

## Mouse ERG K<sup>+</sup> Channel Clones Reveal Differences in Protein Trafficking and Function

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**Background**—The mouse ether-a-go-go-related gene 1a (*mERG1a*, *mKCNH2*) encodes mERG K<sup>+</sup> channels in mouse cardiomyocytes. The mERG channels and their human analogue, hERG channels, conduct I<sub>Kr</sub>. Mutations in hERG channels reduce I<sub>Kr</sub> to cause congenital long-QT syndrome type 2, mostly by decreasing surface membrane expression of trafficking-deficient channels. Three cDNA sequences were originally reported for mERG channels that differ by 1 to 4 amino acid residues (mERG-London, mERG-Waterston, and mERG-Nie). We characterized these mERG channels to test the postulation that they would differ in their protein trafficking and biophysical function, based on previous findings in long-QT syndrome type 2.

**Methods and Results**—The 3 mERG and hERG channels were expressed in HEK293 cells and neonatal mouse cardiomyocytes and were studied using Western blot and whole-cell patch clamp. We then compared our findings with the recent sequencing results in the Wellcome Trust Sanger Institute Mouse Genomes Project (WTSIMGP).

**Conclusions**—First, the mERG-London channel with amino acid substitutions in regions of highly ordered structure is trafficking deficient and undergoes temperature-dependent and pharmacological correction of its trafficking deficiency. Second, the voltage dependence of channel gating would be different for the 3 mERG channels. Third, compared with the WTSIMGP data set, the mERG-Nie clone is likely to represent the wild-type mouse sequence and physiology. Fourth, the WTSIMGP analysis suggests that substrain-specific sequence differences in mERG are a common finding in mice. These findings with mERG channels support previous findings with hERG channel structure–function analyses in long-QT syndrome type 2, in which sequence changes in regions of highly ordered structure are likely to result in abnormal protein trafficking. (*J Am Heart Assoc.* 2014;3:e001491 doi: 10.1161/JAHA.114.001491)

**Key Words:** genetic variability • hERG • long-QT syndrome • mERG • mouse

The mouse ether-a-go-go-related gene 1a (*mERG1a* or *mKCNH2*) and its human analogue (*hERG1a* or *hKCNH2*) encode the  $\alpha$ -subunits that assemble to form rapidly activating delayed rectifier K<sup>+</sup> (ERG or Kv11.1) channels.<sup>1–3</sup> In human heart, hERG (Kv11.1) channels conduct a K<sup>+</sup> current, I<sub>Kr</sub>, that is important for normal cardiac repolarization. Mutations that reduce I<sub>Kr</sub> cause congenital long-QT syndrome type 2 (LQT2)

to result in QT-interval lengthening on the electrocardiogram and increased arrhythmia risk.

LQT2-associated mutations, predominantly missense, are located throughout the hERG channel protein. The hERG channel protein contains regions of highly ordered structures (HOS;  $\alpha$ -helices and  $\beta$ -sheets) in transmembrane and pore domains and in the PAS domain and cyclic nucleotide binding domains in the N- and C-termini, respectively.<sup>4</sup> In addition, it has regions thought not to contain HOS in portions of the N- and C-termini and in some intra- and extracellular linker regions. Many LQT2 missense mutations in regions of HOS result in channels that fail to undergo normal protein trafficking to the surface membrane; rather, the mutated proteins are retained in intracellular compartments, including the endoplasmic reticulum, as misfolded proteins and are degraded.<sup>4–10</sup> Less is known about amino acid sequence changes in regions of non-HOS. These may result in channels that traffic normally but that reduce I<sub>Kr</sub> through altered electrophysiological (gating) properties<sup>11–13</sup> or in channels that gate normally, consistent with polymorphisms.<sup>8,14</sup> Consequently, the location of an amino acid substitution in the I<sub>Kr</sub>

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An accompanying Table S1 is available at <http://jaha.ahajournals.org/content/3/6/e001491/suppl/DC1>

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channel protein structure may provide information about its ability to traffic and function.

There were originally 3 reported *mERG1a* cDNA clones. The first was cloned from a commercial cDNA library by London and colleagues (mERG-London).<sup>3</sup> The second was cloned cDNA prepared from RNA extracted from mouse inner ear by Nie and colleagues (mERG-Nie).<sup>15</sup> A third *mERG1a* clone was derived from the original sequencing more than a decade ago of the mouse genome (mERG-Waterston, Mouse Genome Sequencing Consortium).<sup>16</sup> The mERG-London and mERG-Nie clones have been functionally expressed mainly in *Xenopus* oocytes,<sup>3,15,17,18</sup> whereas the mERG-Waterston clone has not been characterized. When the cDNA and amino acid sequences reported for these 3 mERG clones are compared, they are not identical; rather, they differ from each other by 1 to 4 amino acids (nonsynonymous nucleotide changes). The 3 mERG sequence changes and corresponding wild-type (WT) hERG sequence<sup>19,20</sup> are shown in Table 1. The mERG-London channel sequence contains 2 amino acid substitutions in regions of HOS (455 and 752). Based on findings with human LQT2 missense mutations,<sup>4,8,14</sup> we postulated that when cultured at physiological temperature, mERG-London channels should be trafficking deficient, whereas the mERG-Waterston and mERG-Nie channels should traffic to the cell membrane, and that the voltage dependence of channel gating for the 3 mERG channels would likely be different. We also compared the mERG channels expressed in human (HEK293) cells and mouse (neonatal cardiomyocyte) cells. Consequently, we used the 3 mouse ERG channels to test a structural model for channel trafficking derived from human LQT2 mutations. Finally, we compared our mERG structural findings with the recent Wellcome Trust Sanger Institute Mouse Genomes Project (WTSIMGP) sequencing results.

**Table 1.** Amino Acid Sequence Differences Among mERG Clones

Channel	Amino Acid 455 (in S2 HOS)	Amino Acid 522 (in S3 to S4 Linker)	Amino Acid 752 (in cNBD HOS)	Amino Acid 1006 (C-Terminus)
mERG-London	T455	L522	Y752	N1006
mERG-Nie	A455	L522	C752	D1006
mERG-Waterston	A455	V522	C752	D1006
WT hERG*	A453	L520	C750	D1003

cNBD indicates cyclic nucleotide binding domain; hERG, human ether-a-go-go-related gene; HOS, highly ordered structure; mERG, mouse ether-a-go-go-related gene; WT, wild type.

\*Equivalent WT hERG amino acid position.

## Materials and Methods

### cDNA Constructs

The mERG-London cDNA was graciously provided as a gift by Amber Pond (Purdue University, West Lafayette, IN) and Barry London, MD, PhD (University of Iowa, Iowa City, IA). The mERG-Nie cDNA was graciously provided as a gift from Ebenezer Yamoah (University of California Davis, Davis, CA). The mERG-Waterston sequence was obtained from the Mouse Genome Sequencing Consortium.<sup>16</sup> The mERG-Waterston clone was generated by performing site-directed mutagenesis of the mERG-Nie cDNA using the QuikChange Lightning Site-directed Mutagenesis Kit (Stratagene). Clone sequences were verified using DNA sequencing analysis (University of Wisconsin Biotechnology Center, Madison, WI). Synthesized oligonucleotide primers (Integrated DNA Technologies) were used to clone the WT hERG, mERG-London, mERG-Nie, and mERG-Waterston constructs into the pcDNA 3.1/V5-His mammalian expression vector using the TOPO TA cloning method (Invitrogen). Endotoxin-free cDNA constructs were created for expression in neonatal mouse cardiomyocytes using the EndoFree Plasmid Maxi Kit (Qiagen).

The amino acid and nucleotide changes between the 3 mERG clones are as follows (Table 1). For amino acid 455, mERG-London contains a threonine (T455, nucleotide codon ACT), whereas mERG-Nie and mERG-Waterston contain an alanine (A455, nucleotide codon GCT). For amino acid 522, mERG-Waterston contains a valine (V522, nucleotide codon GTG), whereas mERG-London and mERG-Nie contain a leucine (L522, nucleotide codons CTC and CTG, respectively). For amino acid 752, mERG-London contains a tyrosine (Y752, nucleotide codon TAC), whereas mERG-Nie and mERG-Waterston contain a cysteine (C752, nucleotide codon TGC). For amino acid 1006, mERG-London contains an asparagine (N1006, nucleotide codon AAC), whereas mERG-Nie and mERG-Waterston contain an aspartate (D1006, nucleotide codon GAC).

### Isolation of Neonatal Mouse Cardiomyocytes

Neonatal mouse cardiomyocytes (Taconic Biosciences, Hudson, NY, strain SV129 (129S6/SvEvTac)) were isolated from 1- to 2-day-old pups, as previously published.<sup>21</sup> The animal care and use protocol was approved by the University of Wisconsin-Madison Research Animal Resources Center and met National Institutes of Health guidelines for the health and well-being of the animals. Heart tissue was dissociated into individual cells by digesting 5 times with Hank's Balanced Salt Solution (Mediatech) supplemented with 100 µg/mL collagenase type II (Invitrogen), 1 mg/mL pancreatin (Sigma-Aldrich), and 10% horse serum (Invitrogen). Cells were preplated 3

times in succession for 1 hour each to remove cell debris and fibroblasts.

## Cell Culture and Transfection

HEK293 cells were transiently transfected with 3 µg cDNA (empty vector for control, WT hERG, mERG-London, mERG-Nie, or mERG-Waterston) using Lipofectamine 2000 reagent (Invitrogen). Cells were cultured at either 27°C or 37°C in 5% CO<sub>2</sub> and Minimal Essential Media supplemented with 10% FBS, 1 mmol/L sodium pyruvate, 1 mmol/L nonessential amino acid, and 1% penicillin streptomycin (Invitrogen). Cells were washed 24 hours after transfection and studied after 48 hours of transfection. Neonatal mouse cardiomyocytes were transfected with 5 µg endotoxin-free ERG cDNA (hERG or mERG) and 1 µg green fluorescent protein using an electroporation method.<sup>21</sup> The cardiomyocytes were then plated on laminin-coated plates for Western blot analysis or laminin-coated coverslips for patch-clamp analysis. Neonatal mouse cardiomyocytes were washed 24 hours after electroporation and cultured, as described previously.<sup>21</sup> For some experiments, E-4031 (Alomone Labs) was added to the medium (10 µmol/L) for 24 hours and washed out 1 hour to 2 hours before patch-clamp study.

## Western Blot

HEK293 cells or neonatal mouse cardiomyocytes were washed with PBS and solubilized using a lysis buffer containing 5 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 10% glycerol, (Sigma-Aldrich), 1% Nonidet P-40 (Thermo Scientific), and Complete mini protease inhibitors (Roche). Whole-cell lysates were then collected and centrifuged, and protein quantification was performed using the DC Protein Assay Kit II (Bio-Rad Laboratories). Lysates were prepared and loaded on 7% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes, as described previously.<sup>21</sup> Nonspecific binding sites were blocked using 5% nonfat dry milk and 0.1% Tween-20 in PBS. Membranes were probed with a rabbit anti-ERG primary antibody (1:10 000) containing a C-terminal ERG epitope<sup>5</sup> and then washed with 0.1% Tween-20 in PBS to remove excess antibody. Membranes were probed with a goat anti-rabbit IgG horse radish peroxidase secondary antibody (1:10 000; GE Healthcare Biosciences). Protein bands were visualized using ECL Western blotting detection reagents (GE Healthcare Biosciences).

## Densitometry

Quantitative analysis of hERG or mERG protein bands detected on Western blot was performed using the GS 700

Imaging Densitometer and Molecular Analyst software (Bio-Rad Laboratories). The density of a control lane (empty pcDNA 3.1/V5-His vector) was analyzed and set as the background value. The density of hERG or mERG 135- and 155-kDa protein bands was then analyzed, and the density ratio value (155-kDa protein-band density/135-kDa protein-band density) was generated for each lane on each Western blot.

## Patch Clamp

Functional analysis was performed using the whole-cell patch-clamp method at room temperature (22–23°C) within 3 hours after cells were removed from culture conditions. Pipettes had resistance between 1.5 and 2.5 MΩ when filled with intracellular solution containing 130 mmol/L KCl, 1 mmol/L MgCl<sub>2</sub>, 5 mmol/L EGTA, 5 mmol/L MgATP, and 10 mmol/L HEPES (pH 7.2 by KOH). The extracellular solution contained 137 mmol/L NaCl, 1 mmol/L MgCl<sub>2</sub>, 1.8 mmol/L CaCl<sub>2</sub>, 4 mmol/L KCl, 10 mmol/L glucose, and 10 mmol/L HEPES (pH 7.4 by NaOH). An Axopatch 200B amplifier (Molecular Devices) was used to record membrane currents. Clampfit 10.2 (MDS Analytical Technologies) was used to fit peak tail current amplitudes to a Boltzmann distribution.

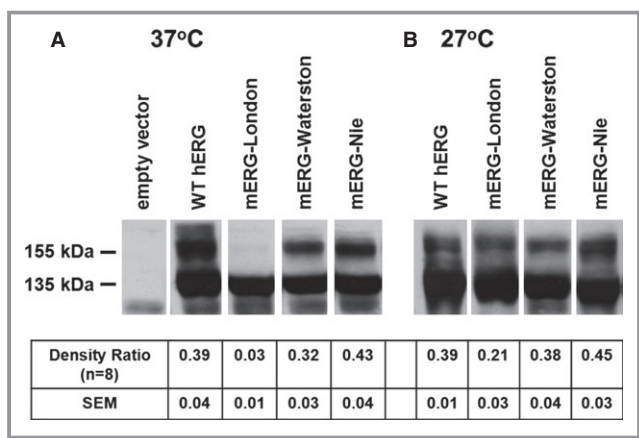
## Data Analysis

Data were analyzed in Origin 7.5 software (OriginLab) and are given as mean±SEM. We performed Shapiro–Wilk tests to assess the normality of each data set. Two-way ANOVA was performed when comparing the different culture conditions between the mERG clones and WT hERG, and the Student *t* test was performed within the WT hERG or mERG clones to test for differences. For both 2-way ANOVA and Student *t* test, *P*<0.05 was considered statistically significant.

## Results

### hERG and mERG Expression in HEK293 Cells

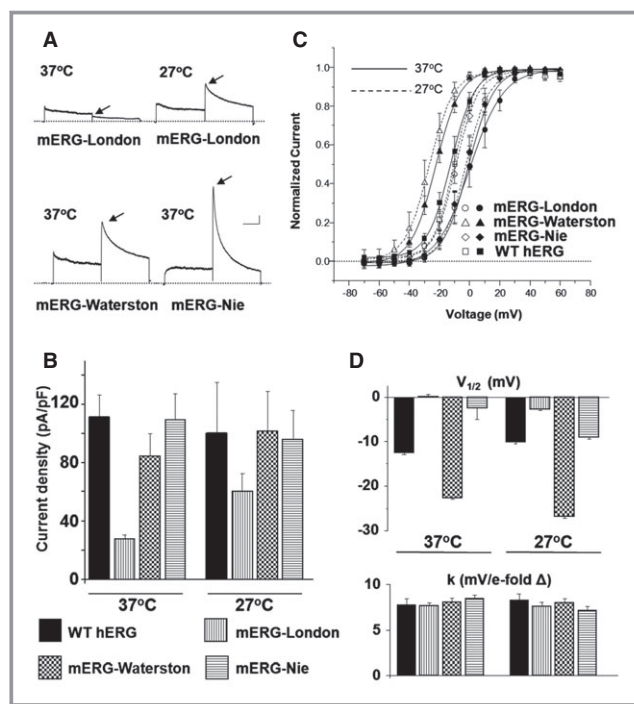
WT hERG channel proteins are synthesized as core-glycosylated, immature, 135-kDa subunits in the endoplasmic reticulum and assembled as tetramers to form channels and are transported (traffic) through the Golgi, where they undergo complex glycosylation to the mature 155-kDa protein found in the cell surface membrane.<sup>5</sup> The N-linked glycosylation consensus motif of N-X-S/T in hERG channel (amino acids N598-S599-S600)<sup>22,23</sup> is also intact in mERG channels.<sup>17</sup> Figure 1A shows a representative Western blot analysis of empty vector, WT hERG, mERG-London, mERG-Waterston, and mERG-Nie channel proteins expressed in HEK293 cells cultured at 37°C. WT hERG, mERG-Waterston,



**Figure 1.** Western blot analysis of HEK293 cells expressing ERG channels. A, Empty vector and ERG clone transfected cells cultured at 37°C. B, ERG clone transfected cells cultured at 27°C. The table shows the ratio of upper (155-kDa) band image density to the lower (135-kDa) band image density. hERG indicates human ether-a-go-go-related gene; mERG, mouse ether-a-go-go-related gene; WT, wild type.

and mERG-Nie lanes show ERG protein bands at 135 and 155 kDa, consistent with the immature and mature ERG channel proteins, respectively. In contrast, mERG-London lane shows a single 135-kDa band for immature ERG protein. The presence of the solitary immature band on Western blot analysis has been associated previously with trafficking-deficient hERG channels found in LQT2 mutations.<sup>5</sup> Many missense LQT2 channels can undergo correction of their trafficking-deficient phenotype including by culturing cells at reduced temperature.<sup>8,24–26</sup> Figure 1B shows a representative Western blot analysis of WT hERG, mERG-London, mERG-Waterston, and mERG-Nie ERG channel proteins expressed in HEK293 cells cultured at 27°C for 24 hours. With this, WT hERG, mERG-London, mERG-Waterston, and mERG-Nie lanes all show the 135- and 155-kDa ERG protein bands. We performed densitometry measurements of the ratio of 155- to 135-kDa band density (n=8 each), shown in the lower part of Figure 1. These data confirm that the 155-kDa band is virtually absent for mERG-London cultured at 37°C (density ratio of 0.03) and that this band increased following culture at 27°C (density ratio of 0.21,  $P<0.05$ ). These data suggest that the mERG-London channel protein, which contains 2 amino acid sequence substitutions located in regions of HOS, is trafficking deficient in HEK293 cells cultured at 37°C and that the trafficking abnormality can undergo correction by culturing at reduced temperature.

Electrophysiological results with the ERG channels expressed in HEK293 cells are shown in Figure 2. Figure 2A shows representative current traces for mERG-London channels expressed in HEK293 cells cultured at 37°C and 27°C (upper traces). From the holding potential of -80 mV, cells were depolarized to 20 mV for 3 seconds and repolarized to



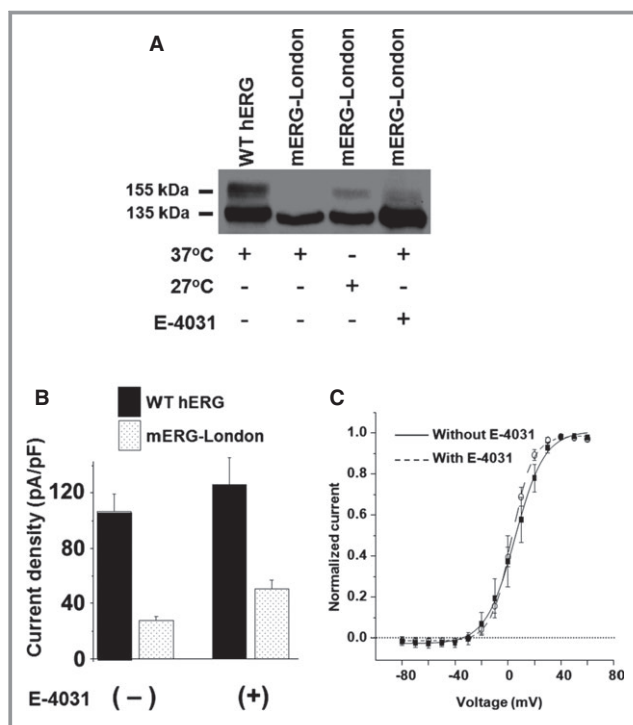
**Figure 2.** Electrophysiological properties of ERG channels in HEK293 cells. A, Representative current recordings of mERG-London cultured at 37°C and 27°C (top) and mERG-Waterston and mERG-Nie cultured at 37°C (bottom). The arrows indicate peak tail current. Dotted line indicates 0 current. Scale is 200 pA by 1 second. B, The mean peak tail current is shown for WT hERG and the mERG channels cultured at 37°C and 27°C (n=6 for each recording). C, I-to-V relations for activation plotted for WT hERG and mERG channels cultured at 37°C and 27°C. Dotted line indicates 0 current. D,  $V_{1/2}$ , and k values of the I-to-V activation relations for WT hERG and mERG channels cultured at 37°C and 27°C. hERG indicates human ether-a-go-go-related gene; mERG, mouse ether-a-go-go-related gene; WT, wild type.

-50 mV for 3 seconds to record tail current (arrow). After culture at 27°C for 24 hours, tail current amplitude of mERG-London channels was increased, confirming that the trafficking abnormality undergoes correction at reduced culture temperature. Figure 2A also shows a representative current trace for mERG-Waterston and mERG-Nie (lower traces) channels cultured at 37°C. Figure 2B shows the mean peak tail current density for WT hERG, mERG-London, mERG-Waterston, and mERG-Nie channels cultured at 37°C or 27°C. The peak tail current density of mERG-London channels was  $27.7 \pm 2.9$  picoamperes per picofarad (pA/pF) at 37°C and increased ~2-fold to  $59.7 \pm 12.7$  pA/pF with culture at 27°C ( $P<0.05$ ). The peak tail current densities of WT hERG, mERG-Nie, and mERG-Waterston channels were similar and did not change with culture temperature ( $P>0.05$ ). Figure 2C shows the activation relations for WT hERG, mERG-London, mERG-Waterston, and mERG-Nie channels cultured at 37°C or 27°C. For each experiment, cells were held at -80 mV, and test steps were applied to -70 to 60 mV in 10 mV increments for



3 seconds, followed by a step to -50 mV for 3 seconds to elicit tail current with the pulse sequence repeated every 10 seconds. For each condition, the peak tail current was normalized to the maximal current and plotted against the test voltage and fit with a Boltzmann distribution to obtain the voltage for half-maximum activation ( $V_{1/2}$ ) and slope factor ( $k$ ) values. The  $V_{1/2}$  and  $k$  values are plotted in Figure 2D and summarized in Table 2. The data show that each channel activates over a different voltage range but with nearly identical slope factors, and culture temperature has little effect.

We also studied whether the mERG-London channels' trafficking abnormality could undergo pharmacological correction (ie, rescue), similar to many missense LQT2 channels.<sup>4,8,26</sup> Figure 3 shows a Western blot analysis of HEK293 cells expressing WT hERG cultured at 37°C (lane 1), mERG-London cultured at 37°C (lane 2), mERG-London cultured at 27°C for 24 hours (lane 3), and mERG-London cultured at 37°C in 10 μmol/L E-4031 for 24 hours (lane 4). The blot shows that the 135-kDa protein band was present for all lanes. For WT hERG, a 155-kDa band was present. For mERG-London at 37°C, the 155-kDa protein band was absent, whereas with culture for 24 hours at 27°C or at 37°C in E-4031, faint 155-kDa protein bands were present. The findings with E-4031 suggest that the mERG-London channel protein undergoes pharmacological rescue (n=3). Figure 3B shows the mean peak tail current density of WT hERG and mERG-London cultured for 24 hours at 37°C without or with E-4031 followed by drug washout for 1 to 2 hours. The voltage protocol was the same used for Figure 2B. Following culture with E-4031 for 24 hours and 1 to 2 hours of drug washout, the peak tail current density of mERG-London channels increased nearly 2-fold (27.7±7.0 to 50.6±6.4 pA/pF, respectively,  $P<0.05$ ), whereas WT hERG channels did not change significantly (106.6±13.2 and 126.2±19.5 pA/pF, respectively;  $P>0.05$ ). Figure 3C shows normalized activation I-V plots of mERG-London channels without and with culture in E-4031 studied using the same voltage protocol used for Figure 2C, and the results show that E-4031 has minimal effects on mERG-London channel-activation gating ( $V_{1/2}$  of 4.6±4.6 and 2.9±1.5 mV,  $P>0.05$ ;  $k$  of 7.7±0.4 and 7.4±0.2 mV/e-fold  $\Delta$ ,  $P>0.05$ , respectively).



**Figure 3.** Pharmacological correction (ie, rescue) of mERG-London. A, A representative Western blot analysis of HEK293 cells expressing WT hERG and the mERG-London channel for different culture conditions. B, Mean peak tail current density of WT hERG and mERG-London without culture (n=6) and with culture in E-4031 (n=4). C, I-to-V relation for activation of mERG-London cultured without and with E-4031 followed by 1 to 2 hours of drug washout (n=4). Dotted line indicates 0 current. hERG indicates human ether-a-go-go-related gene; mERG, mouse ether-a-go-go-related gene; pS/pF, picoamperes per picofarad; WT, wild type.

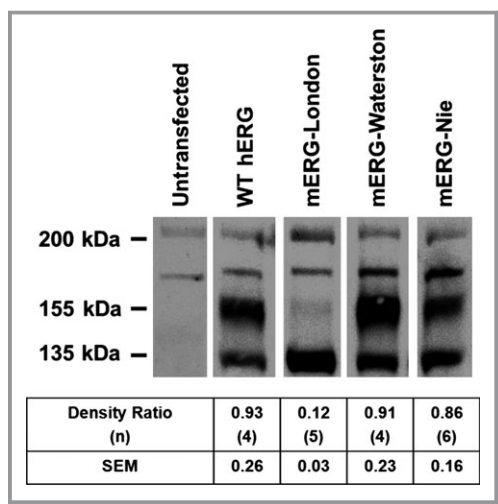
### hERG and mERG Expression in Neonatal Mouse Cardiomyocytes

We next studied ERG overexpression in mouse cardiomyocytes. Figure 4 shows a representative Western blot analysis of untransfected, WT hERG, mERG-London, mERG-Waterston, and mERG-Nie channel proteins expressed in neonatal mouse cardiomyocytes cultured at 37°C. Untransfected cardiomyo-

**Table 2.** Voltage Dependence of ERG Activation in HEK293 Cells

Culture Temperature	WT hERG		mERG-London		mERG-Waterston		mERG-Nie	
	37°C	27°C	37°C	27°C	37°C	27°C	37°C	27°C
$V_{1/2}$ (mV)	-12.5±0.4	-10.2±0.3	0.1±0.5	-2.7±0.4	-22.7±0.4	-26.8±0.4	-2.5±2.5	-9.1±0.3
$k$ (mV/e-fold $\Delta$ )	7.8±0.6	8.3±0.7	7.3±0.4	7.6±0.4	8.1±0.4	8.0±0.5	8.4±0.4	7.2±0.4
n	4	6	10	6	7	8	7	6

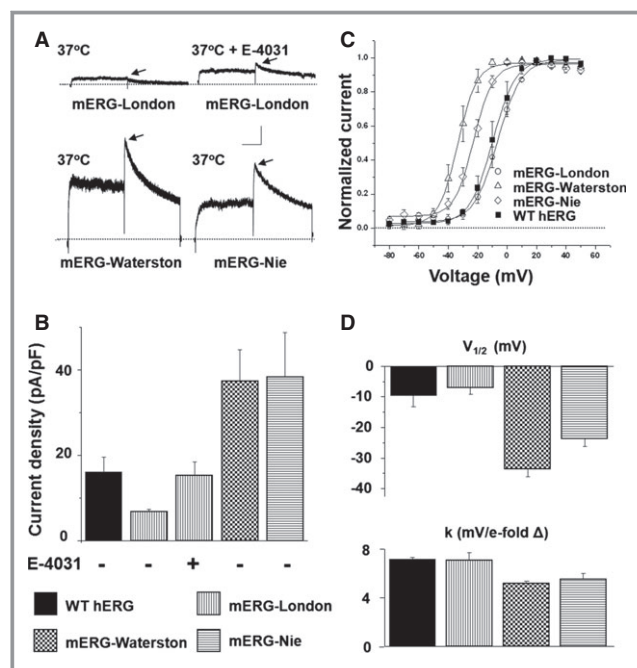
hERG indicates human ether-a-go-go-related Gene; mERG, mouse ether-a-go-go-related gene.



**Figure 4.** Western blot analysis of neonatal cardiomyocytes expressing WT hERG and mERG channels. All cardiomyocytes express 165- and 205-kDa protein bands. Transfection with WT hERG, mERG-Waterston, and mERG-Nie resulted in 135- and 155-kDa bands, whereas transfection with mERG-London showed only the 135-kDa band. The density ratio analysis of 155- to 135-kDa bands is shown below the Western blots. hERG indicates human ether-a-go-go-related gene; mERG, mouse ether-a-go-go-related gene; WT, wild type.

cytes express 2 protein bands at 205 and 165 kDa that have been previously suggested to represent mERG1a.<sup>17,21,27</sup> We have previously shown that these cultured cardiomyocytes express a small-amplitude  $I_{Kr}$ ,<sup>21</sup> although  $I_{Kr}$  contributes minimally to repolarization in the mouse heart.<sup>28</sup> Expression of WT hERG, mERG-Waterston, and mERG-Nie channels resulted in additional protein bands at 135 and 155 kDa, whereas expression of mERG-London channels resulted in a 135-kDa protein band with a minimal 155-kDa band. Densitometry analysis of the ratio of 155- to 135-kDa band density for the transfected cardiomyocytes is shown below the Western blot. As with HEK293 cells, WT hERG, mERG-Waterston, and mERG-Nie channels express both core and complexly glycosylated proteins, whereas the mERG-London channel protein undergoes minimal complex glycosylation, suggesting that it retains a trafficking-deficient phenotype when expressed in neonatal mouse cardiomyocytes.

Electrophysiological results are shown in Figure 5. Figure 5A shows the effect of culturing neonatal mouse cardiomyocytes expressing mERG-London channels at 37°C without and with E-4031 for 24 hours followed by 1 to 2 hours of drug washout (upper traces). From a holding potential of -60 mV to inactivate the most inward current, a depolarizing step to 20 mV was applied for 3 seconds followed by repolarization to -50 mV to record tail current (arrow). Culture in E-4031 increased the tail current amplitude.



**Figure 5.** Electrophysiological properties of ERG channels in neonatal mouse cardiomyocytes. A, mERG-London channel current traces at 37°C for control recordings and after 24 hours of culture in 10  $\mu$ M E-4031 followed by drug washout for 1 to 2 hours (top traces) and control recordings for mERG-Waterston and mERG-Nie channels (bottom traces). The arrows indicate peak tail current. Dotted line indicates 0 current. Scale is 100 pA by 1 second. B, Peak tail current density for WT hERG, mERG-London (without and with culture in E-4031), mERG-Waterston, and mERG-Nie (n=6, 6, 8, 7, 4, respectively). C, I-to-V relations for activation plotted of WT hERG and mERG channels cultured at 37°C. Solid line indicates 0 current. Normalized peak tail I-V plot for WT hERG, mERG-London, mERG-Waterston, and mERG-Nie. Dotted line indicates 0 current. D,  $V_{1/2}$  and k values of the I-to-V activation relations for WT hERG and mERG channels cultured at 37°C. hERG indicates human ether-a-go-go-related gene; mERG, mouse ether-a-go-go-related gene; WT, wild type.

Figure 5A also shows representative current recordings of mERG-Waterston and mERG-Nie channels expressed in cardiomyocytes (lower traces). Figure 5B shows the mean peak tail current density for WT hERG, mERG-London channels without culture in E-4031, mERG-London channels after culture in E-4031 followed by 1 to 2 hours of drug washout, mERG-Waterston and mERG-Nie channels, all cultured at 37°C. The mean peak tail current densities for mERG-London channels without and with culture in E-4031 increased ~2-fold ( $6.8 \pm 0.6$  to  $15.3 \pm 3.2$  pA/pF, respectively), showing pharmacological rescue of mERG-London channels in cardiomyocytes. Figure 5B also shows that the current densities of mERG-Waterston ( $37.3 \pm 7.4$  pA/pF) and mERG-Nie ( $38.3 \pm 10.4$  pA/pF) channels were larger than those found with mERG-London and WT hERG channels ( $16.0 \pm 3.2$  pA/pF). Figure 5C shows normalized activation I-V plots for WT

**Table 3.** Voltage Dependence of ERG Activation in Neonatal Mouse Cardiomyocytes

Proteins	WT hERG	mERG-London	mERG-Waterston	mERG-Nie
Culture temperature	37°C	37°C	37°C	37°C
V <sub>1/2</sub> (mV)	-9.9±0.5	-7.0±0.5	-33.7±0.4	-22.7±0.5
k (mV/e-fold Δ)	7.2±0.6	7.1±0.6	5.2±0.7	5.5±0.5
n	5	4	4	4

hERG indicates human ether-a-go-related gene; mERG, mouse ether-a-go-related gene.

hERG, mERG-London (without culture in E-4031), mERG-Waterston, and mERG-Nie channels using the voltage protocol shown in Figure 2C with the holding potential at -60 mV, and the data were fit with a Boltzmann distribution. The V<sub>1/2</sub> and k results are plotted in Figure 5D and summarized in Table 3. The voltage dependence of mERG-Waterston and mERG-Nie channels shifted negatively compared with WT hERG and mERG-London channels.

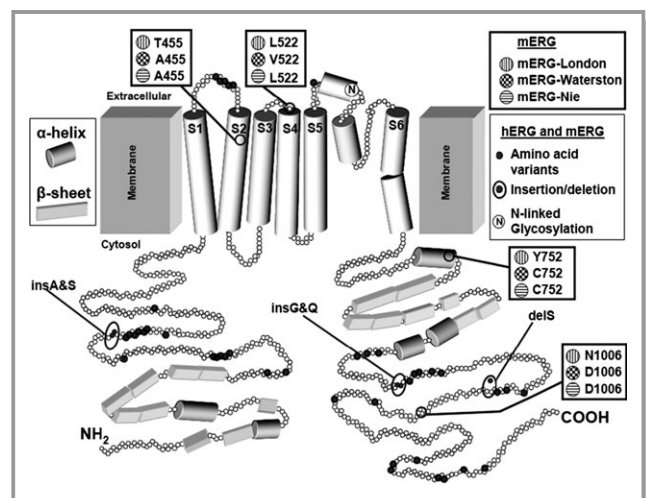
### Discussion

The structural model we tested predicts that hERG LQT2 missense mutations in regions of HOS generally result in abnormal protein trafficking to reduce channel expression in the surface membrane (reduced channel number), whereas amino acid substitutions in regions thought to lack HOS are more likely to traffic normally and alter channel gating or to have minimal or no effects (eg, polymorphisms).<sup>4,14</sup> Consequently, in this study, we hypothesized that mERG-London channels would be trafficking deficient, whereas mERG-Nie and mERG-Waterston channels would traffic to the cell membrane, and that the voltage dependence of channel gating was likely to be different for the 3 mERG channels, given the differing locations of amino acid substitutions. Some LQT2 missense mutations in non-HOS regions are known to result in channels that traffic normally but gate abnormally.<sup>6,9,11,13</sup> Anson and colleagues<sup>14</sup> and Anderson and colleagues<sup>8</sup> studied 8 hERG channel sequence variants in non-HOS regions in the N- and C-termini and the S1 to S2 linker and showed that these channels were WT-like with minimal to no effects on protein trafficking or channel gating consistent with their identification as putative polymorphisms. In addition, >30 hERG amino acid sequence variants thought to represent polymorphisms (see the Gene Connection for the Heart database at <http://www.fsm.it/cardmoc/>) similarly occur in the N- and C-termini mainly in non-HOS regions, although most have not been expressed and studied functionally.

Figure 6 shows a topology cartoon of a mERG1a α-subunit structure.<sup>4,8</sup> The amino acids in regions of HOS are represented by cylinders (α-helices) and bars (β-sheets),

whereas amino acids in non-HOS regions are represented by individual circles. The 3 mERG channels differ from each other at amino acids 455, 522, 752, and 1006 (Table 1), with only the mERG-London channel containing amino acid substitutions in regions of HOS (A455T and C752Y). There are additional differences between mouse and human ERG channels: The mERG channel sequence is 3 amino acids longer (1162 versus 1159) due to 2 insertions of 2 amino acids (insA&S, insG&Q) and a single amino acid deletion (delS), and the mERG and hERG α-subunits differ at an additional 42 amino acid positions located in non-HOS regions (see filled circles in Figure 6). Consequently, the mouse and human ERG channel sequences are highly conserved in regions of HOS but less so in regions thought to lack HOS.

When expressed in HEK293 cells and neonatal mouse cardiomyocytes at 37°C, the mERG-London channel was trafficking deficient, whereas both mERG-Nie and mERG-Waterston channels traffic similarly to WT hERG. The mERG-London channel trafficking abnormality also underwent temperature-dependent correction and pharmacological rescue. The mERG-Waterston and mERG-Nie channels differ by a single amino acid substitution located in the S3 to S4 linker



**Figure 6.** Putative topology map of a mERG1 α-subunit. Modified from Anderson et al<sup>4,8</sup>. hERG indicates human ether-a-go-related gene; mERG, mouse ether-a-go-related gene.

that conveys altered voltage dependence of activation. In hERG channels, engineered mutations in the S3 to S4 linker can alter the  $V_{1/2}$  for activation.<sup>29,30</sup> Our data also show that mERG-Nie channels, which have the most conserved sequence compared with WT hERG channels (differs by the 42 amino acid variants in non-HOS regions and the insertions and deletion), has molecular and functional phenotypes that most closely resemble WT hERG channels. Moreover, we found differences in the expression of ERG channels between HEK293 cells and neonatal mouse cardiomyocytes. A difference is the density ratio of the 155- to 135-kDa bands, which for trafficking-competent channels is close to unity in neonatal mouse cardiomyocytes (Figure 4) but appears to be lower in HEK293 cells (Figure 2). In HEK293 cells, peak tail current densities were larger, and the current densities for WT-hERG, mERG-Nie, and mERG-Waterston channels were similar (Figure 2), whereas in neonatal mouse cardiomyocytes, WT hERG channels generated much less current than the mERG-Nie and mERG-Waterston channels (Figure 5). A possibility is that the mouse ERG channel is optimized for expression in mouse cardiomyocytes, suggesting that the 42 amino acid variants in non-HOS regions and the insertions and deletion may modulate ERG protein surface membrane expression by other mechanisms.

Native mERG channels have been reported to express as 165- and 205-kDa proteins, which are larger molecular masses than hERG protein, and these differences have been proposed to represent more extensive post-translational protein processing.<sup>17,27</sup> In our experiments, mERG and hERG channels expressed as 135- and 155-kDa proteins. The 165- and 205-kDa protein expression pattern was present in all lanes including nontransfected cardiomyocytes (see also, Lin et al<sup>21</sup>). Furthermore, the presence of the 155-kDa band correlated with a robust tail current, similar to hERG channels.<sup>23</sup> These findings suggest that overexpressed mERG channels exhibit properties similar to overexpressed hERG channels.

Genetic variation between inbred mouse strains for some genes encoding ion channels is well recognized. The mouse *KCNJ10* gene, for example, which encodes a brain inward rectifier K<sup>+</sup> channel, has a single amino acid sequence variant that is strain dependent, and this conveys sensitivity to epilepsy in both mice and humans.<sup>31,32</sup> For cardiac ion channels, strain-dependent sequence changes were previously noted for mERG channels but were not investigated.<sup>3</sup> In the present study, our results show that nonsynonymous nucleotide changes in the *mERG1a* gene exert complex effects including the generation of a trafficking-deficient channel that undergoes temperature-dependent correction and pharmacological rescue. The mERG-London clone contains sequence substitutions in regions of HOS, and this may underlie its trafficking-deficient phenotype. Furthermore, the 3 mERG clones generate channels with different voltage

dependence of gating. These findings in mouse ERG channels are in agreement with previous results in human hERG LQT2 mutations and support the structural model we have proposed. Our findings also raise an intriguing question as to which, if any, mERG channel sequence represents the mouse WT phenotype. Each mouse ERG channel sequence we studied was derived from a different mouse inbred strain. The mERG-London channel was cloned from a cDNA library derived from the mouse strain BALB/c that was originally generated >90 years ago. The mERG-Nie channel was cloned using mouse strain C57BL, and the mERG-Waterston channel was derived from mouse strain C57BL/6J with these inbred strains developed >70 years ago. It is not clear whether these differences were present in the original founders for each strain or whether they represent genetic drift or possible sequencing errors. Because inbred mouse is a commonly used animal model to study cardiac arrhythmias,<sup>33–36</sup> strain-specific genetic variability in their cardiac ion channels potentially could influence results of electrophysiology studies and arrhythmia research.

Additional whole-genome information was recently made available through the WTSIMGP (<http://www.sanger.ac.uk/resources/mouse/genomes/>),<sup>37</sup> through which sequencing is available for at least 17 key mouse strains or substrains including BALB/cJ, C57BL/6J, C57BL/6NJ and three 129 strains or substrains. The mERG-Waterston (C57BL/6J) strain sequence is present in the WTSIMGP data set, although the mERG sequence is not identical to the original sequencing of the same strain obtained from the Mouse Genome Sequencing Consortium (V522 to L522).<sup>16</sup> The mERG-London (BALB/c) and mERG-Nie (C57BL) strains (Table 1) are not present in the WTSIMGP data set, whereas sequence data for substrains of both lines are present. As shown in Table S1A, the mERG-Nie sequence for amino acids 455, 522, 752, and 1006 matches the WTSIMGP sequencing findings for mERG for all strains and substrains sequenced. This suggests that the mERG-Nie sequence at amino acids 455, 522, 752, and 1006 may represent the mouse WT sequence with the protein trafficking and biophysical properties we described. However, from our analysis of the WTSIMGP data set, other strain- and substrain-specific sequence changes are present in mERG, with one third of the strains or substrains containing 1 or 2 amino acid substitutions at other locations (see Table S1B). Interestingly, the sequence variants found in the WTSIMGP data set lie in regions of mERG thought to lack HOS. These channels lack expression and biochemical and functional data to allow for comparison of their properties.

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## Disclosures

None.

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