duration compared to WT AP, especially at low frequencies. The I_{Kr} blocker E4031 provoked EADs in WT-iPSC-CM and LQT2-iPSC-CM, with the effect on LQT2-iPSC-CMs being more pronounced. Sotalol, a non-selective β-blocker elicited EADs only in LQT2-iPSC-CM.

These three studies on LQT2 show that LQT-iPSC-CMs of symptomatic patients show a more severe cellular phenotype than those obtained from asymptomatic patients with the same mutation. However, assessing severity in the hiPSC-CMs system is challenging. For instance, blocking I_{Kr} by E-4031 in WT-iPSC-CMs leads to different findings in different studies. In the study of Matsa et al. (2011) no EADs were provoked by the application of E4031, whereas in the study of Ma et al. (2011) and Lahti et al. (2012) EADs could be provoked in >50% of WT-iPSC-CM. This might reflect diversity of hiPSC-CM lines. However, the differences between the outcomes of these studies might also be caused by the use of different concentrations E-4031. Ma et al. (2011) and Lahti et al. (2012) used a concentration of 100 nmol/l and 500 nmol/l, respectively. The concentration used by Matsa et al. (2011) is not known.

LQT3/Conduction disease/BrS

LQT3 is a repolarization disorder caused by gain-of-function mutations in *SCN5A* encoding the cardiac Na^+ channel. These mutations cause an increased persistent Na^+ current which acts to prolong CM repolarization and increase AP duration (Wilde and Bezzina, 2005). On the otherhand, SCN5A mutations associated with loss of channel function cause conduction disease and Brugada Syndrome (BrS). The latter is an arrhythmia syndrome characterized by ST segment elevation in the right precordial leads of the EGC; SCN5A mutations account for around 20% of BrS cases (Wilde and Bezzina, 2005). Loss of Na^+ channel function leads to a decreased peak I_{Na}, which causes slowing of the upstroke velocity of the AP.

Recently, we have, generated an iPSC-CM model of a patient carrying the *SCN5A*-1795insD mutation (Davis et al., 2012). This mutation gives rise to a phenotype of LQT3 as well as BrS and conduction defects, caused by both gain- and loss-of-function effects on the cardiac Na^+ channel, respectively (Remme et al., 2006, 2009). In this study we generated hiPSC-CMs by transducing fibroblasts with lentiviral vectors encoding OCT4, SOX2, KLF4 and C-MYC (Davis et al., 2012). Cardiac differentiation was induced by co-culture with END-2 cells. In line with the known effects of the mutation in a knock-in mouse model, and in line with the clinical presentation in mutation carriers, we observed a decrease in peak I_{Na} and an increase in persistent I_{Na} in the hiPSC-CMs with *SCN5A*-1795insD compared to genetically unrelated control. APs measured in non-spontaneously active hiPSC-CMs displayed a reduced upstroke velocity and a prolonged duration compared to those derived from a genetically unrelated control.

LQT8/Timothy syndrome

LQT8 and Timothy syndrome are caused by mutation in the *CACNA1C* gene encoding the L-type Ca^{2+} channel. Repolarization disease is only one facet of LQT8 as *CACNA1C* mutations also give rise to other features including syndactyly, heart malformations, and autism spectrum disorders (Yazawa et al., 2011). Yazawa et al. (2011) studied hiPSC-CMs of two patients with LQT8. Fibroblasts were isolated from skin biopsies and were reprogrammed using four retroviruses containing SOX2, OCT3/4, KLF4, and MYC. EBs were used in the generation of hiPSC-CMs. EBs from LQT8/Timothy syndrome hiPSC lines contracted at 30 bpm, whereas control hiPSC-line EBs contracted at a rate of 60 bpm. The LQT8-iPSC-CM showed delay in inactivation of $I_{Ca,L}$ and abnormalities in intracellular Ca^{2+} handling, with larger and prolonged Ca^{2+} transients. Importantly, such aspects of $I_{Ca,L}$ mutations can not be revealed when studying the mutation in a heterologous cell system. The APs of LQT8/Timothy syndrome ventricular-like hiPSC-CMs were three times longer than those of WT hiPSC-CMs. Roscovitine, a compound that increases the voltage-dependent inactivation of the voltage dependent Ca^{2+} channel, reverted the delayed inactivation and restored the irregular Ca^{2+} transients associated with LQT8/Timothy syndrome.

CATECHOLAMINERGIC POLYMORPHIC VENTRICULAR TACHYCARDIA

CPVT is characterized by cateholamine/stress-induced ventricular arrhythmias that can lead to sudden cardiac death in young individuals (Priori and Chen, 2011). CPVT is linked to mutations in the *RYR2* gene, encoding an intracellular Ca^{2+} release channel, and mutations in *CASQ2*, encoding a calcium binding protein in the SR which stores Ca^2. RyR2 and CASQ2 play a role in Ca^{2+} cycling and contractile activity of the CM (Priori and Chen, 2011). To date, three groups have published a CPVT hiPSC model (Fatima et al., 2011; Jung et al., 2012; Novak et al., 2012).

Fatima et al. (2011) studied the F243I mutation in the *RYR2* gene. A skin biopsy of a patient with CPVT carrying the F243I mutation in the *RYR2* gene was taken and fibroblasts derived from this biopsy were infected with retroviruses encoding OCT3/4. SOX2, KLF4, and c-MYC. Cardiac differentiation was achieved by co-culturing with END-2 cells. APs were measured in spontaneously beating single hiPSC-CMs and were categorized as ventricular-, atrial-, and nodal-like APs. Isoproterenol was used to evoke the phenotype. In 22 out of 38 CPVT-iPSC-CMs isoprenaline resulted in a negative chronotropic response and 13 cells

exhibited delayed afterdepolarizations (DADs), which are afterdepolarizations after and AP (**Figure 1C**) due to spontaneous SR Ca^{2+} release what activates I_{NCX} (Verkerk et al., 2000a). All control hiPSC-CM showed normal positive chronotropic response. Confocal fluorescence imaging revealed spontaneous local Ca^{2+} release events of higher amplitude and longer duration in CPVT-iPS-CMs. In addition, the CPVT-iPSC-CMs showed a decrease in $I_{Ca,L}$ and Ca^{2+} transients in the presence of forskolin, an adenyl cyclase activator. As the authors state, this is likely due to the large and sustained rise of intracellular Ca^{2+} concentration.

Jung et al. (2012) studied the *RYR2* mutation S406L. Fibroblasts of the patient were transduced with a retroviral vector encoding SOX2, OCT4, KLF4, and c-MYC. To direct the hiPSCs to the cardiac lineage, EB differentiation was used. The CPVT-iPSC-CM showed elevated Ca^{2+} concentrations, a reduced SR Ca^{2+} content, and increased susceptibility to DADs under catecholaminergic stress induced by isoproterenol. Further the authors investigated the ability of dantrolene to rescue the disease phenotype. Dantrolene is a hydrantoin derivative and muscle relaxant, currently used as therapy in cases with malignant hyperthermia, a disorder caused by mutations in the skeletal ryanodine receptor (*RYR1*) (Kobayashi et al., 2009). Dantrolene, restored normal Ca^{2+} spark properties and the arrhythmogenic phenotype.

Novak et al. (2012) studied the effect of the autosomal recessive missense mutation D307H in the *CASQ2* gene. Dermal fibroblasts of two mutation carriers were transduced with a single lentiviral vector containing OCT4, SOX2, KLF4, and c-MYC. Differentiation toward hiPSC-CMs was achieved by EB formation. Spontaneous beating rate of differentiated EBs was significantly lower in CPVT-iPSC-CMs (∼26 bpm) compared to control iPSC-CMs (∼39 bpm). Isoproterenol induced DADs, oscillatory arrhythmic prepotentials (diastolic voltage oscillation, which appear during the late diastolic depolarization) and increased $[Ca^{2+}]_i$.

CONCLUSIONS AND FUTURE PERSPECTIVES

The hiPSC-CM models described in this review show that it is possible to recapitulate *in vitro* in the hiPSC-CM system the disease phenotype of patients with Mendelian cardiac rhythm disorders. Furthermore, different studies have shown that LQT-iPSC-CMs of symptomatic patients show a more severe cellular phenotype than those obtained from asymptomatic patients with the same mutation (Itzhaki et al., 2011a; Matsa et al., 2011; Lahti et al., 2012). Moreover, hiPSC-CMs can recapitulate a phenotype that cannot be shown in a heterologous expression system (Lahti et al., 2012). For example, Lahti et al. (2012) reported a decrease of ∼43% in I_{Kr} density in LQT2- iPSC-CMs with the R176W mutation, which was not revealed in a heterologous expression system. This might reflect, amongst others, differences in cellular environment between the two cell systems or may be due to the effect of high transgene expression as a consequence of the use of a strong promoter in the heterologous expression system. A significant advantage of the hiPSC-CM system is that in this system, in contrast to heterologous expression systems, it also possible to study the effects on the AP and Ca^{2+} cycling.

The responses to some pharmacological agents are studied in hiPSC-CMs. The results are in line with what is seen in patients, healthy human beings, and adult CM. For example β-adrenergic stimulation with isoproterenol leads to a positive chronotropic effect and AP shortening (Zhang et al., 2009; Moretti et al., 2010) and application of β-blockers blunt the effect of isoproterenol (Moretti et al., 2010). Also the AP shortening effects of $I_{K,ATP}$ openers pinacidil and nicorandil and the I_{Ca} channel blocker nifedipine was captured in hiPSC-CM.

Functional I_{Na} (Ma et al., 2011; Davis et al., 2012), $I_{Ca,L}$(Itzhaki et al., 2011a; Ma et al., 2011; Yazawa et al., 2011), I_{Kr} (Itzhaki et al., 2011a; Ma et al., 2011; Matsa et al., 2011; Lahti et al., 2012) and I_{Ks}(Ma et al., 2011) have been demonstrated in hIPSC-CMs and mutations affecting these channels as well as pharmacological ion channel blockade were shown to impact on the AP. While the functional presence of SR, RyRs and the Ca^{2+} binding protein CASQ2was demonstrated (Itzhaki et al., 2011b; Lee et al., 2011; Novak et al., 2012), studies have shown that the coupling between Ca^{2+} influx through L-type Ca^{2+} channels and Ca^{2+} release from the SR through RyRs is poor as a consequence of the lack of t-tubuli in hiPSC-CMS. Thus, the use of hiPSC-CMs to study certain cardiac arrhythmia syndromes, such as CPVT and LQT8, caused by mutations in one of the Ca^{2+} handling proteins is limited to the study of the biophysical properties of the affected protein. While I_{to1}, I_{K1}, and I_f are present in hiPSC-CMs (Ma et al., 2011), their contribution to electrical activity in these cells has not been proven by pharmacological blockade or through the effect of mutations in the respective genes. Openers of $I_{K,ATP}$ shorten the AP (Itzhaki et al., 2011a; Matsa et al., 2011), so functional presence can be presumed but needs to be studied in more detail. The presence of I_{NCX} is also not studied in detail but its functional presence can also be presumed since intact Ca^{2+} handling has been demonstrated (Itzhaki et al., 2011b; Lee et al., 2011). Up till now there is no evidence for the functional presence or absence of $I_{K,ACh}$.

A difficulty in the use of hiPSC-CM models is their immature electrophysiological phenotype, with depolarized MDP or RMP and slow AP upstroke velocities compared to native CMs. The MDP and upstroke velocity of hiPSC-CMs resemble more those of fetal CMs than adult CMs (Davis et al., 2011). Furthermore, most studies report that hiPSC-CM beat spontaneously, which is also characteristic of fetal CMs (Mummery et al., 2003). Of note, in our study on *quiescent* hiPSC-CMs we recorded a more-negative RMP and a faster AP upstroke velocity (Davis et al., 2012). Considering the importance of I_{K1} for setting the RMP, it is likely that the quiescent hiPSC-CMs have a larger I_{K1} than spontaneously beating hiPSC-CMs. It is not known whether quiescent cells were also present among the hiPSC-CM generated in studies in which spontaneously beating hiPSC-CMs were studied. Possibly, in these studies, the spontaneous activity was used as a tool to recognize CMs. A further consideration is that *cultured* adult CMs show a depolarized MDP and slower AP upstroke velocity compared to freshly isolated adult CMs. The depolarized MDP in cultured CMs is known to be caused by progressive decline in I_{K1} (Mitcheson et al., 1998). Another similarity between cultured ventricular myocytes and hiPSC-CMs is that different AP phenotypes are observed. In many hiPSC-CM

studies different AP characteristics are observed and are classified as ventricular-like, atrial-like and nodal-like AP. Similarly, when ventricular CMs are cultured different AP phenotypes are observed after one day in culture, and more pronounced variability is evident after four days in culture (Mitcheson et al., 1998).

Another difficulty is the purity of the population of hiPSC-CMs acquired. With the current techniques it is not possible to acquire a pure population of CMs, the fraction of CMs obtained may vary from 1% to ∼50% of the total cells (Dambrot et al., 2011). Moreover, as discussed above, the CMs that are generated are in fact a mixed population of CMs displaying different AP characteristics. One way in which this issue might be addressed is through the use of selectable markers driven by CM lineage-specific promoter elements. However, while this might be useful in selecting CMs as opposed to other cell types, more research is still required for the identification of promoter elements that may be used in selecting for specific CM types (e.g., ventricular versus atrial). Another solution might be found in chemical enhancement of cardiac differentiation. For instance, ascorbic acid enhances cardiac differentiation and minimizes the interline variance and facilitates the structural and functional maturation of hiPSC-CMs (Cao et al., 2012). From hESC-CM research we know that bone morphogenetic protein (BMP) signaling inhibition after mesodermal formation facilitates cardiac development. In this study, also a possibility for ventricular or atrial specific differentiation is shown (Zhang et al., 2011). Inhibition of retinoid (RA) signaling by noggin leads to CM specification into ventricular cells, whereas RA treatment leads to atrial specification (Zhang et al., 2011). Direction of differentiation to nodal like CMs can be achieved by activation of the $Ca2^{+}$ activated potassium channels of small and intermediate conductance (SKCas) by 1-ethyl-2-benzimidazolinone (Kleger et al., 2010). Ventricular specification and electrophysiological maturation of hESC-CMs may also be promoted by microRNAs (miRs). MiR-499 was shown to promote ventricular specification; miR-1 facilitates electrophysiological maturation (Fu et al., 2011).

In summary, iPSC-CM models recapitulate the phenotype of patients with cardiac arrhythmia syndromes. However, the interpretation of electrophysiological data derived from these cells, should be done with caution, since hiPSC-CMs present immature phenotypes and do not recapitulate all the electrical characteristics of an adult CM. Thus, studies addressing the maturity and purity of the hiPSC-CMs acquired are needed, as well as studies to characterize the electrophysiological and pharmacological characteristcs in more detail. Because of this, there are still limitations for the use of this model system in studies on cardiac rhythm disorders, especially if the disease causing mutation is not known. So far, hiPSC-CMs have only been applied as models to the study of disorders for which mutations in particular genes have been identified. Future studies are likely to demonstrate the potential of these cell systems in pointing us to pathophysiological mechanisms for those cases for which no gene mutations are yet known. Another future application of hiPSC-CMs is the use in cardiac safety pharmacology and the development of new drugs. hiPSC-CMs provide researchers with CMs of human origin which are better-suited than CMs of animal origin or heterologous cell systems. Furthermore, hiPSC-CMs provide us with the opportunity to test drugs in disease-specific CMs instead of healthy CMs. Thus, in future studying hiPSC models might lead to novel insights in pathophysiology, improve understanding of genotype-phenotype relationships and could be used in the development and testing of pharmacological agents to treat human cardiac disease.

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Cardiac electrophysiology in mice: a matter of size

Sven Kaese[1] and Sander Verheule[2]*

[1] Division of Experimental and Clinical Electrophysiology, Department of Cardiology and Angiology, University Hospital Münster, Münster, Germany
[2] Department of Physiology, Faculty of Medicine, Maastricht University, Maastricht, Netherlands

Edited by:
Carol Ann Remme, University of Amsterdam, Netherlands

Reviewed by:
Morten B. Thomsen, University of Copenhagen, Denmark
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Yoram Etzion, Ben-Gurion University of the Negev Beer-Sheva, Israel

***Correspondence:**
Sander Verheule, Department of Physiology, Universiteitssingel 50, P.O. Box 616, 6200 MD, Maastricht, Netherlands.
e-mail: s.verheule@maastrichtuniversity.nl

Over the last decade, mouse models have become a popular instrument for studying cardiac arrhythmias. This review assesses in which respects a mouse heart is a miniature human heart, a suitable model for studying mechanisms of cardiac arrhythmias in humans and in which respects human and murine hearts differ. Section I considers the issue of scaling of mammalian cardiac (electro) physiology to body mass. Then, we summarize differences between mice and humans in cardiac activation (section II) and the currents underlying the action potential in the murine working myocardium (section III). Changes in cardiac electrophysiology in mouse models of heart disease are briefly outlined in section IV, while section V discusses technical considerations pertaining to recording cardiac electrical activity in mice. Finally, section VI offers general considerations on the influence of cardiac size on the mechanisms of tachy-arrhythmias.

Keywords: arrhythmias, atrial fibrillation, conduction, mouse, scaling, ventricular fibrillation

SCALING OF CARDIAC PHYSIOLOGY TO BODY MASS

Body mass varies between small mammals like the mouse (0.03 kg, see **Table 1**) to the largest mammal, the blue whale (30,000 kg) by a factor of 10^6 (Noujaim et al., 2004). Very diverse physiological parameter such as basal metabolic rate, life span, left ventricular ejection time, and ECG time intervals (RR, PR, QRS, and QT) scale with body mass (BM) (West et al., 1997, 1999; Noujaim et al., 2004, 2007; Popovic et al., 2005). The "universal law of allometric scaling" for a parameter **P** is expressed by the allometric equation: $\mathbf{P} = \mathbf{aBM^b}$ (West et al., 1997, 1999; Noujaim et al., 2007), where ***a*** is a normalization constant and ***b*** represents the scaling exponent. Frequently, ***b*** is a multiple of 1/4. Basal metabolic rate scales as $BM^{3/4}$ (West et al., 1997, 1999) and is thought to be related to the ratio of body surface area to body volume. A larger body surface-to-volume ratio results in faster temperature loss of the body and thus requires a higher basal metabolic rate to maintain body temperature. Mice, with a high surface-to-volume ratio, tend to have a relatively high basal metabolic rate (see **Table 1**). Adequate blood pressure is required to maintain homeostasis and basal metabolic rate. The mean arterial pressure does not scale with body mass and is thus similar between mice and humans (West et al., 1997; Janssen and Smits, 2002). By contrast, cardiac output (CO, exponent 3/4), cardiac stroke volume (SV, exponent 1), blood volume (exponent 1) and circulation time (exponent 1/4) (West et al., 1997; Janssen and Smits, 2002) scale with BM. In mice, CO amounts to 0.0085–0.027 L/min (**Table 1**) and thus the murine blood volume of 0.002–0.003 L can circulate around 3–14 times per minute. Humans have a CO of 4–8 L/min and a blood volume of 4.5–5.5 L (**Table 1**), which circulates circa 1–2 times per minute. The cardiac index (CI) relates (CO = SV*HR) to body surface area (CI = CO/body surface area). This hemodynamic parameter represents cardiac performance normalized to body surface area and thus indirectly to basal metabolic rate. The cardiac index is tenfold higher in mice than in humans (Janssen and Smits, 2002), reflecting a higher basal metabolic rate in mice. Normalized SV is similar between mice and humans at around 1 µl/g body weight. Therefore, the difference in HR is mainly responsible for differences in CI.

Over the entire range of BM form mice to whales, cardiac mass amounts 0.6% of body mass (Prothero, 1979) and cardiac gross anatomy is remarkably similar, with comparable pacemaking and conduction structures (Wessels and Sedmera, 2003). Using the same biochemical processes to ensure optimal electromechanical function, hearts of very different sizes have to maintain sufficient cardiac function under a variety of conditions. Resting heart rates in conscious mice (measured by telemetry) range from 550 to 725 beats per minute (bpm) (Kass et al., 1998; Gehrmann et al., 2000; Janssen and Smits, 2002; Fabritz et al., 2010), corresponding to a sinus cycle length (SCL) of 80–110 ms. Because cardiac conduction velocities are not strongly dependent on BM (**Table 1** and **Figure 1**), the timing intervals of the ECG scale with the propagation distance, i.e., cardiac size. The ECG time intervals RR, PR, QRS, and QT scale with $BM^{1/4}$ (Noujaim et al., 2004, 2007) and consequently heart rate scales with body mass with an exponent of -1/4 (West et al., 1997; Janssen and Smits, 2002; Noujaim et al., 2004). The PR interval represents electrical conduction from the atrium through the AV node and His-Purkinje system to the ventricles and thus can be seen as a fractal network with three separate components, the PA, AH, and HV subintervals, that also scale with body mass with an exponent of 1/4 (Noujaim et al., 2004). Hence, percentage of each subinterval to the PR interval is constant and independent of body and heart size (Noujaim et al., 2004).

Table 1 | Comparison of mouse and human (cardiac) physiology.

	Human	References	Mouse	References
GENERAL				
Body mass (kg)	58–85	de la Grandmaison et al., 2001; Stein et al., 2002; Noujaim et al., 2004; Johnstone et al., 2005; Kasper et al., 2008; Later et al., 2010; Barnes et al., 2012	0.015–0.043	Sheng et al., 1999; Verheule et al., 1999; VanderBrink et al., 2000; Janssen and Smits, 2002; Speakman et al., 2002; Noujaim et al., 2004; Xiao et al., 2004; Muller et al., 2005; Hong et al., 2008; Barwe et al., 2009; Brands et al., 2010; Carlstrom et al., 2010; Gros et al., 2010; Carroll et al., 2011
Lifespan (year)	70–80	Zhang and Zhang, 2009	2–2.5	Speakman et al., 2002; Zhang and Zhang, 2009
Basal metabolic rate (kJ/d)	6279	Johnstone et al., 2005	15.6	Speakman et al., 2002
Basal metabolic rate (O2 consumption L/(kg* h)	0.9	Janssen and Smits, 2002	0.8–3	Desai et al., 1997; Janssen and Smits, 2002
HEART				
Heart weight (g)	261–366	de la Grandmaison et al., 2001; Cunha et al., 2002; Janssen and Smits, 2002; Later et al., 2010	0.12–0.17	Muller et al., 2005; Hong et al., 2008; Barwe et al., 2009; Carlstrom et al., 2010; Carroll et al., 2011
Heart weight/body weight ratio (kg/kg)	0.004–0.006	de la Grandmaison et al., 2001; Cunha et al., 2002; Later et al., 2010	0.004–0.005	Muller et al., 2005; Hong et al., 2008; Barwe et al., 2009; Carlstrom et al., 2010; Carroll et al., 2011
HEMODYNAMIC				
Stroke volume (mL)	50–100	Janssen and Smits, 2002; Meijler et al., 2005; Kasper et al., 2008	0.015–0.05	Janssen and Smits, 2002; Fabritz et al., 2010; Gros et al., 2010; Hyyti et al., 2010; Maslov et al., 2010
Cardiac output (L/min)	4–8	Janssen and Smits, 2002; Mestas and Hughes, 2004; Meijler et al., 2005; Kasper et al., 2008	0.005–0.03	Janssen and Smits, 2002; Mestas and Hughes, 2004; Gros et al., 2010; Maslov et al., 2010
Blood pressure (mean arterial pressure, mmHg)	88–100	Mancia et al., 1983; Janssen and Smits, 2002; Barnes et al., 2012; Damkjaer et al., 2012	73–125	Kass et al., 1998; Sheng et al., 1999; Janssen and Smits, 2002; Brands et al., 2010; Carlstrom et al., 2010
Blood volume (L)	5–6	Janssen and Smits, 2002; Mestas and Hughes, 2004; Kasper et al., 2008	0.002–0.03	Sheng et al., 1999; Janssen and Smits, 2002; Mestas and Hughes, 2004
CARDIAC EP				
Heart rate (beats/min)	56–101	Edvardsson et al., 1984; Franz et al., 1987; Janssen and Smits, 2002; Stein et al., 2002; Noujaim et al., 2004; Monnig et al., 2005; Zhang and Zhang, 2009; Barnes et al., 2012; Rich et al., 2012	500–724	Kass et al., 1998; Gehrmann et al., 2000; Janssen and Smits, 2002; Xiao et al., 2004; Fabritz et al., 2010
PR interval (ms)	120–200	Stein et al., 2002; Waldek, 2003; Noujaim et al., 2004; Kasper et al., 2008; Grecu et al., 2009	30–56	Thomas et al., 1998; Jalife et al., 1999; Morley et al., 1999; Vaidya et al., 1999; VanderBrink et al., 1999, 2000; Verheule et al., 1999; Gehrmann et al., 2000; Maguire et al., 2000; Saba et al., 2000; Tamaddon et al., 2000; Wehrens et al., 2000; Li et al., 2004, 2009; Noujaim et al., 2004; Saba et al., 2005; Zhang et al., 2005; Sawaya et al., 2007; Mancarella et al., 2008; Stein et al., 2009
QRS duration (ms)	84–110	Kasper et al., 2008; Grecu et al., 2009; Rich et al., 2012	9–30	Thomas et al., 1998; Jalife et al., 1999; Morley et al., 1999; Vaidya et al., 1999; VanderBrink et al., 1999, 2000; Verheule et al., 1999; Gehrmann et al., 2000; Maguire et al., 2000; Saba et al., 2000; Tamaddon et al., 2000; Wehrens et al., 2000; Schrickel et al., 2002; Alcolea et al., 2004; Xiao et al., 2004; Saba et al., 2005; Sawaya et al., 2007; Schrickel et al., 2007; Stein et al., 2009; Gros et al., 2010
QT (ms)	385	Rich et al., 2012	29–109	Thomas et al., 1998; Jalife et al., 1999; Morley et al., 1999; Vaidya et al., 1999; VanderBrink et al., 1999; Gehrmann et al., 2000; Maguire et al., 2000; Saba et al., 2000; VanderBrink et al., 2000;

(Continued)

Table 1 | Continued.

	Human	References	Mouse	References
				Wehrens et al., 2000; Schrickel et al., 2002; Li et al., 2004; Xiao et al., 2004; Saba et al., 2005; Schrickel et al., 2007; Fabritz et al., 2010; Gros et al., 2010
QTc (ms)	398–430	Stein et al., 2002; Rich et al., 2012	30–124	Gehrmann et al., 2000; Maguire et al., 2000; Saba et al., 2000; Schrickel et al., 2002, 2007; Li et al., 2004; Zhang et al., 2005; Sawaya et al., 2007; Stein et al., 2009; Gros et al., 2010
Atrial ERP (ms)	172–245	Chiamvimonvat et al., 1998; Schauerte et al., 2001; Dorian et al., 2007; Roberts-Thomson et al., 2009; Stiles et al., 2010	23–71	Thomas et al., 1998; VanderBrink et al., 1999; Verheule et al., 1999; van Veen et al., 2005; Zhang et al., 2005; Sawaya et al., 2007; Schrickel et al., 2007; Li et al., 2009; Odening et al., 2009
Atrial CV (cm/s)	88	Hansson et al., 1998	30–60	Thomas et al., 1998; Verheule et al., 1999; Alcolea et al., 2004; van Veen et al., 2005
AV Wenckebach CL (ms)	329–453	Chiamvimonvat et al., 1998; Schauerte et al., 2001; Stein et al., 2002; Kose et al., 2004; Dorian et al., 2007; Grecu et al., 2009	66–133	Thomas et al., 1998; Hagendorff et al., 1999; VanderBrink et al., 1999; Verheule et al., 1999; Maguire et al., 2000; Saba et al., 2000; VanderBrink et al., 2000; Korte et al., 2002; Schrickel et al., 2002; Saba et al., 2005; Zhang et al., 2005; Sawaya et al., 2007; Schrickel et al., 2007; Li et al., 2009; Blana et al., 2010
Ventricular ERP (ms)	223–257	Edvardsson et al., 1984; Chiamvimonvat et al., 1998; Schauerte et al., 2001; Dorian et al., 2007	33–80	Thomas et al., 1998; Verheule et al., 1999; Kovoor et al., 2001; Korte et al., 2002; Saba et al., 2005; van Veen et al., 2005; Zhang et al., 2005; Sawaya et al., 2007; Schrickel et al., 2007; Li et al., 2009; Stein et al., 2009
Ventricular CV (cm/s)	80	de Bakker et al., 1993	30–60	Thomas et al., 1998; Morley et al., 1999; Alcolea et al., 2004; van Veen et al., 2005

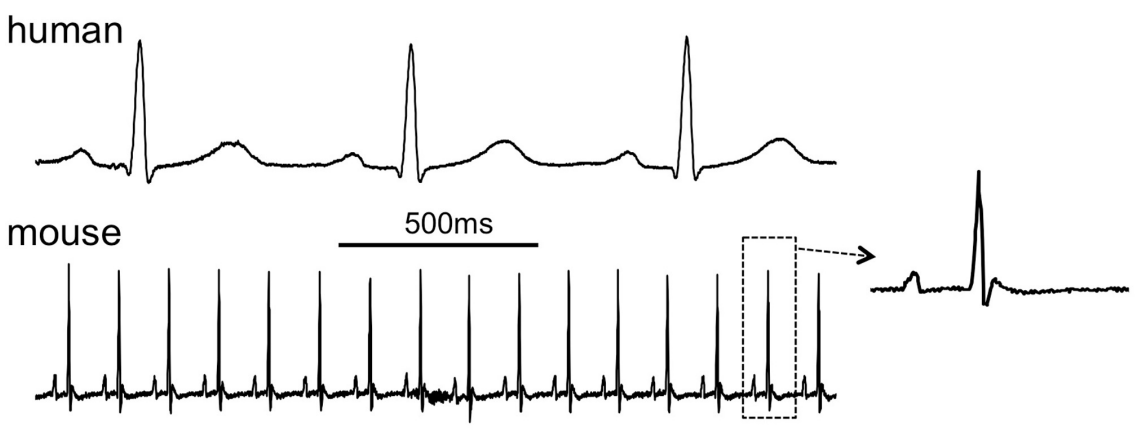

FIGURE 1 | Comparison of the human and murine ECG, 2-s traces showing lead I. Inset shows a single complex from the mouse ECG (human ECG is patient 121 in the PTB database at www.physionet.org, mouse ECG courtesy of Dr. Ricardo Carnicer, Oxford University).

Mice can increase their HR by around 30–40% (Vornanen, 1992; Kass et al., 1998; Fabritz et al., 2010), whereas humans HR can increase by 300%. Thus, an increase in frequency can contribute far less to increase cardiac output (CO) in mice than in humans. The ratio of maximal to resting heart rate scales to BM with the following equation $HR_{max}/HR_{rest} = 1.87 \times BM^{-0.1}$ (Kass et al., 1998). Actin-myosin crossbridge kinetics have an estimated maximal rate of around 1000 bpm, corresponding to a cycle length (CL) of 60 ms (Kass et al., 1998). Maximal heart rates observed in mice are in a range of 725–815 bpm (Vornanen, 1992; Kass et al., 1998; Fabritz et al., 2010). Thus, an increase in murine HR is limited as the maximal HR is close to the maximal

actin-myosin crossbridge frequency. In addition, studies in mice have found an AV Wenckebach period of 66–103 ms (**Table 1**). With an increase in HR, this Wenckebach period is reached relatively quickly, also limiting the increase in CO. For efficient cardiac pump function and CO, optimal timing of AV conduction is required. Late diastolic left ventricular filing time scales with heart length and with an 1/3 exponent to cardiac or body mass, whereas AV delay scales with an ¼ exponent of heart or body mass (Noujaim et al., 2004; Meijler et al., 2005). In mice, an increase in heart rate decreases diastolic left ventricular filing time and thus gradually reduces ventricular end-diastolic volume. Finally, mice at physiological HR have a small force-frequency reserve compared to humans (Georgakopoulos and Kass, 2001). Consequently, ventricular filing is limited at high heart rates in mice, further impeding CO enhancement and cardiac contractility. Taken together, these factors explain why mice can raise their CO only over a small range, whereas humans can increase their CO by a factor 5–6. This difference should be taken into account when HR regulation (e.g., during exercise) is relevant.

CARDIAC ACTIVATION IN MICE

SINOATRIAL NODE

The lateral and medial limb of the crista terminalis at the orifice of the superior caval vein frame the murine SA node region (Verheijck et al., 2001; Mommersteeg et al., 2007). During development, the SA is node is distinguished from the adjacent atrial myocardium by the absence of the transcription factor Nkx2.5 and the presence of the transcription factors Tbx3 and Tbx18, both in humans and mice (Christoffels et al., 2006; Mommersteeg et al., 2007; Sizarov et al., 2011). These differences in transcription factor profiles initiate diverging gene expression programs, leading to differences in e.g., connexin and ion channel expression (Hoogaars et al., 2007). Histologically, the SA node consists of a large head and a smaller tail along the crista terminalis (Wiese et al., 2009). Characteristic sinus node APs (a marked diastolic depolarization and low upstroke velocity) are found in a region 300 μm in parallel and 150 μm perpendicular to the crista terminalis (Opthof, 2001; Verheijck et al., 2001). A morphologically "nodal" cell type is present in a somewhat larger region 500 μm parallel to the crista (Opthof, 2001; Verheijck et al., 2001). Surprisingly, Glukhov et al. have shown that in the mouse, the location of the dominant pacemaker can shift during (para)sympathomimetic treatment within a still larger region of approximately 5 × 2 mm between the superior and inferior caval veins (Glukhov et al., 2010).

The sinoatrial conduction time, defined as the conduction time from the primary, dominant sinus node cells to atrial myocardium is 4–5 ms and the transition from a nodal to an atrial AP morphology takes place over a very short distance of about 100 μm (Opthof, 2001; Verheijck et al., 2001).

SA nodal APs in mice have an amplitude, maximum diastolic potential and upstroke velocity comparable to those in humans, but a much shorter APD, probably due to differences in delayed rectifier currents (Opthof, 2001). The SCN5A encoded cardiac Nav1.5 sodium channel affects SA node pacemaker function, as heterozygous deletion of the SCN5A gene in mice caused sinus bradycardia and exit block (Lei et al., 2005). Similarly, sinus bradycardia and sinus pauses are prevalent in LQT3 patients with certain SCN5A mutations (Veldkamp et al., 2003). However, SCN5A-related alterations in sinus node function may be caused by the involvement of Nav1.5 in extracellular matrix remodeling, rather than a primary electrical effect (Hao et al., 2011). The α_{1D} form of the L-type Ca^{2+} channel is expressed in the SA node and contributes to diastolic depolarization and pacemaker activity, and deletion of this gene leads to sinus bradycardia (Zhang et al., 2002; Mangoni et al., 2003; Qu et al., 2005).

The murine SA node expresses hyperpolarization-activated, nonselective cation channels from the HCN family. HCN4 is the dominant isoform, although smaller amounts of HCN1 and HCN2 are also expressed (Liu et al., 2007; Ludwig et al., 2008; Herrmann et al., 2011). Specific mutations in the HCN4 gene can cause bradycardia in humans (Milanesi et al., 2006). HCN4 knock-out mice have produced diverging results (Herrmann et al., 2012). Mice with conditional ubiquitous HCN4 deletion in the adult stage were not bradycardic, even at less than 10% of normal HCN4 levels, but did show sinus pauses that were activity dependent and more prevalent at low heart rates (Herrmann et al., 2007; Hoesl et al., 2008). I_f in isolated SA nodal cells from these mice was decreased by 75% and these cells often did not show pacemaker activity under baseline conditions, but did become spontaneously active in response to adrenergic stimulation (Herrmann et al., 2007; Ludwig et al., 2008). However, another mouse model with heart-specific conditional deletion of HCN4 did show profound sinus bradycardia (Baruscotti et al., 2011). HCN2 deficient mice have a sinus dysarrhythmia leading to a marked increase in RR variability, although the response to adrenergic and cholinergic stimulation was preserved (Ludwig et al., 2003).

In mice, Cx45 is expressed in SA node center (Opthof, 2001; Verheijck et al., 2001). On the epicardial side, the murine SA node is entered by strands of cells expressing Cx40 and Cx43 (Verheijck et al., 2001). Also in humans, atrial myocardial cells with Cx43 expression protrude into the Cx43 negative SA nodal area (Oosthoek et al., 1993). The murine SA node architecture containing different types of gap junctions and connective tissue around the nodal area are hypothesized to shield the SA node against hyperpolarizing influence of surrounding atrial myocardium and thus to allow pacemaker activity and conduction from the SA node to atrial myocardium (Verheijck et al., 2001).

ATRIAL ACTIVATION

Various studies have shown atrial conduction patterns in mice. Atrial activation starts near the expected location of the sinoatrial node close to the superior vena cava and from there spreads rapidly over the right and left atria (Leaf et al., 2008; Mathur et al., 2009; Blana et al., 2010; Kirchhof et al., 2011; Swaminathan et al., 2011). It is not clear whether mice have a true Bachmann's bundle, which in larger species is a thick, highly anisotropic structure consisting of many parallel fibers, serving as the largest and fastest conduction pathway between the atria. The total atrial conduction time amounts to approximately 15 ms (Blana et al., 2010; Swaminathan et al., 2011). The atria express both Cx40 and Cx43. The specific role of Cx40 is unclear, with observations in $Cx40^{-/-}$

mice ranging from a reduced CV (Verheule et al., 1999) to a reduced conduction heterogeneity without a change in CV (Leaf et al., 2008). The atrial ERP shows little rate-dependence and in some cases it may be longer than the AV nodal ERP (Etzion et al., 2008).

ATRIOVENTRICULAR NODE

The murine AV node has similar properties to the human AV node (VanderBrink et al., 1999). In some humans, the AV node shows "dual AV physiology" with a distinct slow and fast pathway, giving rise to a discontinuous AV conduction curve during premature atrial stimulation. The prevalence of dual AV nodal physiology increases during postnatal development and around 40% of adult humans display dual AV nodal physiology (Denes et al., 1973; Thapar and Gillette, 1979; Cohen et al., 1997). A maturation study in mice demonstrated that dual AV nodal physiology and AV nodal echo beats also become more frequent with increasing age, but without inducibility of AV nodal reentry tachycardia (Maguire et al., 2000). In contrast, another study of the murine AV node did not find dual AV nodal physiology (VanderBrink et al., 1999). As in larger species, the AV node shows "facilitation" during an acceleration in rate, and this property is lost in Cx40 deficient mice (Zhu et al., 2005).

The areas bordering the AV node show a complex profile of Cx expression, which has been studied in mice and humans (Aanhaanen et al., 2010; Greener et al., 2011). Myocytes in the compact node, the area responsible for the major part of the AV delay, express Cx40 and Cx45 in both mice and humans. In addition, Cx30.2 is expressed in mice, but its orthologue Cx31.9 is not present in the human AV node. In $Cx40^{+/-}$ mice and $Cx45^{+/-}$ mice, AV conduction is not affected (Hagendorff et al., 1999; Verheule et al., 1999; Kruger et al., 2006). The AV delay increases in $Cx40^{-/-}$ mice (Hagendorff et al., 1999; Verheule et al., 1999) and increases further in $Cx40^{-/-}Cx45^{+/-}$ mice (Kruger et al., 2006), indicating that both connexins contribute to AV conduction. Intriguingly, the AV delay is shorter in $Cx30.2^{-/-}$ mice and normal in $Cx30.2^{-/-}Cx40^{-/-}$ mice (Kreuzberg et al., 2006; Schrickel et al., 2009). Thus, Cx30.2 may represent an arrangement to decelerate AV nodal conduction that is specific to mice.

In humans, the homeobox transcription factor Nkx2-5 is expressed in the AV node and heterozygous mutations caused congenital atrioventricular conduction abnormalities, e.g., AV block (Schott et al., 1998). A murine model with a heterozygous Nkx2-5 mutation has a hypoplastic AV node (Jay et al., 2004). Furthermore, overexpression of a human mutation of Nkx2-5 in transgenic mice led to heart failure and progressive AV conduction defects, possibly due to reduced Cx40 and Cx43 expression (Kasahara et al., 2001). Similarly, haploinsuffiency in mice of the transcription factor Tbx5, which has a synergystic effect with Nkx2-5 on gene transcription, causes abnormalities similar to those in the human Holt-Oram syndrome, including decreased Cx40 expression and AV block (Bruneau et al., 2001; Moskowitz et al., 2007).

VENTRICULAR ACTIVATION AND REPOLARIZATION

The ventricular conduction system lineage is established during development by expression of the transcription factors Nkx2-5 and Tbx5, (Jay et al., 2004; Moskowitz et al., 2007) activating a gene program that includes Cx40, the major gap junction protein in the His bundle and proximal conduction system. Deletion of Cx40 leads to conduction slowing in the ventricular conduction system (Simon et al., 1998; Bevilacqua et al., 2000; VanderBrink et al., 2000). The murine left bundle branch is thicker than the right bundle branch, as evidenced by Cx40 expression patterns (van Rijen et al., 2001). This is supported by studies that suggested that the left bundle brunch contains more parallel fibers and thus probably has a higher conduction reserve than the right bundle branch, possibly explaining why the right bundle branch is more prone to conduction block (Alcolea et al., 2004). Similarly, clinical studies tend to show a higher prevalence of conduction block in the right bundle branch than in the left bundle branch (Tuzcu et al., 1990; Newby et al., 1996; Golshayan et al., 1998).

The activation pattern of ventricular myocardium differs between mice and humans. In mice, on study has reported that ventricular epicardial activation starts with clearly defined breakthroughs in the right ventricle and subsequently in the left ventricle (Tamaddon et al., 2000). Another study has shown a first breakthrough at the left ventricular apex, followed shortly by a right ventricular breakthrough (Nygren et al., 2000). By contrast, studies in humans found first depolarization at the left ventricular side of the interventricular septum, conducted by septal left bundle branch fibers and corresponding to the Q wave in the ECG (Durrer et al., 1970). Septal right bundle branch fibers cause subsequent right ventricular activation (Durrer et al., 1970). Within the interventricular septum, mice show an activation pattern from base to apex, probably due to an electrical connection from the common bundle into the ventricular septum (van Rijen et al., 2001). By contrast, humans show intraventricular septal activation from left to right and from apex to base (Durrer et al., 1970). Conduction velocity is slower in the midseptal than in the proximal region of the murine bundle branches, due to regional differences in bundle geometry and connexin expression (van Veen et al., 2005). During murine intrauterine development, the epicardial ventricular activation pattern switches from basico-apical to apico-basal, in correspondence with maturation of the conduction system (Rentschler et al., 2001; Gourdie et al., 2003).

Within the mouse ventricles, repolarization is earlier in the epicardium than in the endocardium due to a shorter epicardial action potential (Knollmann et al., 2001), and earlier at the apex than at the base (Killeen et al., 2007b; London et al., 2007). Transmural and apico-basal gradients in I_{to} are thought to be the main determinant of heterogeneity of the murine ventricular APD (Rossow et al., 2006; Wang et al., 2006; Killeen et al., 2007b; London et al., 2007).

Cx43 is the main gap junction protein in the ventricular working myocardium, but its expression can be decreased by ∼80% before a marked decrease in CV and increase in vulnerability to arrhythmias becomes apparent (Danik et al., 2004; van Rijen et al., 2004), as would be expected based on mathematical models (Shaw and Rudy, 1997). However, more modest reductions in gap junctional coupling may have significant effects when they occur in conjunction with other factors such as reduced excitability or fibrosis (van Veen et al., 2005; Stein et al., 2009). Similar conjunctions of disease processes are common in

human pathology, but poorly represented by monogenic mouse models.

ARRHYTHMIAS LINKED TO ABNORMAL CARDIAC DEVELOPMENT

Some studies have linked human arrhythmias to gene programs active during prenatal cardiac development. The developing mouse heart contains muscular atrioventricular connections over the annulus fibrosus (Rentschler et al., 2001). Persistence of these connecting fibers bypass the AV node and cause ventricular pre-excitation syndromes similar to Wolff-Parkinson-White syndrome (Rentschler et al., 2001) Patients suffering from Wolff-Parkinson-White syndrome showed mutations in the PRKAG2 gene, which encodes the gamma-2 subunit of the AMP-activated protein kinase (Blair et al., 2001; Gollob et al., 2001). In mice, overexpression of one of the human mutations of the PRKAG2 gene caused ventricular preexcitation and an altered annulus fibrosis structure, disrupted by glycogen-filled myocytes that may function as accessory pathways (Arad et al., 2003). Similarly, inactivation of Tbx2, a transcription factor involved in the development of the atrioventricular canal, leads to malformation of the annulus fibrosis and ventricular preexcitation (Aanhaanen et al., 2011). Mutations in Tbx3 are linked to ulnar-mammary syndrome in humans. In mice, a reduction in Tbx3 activity leads to SA node dysfunction, AV block and ventricular preexcitation (Frank et al., 2012).

Gittenberger-de Groot and co-workers have presented extensive evidence for the relationship between nodal pacemaker tissue and the myocardial sleeves in the developing embryo (Douglas et al., 2011) For example, podoplanin is a myocardial marker expressed in cells of the developing pacemaking system, which later differentiate e.g., to the sinoatrial node (SA node) and atrioventricular node (AV node) (Gittenberger-de Groot et al., 2007). Podoplanin expression is also present in surrounding myocardium of the common pulmonary vein (PV) (Gittenberger-de Groot et al., 2007), an area that can later become important in the genesis of atrial fibrillation in humans (Haissaguerre et al., 1998). These embryological relationships have been proposed account for ectopic activity in the myocardial sleeves of the pulmonary veins (Jongbloed et al., 2004). However, the sinus node originates from a different type of cardiac precursor cells (i.e., Tbx18-positive and Nkx2.5-negative) than the PV myocardium (Christoffels et al., 2006). In addition, a nodal phenotype would be more likely to give rise to relatively slow, gradually appearing and disappearing pacemaking activity, as has been described in guinea pig pulmonary veins (Cheung, 1981). By contrast, human PVs show paroxysms of arrhythmic activity that initiate and terminate suddenly, with very rapid rates that are more compatible with triggered activity or microreentry than with a pacemaking activity in a latent nodal region (Haissaguerre et al., 1998).

CARDIAC CELLULAR ELECTROPHYSIOLOGY

In general, the duration of cardiac action potentials increases with body size and is approximately 50 ms in mouse ventricles (Danik et al., 2002), compared to 250 ms in humans (Edvardsson et al., 1984). The morphology of the action potential reflects the contribution of numerous depolarizing and repolarizing currents. Even when the same type of ion channel is expressed in human and mice, its contribution to the morphology of the action potential may differ substantially, given the large difference in APD. Both in mice and humans, the refractory period ends before complete repolarization of the AP (Knollmann et al., 2001; Fabritz et al., 2003; Sabir et al., 2007a,b). Based on recordings of monophasic action potentials, the ERP in mouse ventricles corresponds to the APD80 (Knollmann et al., 2001).

Murine and human ventricular APs are both characterized by a fast depolarizing phase (phase 0), the AP upstroke, which is generated by the large, rapidly activating sodium current I_{Na} (Nerbonne, 2004). The subsequent repolarization phase is a delicate balance of several depolarizing currents (I_{CaL}, I_{NCX}) and repolarizing potassium currents. In the human ventricle, a fast repolarizing phase (phase 1) is followed by the action potential plateau (phase 2) (Nerbonne, 2004). In the mouse heart, the L-type Ca^{2+} current (I_{CaL}) contributes less to the ventricular AP than in humans (Sabir et al., 2008) and therefore the murine AP shows a gradual repolarization rather than a distinct plateau phase. In the mouse ventricles, α_{1C} channels are primarily responsible for I_{CaL}. By contrast, the I_{CaL} of supraventricular myocardium consists of both α_{1C} and α_{1D} Ca^{2+} channels (Zhang et al., 2005; Mancarella et al., 2008; Zhang et al., 2011). The α_{1D} Ca^{2+} channel makes a significant contribution to Ca^{2+} influx and Ca^{2+} induced Ca^{2+} release from the SR in atrial myocytes (Mancarella et al., 2008).

In humans, the plateau phase ends when the balance shifts from I_{NCX} and the slowly inactivating I_{CaL} to slowly activating potassium currents, giving rise to the final repolarization (phase 3) until the resting potential (phase 4) is restored. In the human ventricle, the rapid and slow delayed outward rectifier K^+ currents (I_{Kr} and I_{Ks}) are predominantly responsible for phase 3 repolarization (Li et al., 1996). In contrast, the much faster repolarization in mice ventricles is mediated by transient outward K^+ currents with a fast and slow recovery from inactivation ($I_{to,f}$ and $I_{to,s}$), a slowly inactivating K^+ current ($I_{K,slow1}$ and $I_{K,slow2}$) and a non-inactivating, steady state current (I_{ss}) (Guo et al., 1999; Xu et al., 1999; Zhou et al., 2003; Brouillette et al., 2004; Sabir et al., 2008). In humans, the $I_{to,f}$ current is mainly involved in phase 1 repolarization, with more prominent expression in the epicardium (Nerbonne, 2004). In mice, $I_{to,f}$ is expressed throughout the left and right ventricles, whereas $I_{to,s}$ is confined to the septal myocardium (Xu et al., 1999). The equivalents of murine I_{ss} and I_{Kslow} have not been detected in human ventricles (Sabir et al., 2008). Studies in mice did observe the rapid and slow delayed rectifier K^+ currents (I_{Kr} and I_{Ks}), but their contribution to repolarization under physiological conditions is probably negligible or minor (Babij et al., 1998; Drici et al., 1998; Balasubramaniam et al., 2003; Nerbonne, 2004; Salama et al., 2009). In the ventricles of mice and humans, the inwardly rectifying K^+ channel (I_{K1}) plays a similar role in stabilizing the resting membrane potential and determining terminal repolarization (Babenko et al., 1998; Flagg and Nichols, 2001; Lopatin and Nichols, 2001; Nerbonne et al., 2001; Nerbonne, 2004). During postnatal development, the APD shortens progressively as a result of the upregulation of a number of potassium currents, both in the atria (Trepanier-Boulay et al., 2004) and in the ventricles (Grandy et al., 2007).

In the adult mouse heart, atrial and ventricular action potentials are very similar in morphology [for a detailed review of all underlying currents see Nerbonne (2004)].

Genetically engineered mice have revealed surprising contributors to the cardiac action potential. For example, deletion of the calcium-activated potassium channel SK2 prolongs the APD in atrial myocytes and leads to AF (Li et al., 2009), although SK channels do not seem to contribute to the normal atrial action potential in larger species (Nagy et al., 2009). SK channel function may be affected by the tendency of SK subunits to form heteromers (Tuteja et al., 2010), and its functional contribution may increase under pathological conditions, also in the ventricular myocardium (Chua et al., 2011). In the mouse ventricles, deletion of the "pacemaker current" HCN3 leads to an increase in T wave amplitude due to an acceleration of the terminal repolarization in epicardial myocytes (Fenske et al., 2011).

The main Ca^{2+} extrusion mechanism in cardiomyocytes is the Na^{+}/Ca^{2+} exchanger (NCX), which generates a net depolarizing current due to its stoichiometry (Bers, 2002; Pott et al., 2004). In most respects, mice overexpressing NCX and cardiac-specific NCX knockouts show opposite cardiac phenotypes, although cardiac hypertrophy was observed in both (Henderson et al., 2004; Pott et al., 2004; Goldhaber et al., 2005; Reuter et al., 2005; Pott et al., 2012). Due to the calcium-dependence of I_{CaL} inactivation, NCX overexpressing mice and cardiac-specific NCX knockouts had accelerated and decelerated inactivation kinetics of I_{CaL}, respectively, leading to APD prolongation and shortening, respectively (Pott et al., 2004; Goldhaber et al., 2005; Reuter et al., 2005; Pott et al., 2007). NCX overexpression increased and cardiac-specific NCX deletion decreased vulnerability to ischemia/reperfusion injury, supporting the role of calcium influx through the reverse-mode NCX under these circumstances (Pott et al., 2004; Imahashi et al., 2005). In hearts of larger mammals, like dog, rabbit, and human, reuptake into the sarcoplasmic reticulum and elimination by the NCX account for approx. 70% and 30% of Ca^{2+} removal, respectively, while in mice and rats up to 90% of Ca^{2+} is taken back up into the sarcoplasmic reticulum (Bassani et al., 1994; Nishimaru et al., 2001). Under steady state conditions (i.e., a stable diastolic SR calcium load), the amount of calcium extruded by the sodium-calcium exchanger (NCX) equals the amount of calcium entering through L-type calcium channels. Increased NCX expression is thought to contribute to arrhythmogenesis in failing human and rabbit hearts (Studer et al., 1994; Lindner et al., 1998; Pieske et al., 1999; Pogwizd et al., 1999, 2001; Schillinger et al., 2000), and EADs, DADs and episodes of ventricular tachycardia were inducible in NCX-overexpressing mice (Pott et al., 2012). Increased NCX expression causes reduced twitch calcium transients and SR calcium load in rabbit ventricles (Ranu et al., 2002). In contrast, increased NCX expression in mice does not affect twitch Ca^{2+} transients and SR calcium load (Adachi-Akahane et al., 1997; Terracciano et al., 1998; Yao et al., 1998). This difference between mice and larger mammals may be caused by a higher cytoplasmic sodium concentration ($[Na]_i$) in murine myocytes (Shattock and Bers, 1989; Yao et al., 1998; Despa et al., 2002), which would impair extrusion via NCX (Bers, 2002). A negative force-frequency relationship has been reported for murine ventricles, often with high Ca^{2+} concentration of the SR, even under low stimulation frequencies (Bers, 2002). Again, the high $[Na]_i$ may impair Ca^{2+} extrusion via NCX and explain why an increasing in heart rate hardly affects SR calcium load in mice (Shattock and Bers, 1989; Yao et al., 1998). In addition, refractoriness of excitation contraction coupling and smaller fractional SR Ca^{2+} is observed during high heart rates (Bers et al., 1993). Both mechanisms probably contribute to negative force-frequency relationship in mouse ventricular myocytes (Bers, 2002).

CHANGES IN CARDIAC ELECTROPHYSIOLOGY IN MOUSE MODELS OF HEART DISEASE

MYOCARDIAL INFARCTION

Mouse models of myocardial ischemia and infarction are commonly generated by ligation of the left anterior descending coronary artery (LAD). Temporary ligation of the LAD with subsequent reperfusion has been used as a model of ischemia-reperfusion injury, allowing determination of the infarct size in relation to the area at risk (Michael et al., 1995). In Langendorff-perfused mouse hearts, regional ischemia and reperfusion increased the incidence of induced and spontaneous ventricular tachycardia (VT) (Koyama et al., 2003; Inagaki et al., 2005; Anzawa et al., 2006; Maass et al., 2009; He et al., 2012). Depending on the exact inschemia-reperfusion protocol, ventricular fibrillation (VF) may also be observed (Koyama et al., 2003; Inagaki et al., 2005; He et al., 2012), especially when catecholamines are added to the perfusate (Stables and Curtis, 2009). In open-chest experiments, episodes of ventricular tachycardia (VT) were observed both during regional ischemia and reperfusion (Sakamoto et al., 1999; Anyukhovsky et al., 2011). Mice with a permanent ligation of the LAD show infarct-related changes in T-wave morphology, reduced cardiac function, and increased inducibility of atrial and ventricular arrhythmias (Gehrmann et al., 2001). In conscious mice with a myocardial infarction (MI), telemetric monitoring revealed frequent premature ventricular beats, but spontaneous VT episodes were rare (Betsuyaku et al., 2004). Heart rate variability (HRV) a marker of autonomic nervous system activity and a predictor of arrhythmias in human MI patients, was not affected by MI in mice (Gehrmann et al., 2001) Decreased HRV is thought to result from an enhanced sympathetic and decreased parasympathetic activity, but in contrast to humans, mice already show a predominant sympathetic tone, since parasympathetic blockade does not increase in HR (Mansier et al., 1996; Wickman et al., 1998) but sympathetic blockade caused a pronounced decrease in HR (Janssen et al., 2000; Just et al., 2000). For a detailed review on autonomic regulation of cardiac function in mice, see (Janssen and Smits, 2002).

PRESSURE OVERLOAD

Transverse aortic constriction (TAC) by banding the proximal aorta has become a standard method to generate chronic pressure overload in mice, leading to LV hypertrophy with decreased fractional shortening within 4–5 weeks (Hill et al., 2000). TAC leads to an increase in QRS duration and a decrease in RV longitudinal conduction velocity (Boulaksil et al., 2010). Furthermore, these mice showed increased interstitial fibrosis and Cx43 heterogeneity (Boulaksil et al., 2010), and an increased inducibility

of polymorphic ventricular tachyarrhythmias (Boulaksil et al., 2010; Jansen et al., 2012; Vinet et al., 2012). The inducibility and stability of atrial fibrillation also increase as a result of TAC (Liao et al., 2010). In addition to the working myocardium, pressure overload may also affect Cx and HCN expression in the ventricular conduction system and thereby alter the ventricular activation pattern (Harris et al., 2012).

METHODS FOR STUDYING MURINE CARDIAC ELECTROPHYSIOLOGY

Different mouse strains show considerable differences in baseline electrocardiographic parameters (Wehrens et al., 2000; Waldeyer et al., 2009) and in processes such as for example wound healing after myocardial infarction (van den Borne et al., 2009), underscoring the importance of a homogeneous genetic background in assessing the role of a genetically modified gene. Studying murine cardiac physiology has required the miniaturization of instruments and techniques originally developed for human studies. This section will briefly outline a number of techniques that are currently available.

SURFACE ELECTROCARDIOGRAMS

As shown in **Figure 1**, murine and human ECGs show some salient differences that complicate comparison of ECGs from both species (Sabir et al., 2008). In both species, the ECGs show P waves, reflecting spread of atrial depolarization (Sabir et al., 2008). Subsequently, both ECGs show an isoelectric PQ interval that reflects impulse conduction from the atria through the AV node and the His-Purkinje system to the ventricles (Schrickel et al., 2002; Sabir et al., 2008). In mice and humans, the ensuing QRS complex reflects ventricular depolarization (Schrickel et al., 2002; Sabir et al., 2008). The normal murine ECG shows a distinct J wave at the end of the QRS complex (Liu et al., 2004). By contrast, the human ECG infrequently displays a slight J wave, but it can become more prominent during hypothermia (Osborn, 1953). Because of the triangular shape of the murine ventricular action potential, with a gradual time course of repolarization, mouse ventricles do not have a distinct "moment of repolarization." As a result, the T wave in the murine ECG is often poorly visible (Liu et al., 2004; Sabir et al., 2008). In some murine studies, the QT interval was defined as the period between the start of the Q wave to the point of return to the isoelectrical line (Verheule et al., 1999; Schrickel et al., 2002). However, correlation of monophasic action potential recordings with the surface ECG indicates that the terminal repolarization of the ventricles may extend into the subsequent P-wave (Danik et al., 2002; Liu et al., 2004). The absence of a clear T wave impedes investigation of murine ventricular repolarization in the surface ECG (Danik et al., 2002). Within the physiological range, the relative degree of rate dependence of the ventricular APD is comparable between mice and humans (Knollmann et al., 2007). The murine QT interval can be corrected for heart rate using Bazett's formula, which is used in human ECG analysis, or (preferably) by a related formula specifically modified for mice (Mitchell et al., 1998).

Small implantable ECG transmitters permit investigation of spontaneous arrhythmias in conscious mice during normal physical activity and during short-term and long-term drug application (Gehrmann and Berul, 2000; Gehrmann et al., 2000; Fabritz et al., 2010). This technique also allows investigation of acute and chronic exercise stress on arrhythmogenesis by using standardized protocols such as e.g., swimming exercise (Berul, 2003; Fabritz et al., 2010). In determining the degree of exercise, it should be take into account that mice will voluntary run a respectable distance of 6 km per day (de Waard et al., 2007).

PROGRAMMED ELECTRICAL STIMULATION IN INTACT MICE

In intact mice under anaesthesia, transesophageal catheters can be used for programmed electrical stimulation of the atria for arrhythmia induction and determination of sinus node recovery time, AV nodal ERP and Wenckebach period (Schrickel et al., 2002; Berul, 2003; Verheule et al., 2004). Due to the lack of direct contact with the atrium and the distance between stimulation poles, stimulus artefacts during transesophageal stimulation are large, obscuring the atrial complex. Therefore, the atrial ERP can not be determined reliably using this technique (Etzion et al., 2008). Because transesophageal stimulation allows survival of the animal, it can be used for longitudinal studies of cardiac electrophysiology over a longer time period. The ventricles cannot be paced using transesophageal stimulation, but Gutstein et al. have presented a method for subdiaphragmatic ventricular stimulation that allows repeated studies in individual mice (Gutstein et al., 2003).

Pacing (and recording) electrodes can be attached either epicardially in open chest experiments (Berul et al., 1996; Verheule et al., 1999) or endocardially using a transvenous access route (VanderBrink et al., 1999; Saba et al., 2000). The latter also permits the recording of his bundle potentials (VanderBrink et al., 1999; Saba et al., 2000). Programmed electrical stimulation using epi- or endocardial electrodes allows assessment of SA node and AV node function, refractory periods, Wenckebach periodicities and vulnerability to atrial and ventricular arrhythmias (Gehrmann et al., 2000; Saba et al., 2000; Berul, 2003). Given the small dimensions of the mouse heart, close apposition of the poles of the stimulation electrode is necessary to reduce stimulus artefacts and allow recording of local electrograms (Verheule et al., 1999; Etzion et al., 2008).

MAPPING IN PERFUSED MOUSE HEARTS

Isolated Langendorff perfused hearts allow detailed studies of cardiac electrophysiology without neurohumoral and autonomic influences, under artificial conditions that can easily be manipulated. Perfused hearts allow pacing and recording of electrograms and monophasic action potentials (Fabritz et al., 2003; Blana et al., 2010). In addition, the Langendorff setup offers the opportunity to perform high-density epicardial mapping using multi-electrode arrays to record activation patterns (van Rijen et al., 2001; Verheule et al., 2004). Sampling frequency has to be sufficiently high in mapping experiments. For example, with a relatively simple 5×5 electrode array covering 2×2 mm (about the size of an atrial free wall), the inter-electrode distance would be 0.4 mm. Assuming a conduction velocity of 0.5 m/s, (Verheule et al., 1999, 2004) the time difference between electrodes in the

propagation direction would be 0.8 ms. Accurate determination of local conduction velocities would then require a sampling frequency of at least 2.5 kHz (1/0.8 × 2).

With currently available technology, the highest spatio-temporal resolution can be attained using optical mapping (up to 100 × 100 pixels at a sampling rate of up to 10 kHz). Using this technique, detailed activation mapping has been reported in various mouse models (de Diego et al., 2008; Blana et al., 2010; Lang et al., 2011). In addition, the acquired optical signals are linearly dependent on the transmembrane potential and therefore represent an "ensemble action potential," providing information on the time course of repolarization and diastolic intervals. Furthermore, activation patterns during arrhythmias, e.g., rapid reentry, can be studied in detail (Vaidya et al., 1999; Baudenbacher et al., 2008). However, to suppress motion artefacts, most studies have used excitation-contraction uncouplers such as butadione monoxime, cytochalasin D or blebbistatin, all of which can affect cardiac electrophysiology in mice. 2,3-Butadione monoxime (BDM) markedly shortens the APD (Biermann et al., 1998). In mice, Cytochalasin D prolongs APD and reduces CV (Baker et al., 2004). Blebbistatin has little effect on the AP morphology, (Fedorov et al., 2007) but can suppress arrhythmias by inhibiting myofilament calcium sensitivity (Baudenbacher et al., 2008).

THE ISSUE OF CARDIAC SIZE IN TACHY-ARRYTHMIAS

In 1914, Garrey postulated that a certain size of myocardial tissue, a "critical mass," is required to sustain reentrant arrhythmias (Garrey, 1924). This led to the belief that the mouse heart would be "too small to fibrillate." This belief has been challenged by murine studies showing atrial (Schrickel et al., 2002; Verheule et al., 2004) and ventricular fibrillation (Vaidya et al., 1999). Specific mouse models of atrial (Nattel et al., 2005; Schotten et al., 2011) and ventricular (Sabir et al., 2008) arrhythmias have been expertly reviewed elsewhere. Similarly, the general mechanisms of atrial (Nattel et al., 2005; Schotten et al., 2011) and ventricular (Antzelevitch, 2001) arrhythmias have been reviewed extensively. Instead, this last section will discuss the general issue of cardiac size in the context of arrhythmias. With respect to the propagation pattern, arrhythmias can be either "hierarchical," with one or more localized sources driving the arrhythmia or "anarchical" where no particular site is driving the arrhythmia. Whereas the former is amenable to targeted ablation strategies, the latter is not. With respect to the underlying mechanism, an arrhythmia will be based on cellular proarrhythmic events giving rise to premature, ectopic action potentials or on reentrant conduction (Antzelevitch, 2001; Sabir et al., 2008). Regardless of the system of classifying arrhythmias, in all cases the size of the substrate is likely to affect the initiation, stability and pattern of the arrhythmia, as discussed below.

REENTRY

The simplest form of reentry is a single reentrant wave circling around an anatomic obstacle or area of functional block. The "wavelength" of such a wave is the product of CV and ERP. (Wiener and Rosenblueth, 1946) With the normal CV and ERP in the mouse heart, this wavelength (WL) would be approximately

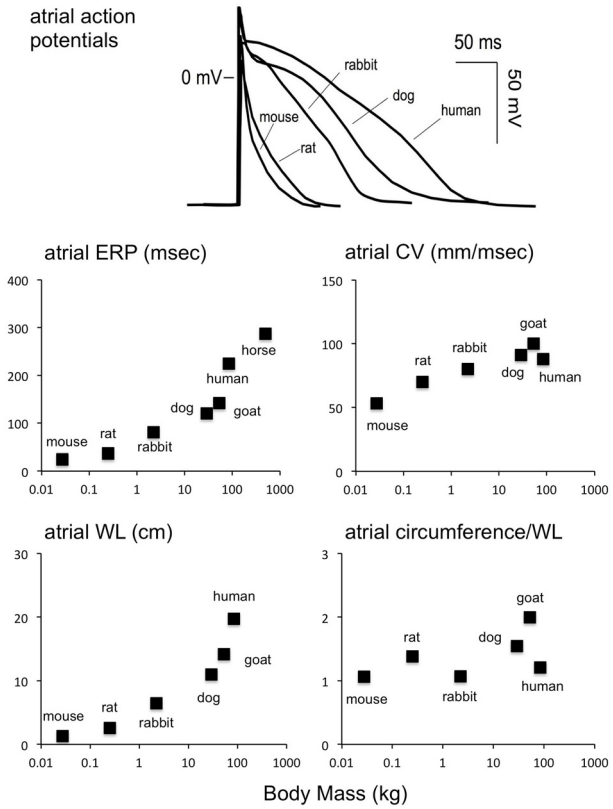

FIGURE 2 | Scaling of atrial electrophysiology to body mass. Upper panel: atrial action potentials in various species (Schotten et al., 2011). Middle row, left panel: atrial ERP as a function of body mass (mouse Verheule et al., 1999, rat Chang et al., 2002, rabbit Fedorov et al., 2007, dog Fareh et al., 1998, goat Blaauw et al., 2004, human Roberts-Thomson et al., 2009, horse Van Loon et al., 2002). Middle row, right panel: atrial CV as a function of body mass [mouse Leaf et al., 2008, rat Haugan et al., 2005, rabbit de Groot et al., 2003, dog Fareh et al., 1998, goat Verheule et al., 2010, human Hansson et al., 1998, horse (NA)]. Bottom row, left panel: wavelength (product of CV and ERP) as a function of body mass. Bottom row, right panel: ratio of atrial circumference to wavelength. The atrial circumference was calculated by taking the LA diameter and approximating the total circumference as 2*2*π*LA radius (mouse Blana et al., 2010, rat Boixel et al., 2003, rabbit Hirose et al., 2005, dog Stepien et al., 1998, goat Wijffels et al., 1997, human Monnig et al., 2005, horse Menzies-Gow et al., 2005). Interestingly, the number of reentrant waves that can fit on the atrial circumference does not scale strongly with the body mass.

1.2 cm in for the atrium, close to the normal estimated atrial circumference (**Figure 2**). However, this same argument holds true for larger species, as the ratio between estimated atrial circumference to WL is almost completely independent of body mass (**Figure 2**). According to the WL theory, a decrease in CV or ERP would make reentry more likely. Indeed, shortening the ERP by applying acetylcholine increases AF stability in mice, as it does in larger species (Kovoor et al., 2001; Wakimoto et al., 2001).

A shorter WL would also allow more fibrillation waves to propagate simultaneously within the atria or ventricles. According to the "multiple wavelet theory," fibrillation can persist without any particular site or circuit dominating the arrhythmia, as

long as a sufficiently large number of irregularly propagating fibrillation waves can coexist within a substrate (Allessie, 1998). The likelihood of multiple wavelet reentry increases when WL becomes smaller (Rensma et al., 1988; Allessie et al., 1996). However, several studies have shown that structural inhomogeneities can lead to "zig-zag" conduction, allowing "microreentry" to occur in much smaller circuits than would be expected based on calculation of the macroscopically determined WL (de Bakker et al., 1993; Spach and Josephson, 1994; Koura et al., 2002). To date, there is no direct evidence that "anarchical" multiple wavelet reentry can occur in mouse hearts. The small substrate size represented by the mouse heart may limit the number of coexisting fibrillation waves and thus make multiple wavelet reentry less likely. For example, the degree of fibrosis necessary to create a substrate for multiple wavelet reentry in a mouse heart may have to be larger than that required in a larger heart. AF stability was indeed greatly increased in a mouse model of selective atrial fibrosis (Verheule et al., 2004), but fibrillatory conduction in this model may be based on triggered activity rather than multiple wavelet reentry (Choi et al., 2012).

Spiral wave reentry is a specific form of reentry that has been described mathematically and observed experimentally in several types of excitable media (Jalife et al., 2002; Comtois et al., 2005). Vaidya et al. demonstrated sustained spiral wave reentry around a single core in murine ventricles with a period of wavefront rotation of 72 ms and frequency of 14 Hz (Vaidya et al., 1999). This study also detected figure-of-8 reentry with a rotation period of 66 ms, demonstrating that the murine ventricle is able to sustain two stable reentrant waves (Vaidya et al., 1999). It is important to note that calculated wavelengths in this study were much larger than size of the mouse heart and that APD showed almost no frequency dependence (Vaidya et al., 1999). Computer simulations suggested that the core during vortex reentry altered repolarization in adjacent myocardium, leading to reduced APDs near the core and longer APDs at larger distance to the core (Beaumont et al., 1998). This hypothesis was supported by the observation that during occurrence of spiral wave reentry, the average APD of the entire preparation was reduced and that pacing at the frequency of the spiral wave produced longer APDs (Vaidya et al., 1999). Thus, wavelength calculations based on external electrical stimulation probably have a poor predictive value for generation and maintenance of spiral wave reentry in the murine heart (Vaidya et al., 1999).

Studies in mammalian species from mouse to horse show scaling of VF frequency with BM, following the equation $VF_{frequency} = 18.9 \times BM^{-1/4}$ (Noujaim et al., 2007). Noujaim et al. demonstrated stable VF with vortex-like reentry and highest dominant frequency (DF) in the mouse heart with 38 Hz and reduction of DF with growing BM, as reflected by a DF of 6.8 Hz in the human heart (Noujaim et al., 2007). However, in larger mammalian hearts, the rapid activity of a rotor in one region does not conduct in a 1:1 fashion to the rest of the substrate. In most cases, and certainly within the complex anatomy of the atria, the waves emanating from that "mother" rotor will break up and spin off "daughter" wavelets to the rest of the atrium (Chen et al., 2000). Thus, the overall conduction pattern will be characterized by interaction of the rotor and fibrillatory waves in the rest of the substrate. When a rotor is present in a relatively small medium, the interaction between the rotor and the rest of the substrate is therefore likely to be different (Zou et al., 2005).

ECTOPIC ACTIVITY

Cellular proarrhythmic events can give rise to ectopic activity. Accelerated diastolic depolarization of myocytes with pacemaker activity can cause a latent pacemaker region to act as an ectopic focus (enhanced automaticity). By contrast, triggered activity results from "afterdepolarizations" triggered by a normal action potential. Early afterdepolarizations (EADs) develop during phase 2–3 of the AP and are precipitated by a prolonged APD during which the L-type Ca^{2+} channels recover and switch from the inactivated to the open state to produce a depolarizing current (Sabir et al., 2008). Delayed afterdepolarizations (DADs) occur during phase 4 of the AP in situations of intracellular Ca^{2+} overload, evoked by e.g., beta-adrenergic stimulation, ischemia, and hypokalaemia. In this case, the excess of intracellular Ca^{2+} is transported by the NCX, causing a depolarizing current (Schotten et al., 2011). As discussed in section III, to function at high heart rates, mouse myocytes have a short action potential and some differences in calcium handling compared to myocytes from larger mammalian hearts, and these factors may affect the generation of EADs and DADs. Nevertheless, several mouse models have shown arrhythmias initiated by afterdepolarizations (see e.g., Fabritz et al., 2003; Killeen et al., 2007a; Choi et al., 2012; Li et al., 2012; Pott et al., 2012).

Regardless of its exact nature (diastolic depolarization, early or delayed after depolarization), a proarrhythmic mechanism active in a single myocyte is unlikely to lead to a propagated response in the intact tissue, because the current generated by that myocyte will leak away to its neighbors without reaching the action potential threshold. Thus, the occurrence of a proarrhythmic event will have to be synchronized in a sufficiently large group of myocytes in order to reach the threshold and be able to propagate away from that area. This holds true both for an ectopic pacemaker region (Joyner et al., 2000) and for an area displaying triggered activity (Sato et al., 2009). Does an ectopic focus in a mouse heart have to same relative size to the rest of the heart as a focus in larger mammals? Mouse ventricular myocytes (90 μm in length, 14 μm in diameter) (Toischer et al., 2010) are smaller than dog myocytes (130 μm in length, 30 μm in diameter) (Clemo et al., 1998). Assuming myocytes to be roughly cylindrical, this would translate to a ratio of 8:1 in myocyte volume between dogs and mice. While considerable, this ratio is much smaller than the 1100:1 ratio in ventricular weight (or volume) between these species (Bienvenu and Drolet, 1991), implying that the mouse ventricle contains far fewer myocytes (by about a factor 150 in this crude estimate). No direct measurements of electrotonic interactions in mouse ventricles are available, but at 0.6 m/s, the conduction velocity in mouse ventricles is close to that in larger species, suggesting that electrotonic interactions are comparable (Gutstein et al., 2001). These indirect arguments would indicate that an ectopic focus in mouse ventricles may not be much smaller than that in larger hearts, and would thus occupy a relatively larger area of the heart. During rapid focal activity, this size-relation would affect the pattern of fibrillation (i.e., wavebreak). In addition, the small size of the mouse heart would limit the number of localized arrhythmogenic sources that can coexist. Propagation from an ectopic

focus is most likely when electrical coupling gradually increases from the focus to the surrounding muscle, because a high degree of electrical coupling would effectively silence the focus (Joyner et al., 2007). Thus, structural remodeling (e.g., fibrosis) would allow a smaller focus to successfully conduct its rapid rate to the rest of the heart.

Apart from the role of cardiac size, various other caveats apply to extrapolation of arrhythmias in mouse models to clinically observed human arrhythmias. A very high degree of overexpression or underexpression of a particular gene may lead to indirect alterations that would not be observed as a result of more moderately altered expression of that gene in human pathology. In addition, overexpression or deletion of a gene during the prenatal period may lead to a disruption of cardiac development and result in secondary changes that do not reflect the role of that gene in the adult human heart. All cautionary considerations notwithstanding, mouse models have displayed numerous types of arrhythmias that bear a striking electrocardiographic resemblance to human arrhythmias. These include various tachyarrhythmias such as atrial flutter (Zhang et al., 2005), atrial fibrillation (Verheule et al., 2004), (polymorphic) ventricular tachycardia (Fabritz et al., 2003; van Rijen et al., 2004; Cerrone et al., 2007) and ventricular fibrillation (Cerrone et al., 2005), bradyarrhythmias such as sinus bradycardia (Lei et al., 2005) and conduction disorders such as AV block (Zhang et al., 2011), bundle branch block (van Rijen et al., 2001), accessory atrioventricular pathways (Arad et al., 2003) and long QT (Salama and London, 2007; Ruan et al., 2009; Sanguinetti, 2010) and Brugada (Martin et al., 2010) syndromes.

CONCLUSIONS

Over the last two decades, mice have become a widely used model instrument in research into cardiac arrhythmias. Progress in miniaturization of techniques has allowed detailed studies of electrophysiological parameters of the murine heart. Genetic engineering has allowed the generation of mouse models carrying human mutations that cause arrhythmias and has contributed to understanding the role of specific genes, proteins and ionic channels in arrhythmogenesis. In general, mouse models have broadened our knowledge of cardiac electrophysiology and mechanisms underlying atrial and ventricular arrhythmias in humans. Mouse models can mimic human cardiac arrhythmias despite differences in cardiac electrophysiology. However, some mechanisms of arrhythmias in mice may differ from those in humans and therefore have to be extrapolated to the human situation with caution.

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