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# Inhibition of nonsense-mediated mRNA decay by antisense morpholino oligonucleotides restores functional expression of hERG nonsense and frameshift mutations in long-QT syndrome

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## ABSTRACT

Mutations in the human *ether-a-go-go-related gene* (*hERG*) cause long-QT syndrome type 2 (LQT2). We previously described a homozygous LQT2 nonsense mutation Q1070X in which the mutant mRNA is degraded by nonsense-mediated mRNA decay (NMD) leading to a severe clinical phenotype. The degradation of the Q1070X transcript precludes the expression of truncated but functional mutant channels. In the present study, we tested the hypothesis that inhibition of NMD can restore functional expression of LQT2 mutations that are targeted by NMD. We showed that inhibition of NMD by RNA interference-mediated knockdown of UPF1 increased Q1070X mutant channel protein expression and hERG current amplitude. More importantly, we found that specific inhibition of downstream intron splicing by antisense morpholino oligonucleotides prevented NMD of the Q1070X mutant mRNA and restored the expression of functional Q1070X mutant channels. The restoration of functional expression by antisense morpholino oligonucleotides was also observed in LQT2 frameshift mutations. Our findings suggest that inhibition of NMD by antisense morpholino oligonucleotides may be a potential therapeutic approach for some LQT2 patients carrying nonsense and frameshift mutations.

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## 1. Introduction

Long-QT syndrome is a disease associated with delayed cardiac repolarization and prolonged QT intervals on the electrocardiogram, which can lead to ventricular arrhythmias and sudden death [1]. The congenital long-QT syndrome type 2 (LQT2) is caused by mutations in the human *ether-a-go-go-related gene* (*hERG*) [2]. *hERG* encodes the pore-forming subunit of the rapidly activating delayed rectifier K<sup>+</sup> channel (*I<sub>Kr</sub>*) in the heart [3–5]. Over 500 *hERG* mutations have been identified in patients with LQT2 [6–11]. Approximately 30% of LQT2 mutations are nonsense or frameshift mutations that introduce premature termination codons (PTCs). We have recently shown that hERG nonsense mutations lead to the degradation of mutant mRNA transcripts by nonsense-mediated mRNA decay (NMD) [12,13]. NMD prevents the production of truncated and potentially harmful proteins by eliminating abnormal mRNA transcripts carrying PTCs [14]. However, the elimination of mutant mRNAs that would otherwise produce partially or fully functional proteins may lead to a severe clinical

phenotype [14]. Thus, interventions preventing the degradation of PTC-containing transcripts may be therapeutically useful [15,16].

We recently reported a homozygous hERG nonsense mutation Q1070X that causes profound QT prolongation and severe ventricular arrhythmias [13]. We showed that hERG channels with the Q1070X mutation traffic normally to the plasma membrane and generate hERG current when expressed from hERG cDNA. However, using a minigene construct, we found that the Q1070X mutation causes a marked decrease in mutant mRNA transcripts by the NMD pathway. Thus, in homozygous Q1070X patients, most hERG mRNA transcripts are degraded by NMD before they form functional channels leading to a severe clinical phenotype [13,17]. Because the Q1070X mutant is able to form functional hERG channels if the mutant mRNA transcripts are not eliminated by NMD, inhibition of NMD might restore functional expression of this truncated channel and therefore provide a potential treatment for homozygous hERG Q1070X patients.

In the present study, we tested the hypothesis that inhibition of NMD can restore functional expression of the hERG mutations associated with long-QT syndrome. We report that inhibition of NMD by RNA interference (RNAi)-mediated knockdown of UPF1 increased Q1070X mutant channel protein level and hERG current amplitude. More importantly, we show that specific inhibition of downstream intron splicing by antisense morpholino oligonucleotides (MO) prevented NMD of Q1070X mutant mRNA, leading to the functional expression of Q1070X mutant channels. This strategy was

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also used to restore the functional expression of two LQT2 frameshift mutations. Our findings suggest that inhibition of NMD by antisense MO may represent a novel therapeutic approach for some PTC-containing mutations in LQT2 and other NMD-related diseases.

## 2. Materials and methods

### 2.1. Plasmid constructs and transfection

The hERG minigene composed of hERG cDNA exons 1–10 and hERG genomic DNA from intron 10 to poly(A) site was constructed by replacing hERG cDNA C-terminal fragment with an intron-containing hERG genomic DNA fragment obtained from a human BAC clone consisting of the entire hERG gene (RP11-166D23). The minigene was subcloned into a modified pcDNA5 vector in which the BGH poly(A) signal was deleted. Thus, the native poly(A) signal of hERG gene is used for the formation of the poly(A) tail of hERG mRNA. The Q1070X, R1005fs + 50X and Q1010fs + 45X mutations were generated by site-directed mutagenesis using the pAlter *in vitro* mutagenesis system (Promega, Madison, WI). Flp-In HEK293 cells (Invitrogen, Carlsbad, CA) were stably transfected with pcDNA5 hERG cDNA or minigene constructs and selected with 100 µg/ml hygromycin B. Flp-In HEK293 cells, which contain a single integrated Flp recombination target site, allow stable integration of a single copy of the cDNA or minigene construct at a specific genomic site and, subsequently, similar expression in all cell clones.

### 2.2. Morpholino oligonucleotide treatment

MOs were synthesized by Gene Tools (Philomath, OR). The antisense MO was designed to target the 5' splice site of intron 14, 5'-aagcaggctggagcttacCTGAGA-3' (upper case indicates exonic sequences and lower case indicates intronic sequences). An invert MO with the same sequence but in a reverse orientation was used as a control. The Endo-Porter delivery system (Gene Tools) was used to deliver the MOs into the cells.

### 2.3. RNase protection assay

RNA isolation and RPA were performed as previously described [12]. Briefly, antisense RNA riboprobes were transcribed *in vitro* in the presence of biotin-14-CTP. The probe designed to detect the mRNA levels of the hERG minigene contained 277 nt spanning the region of exons 12 and 13. The total length of the probe was 409 nt and contained sequences from the pCRII vector at both ends. The probe designed to detect intron 14 splicing events contained 222 nt spanning the region of exon 14 and intron 14. The total length of the probe was 336 nt and contained sequences from the pCRII vector at both ends. The probe for the hygromycin B resistance gene contained 158 nt of the gene and 70 nt from the pGEM vector. Yeast RNA was used as a control for the complete digestion of the probes by RNase. The relative intensity of each band was quantified using Scion Image software (Scion Corp., Frederick, MD).

### 2.4. Immunoblot analysis

Immunoblot analysis was performed as previously described [18]. The cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred onto nitrocellulose membranes. The membranes were probed with an anti-hERG antibody against the N terminus of hERG protein (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The expression level of hygromycin B phosphotransferase (HPH) encoded by the hygromycin B resistance gene in pcDNA5 vector was used as a loading control [19]. The polyclonal anti-HPH antibody was custom generated by Genscript (Piscataway, NJ) using a peptide antigen corresponding to the last 14

amino acids of HPH. The anti-HPH antibody was affinity purified and used at a 1:1000 dilution. The intensity of protein bands was quantified using Scion Image software.

### 2.5. RNA interference

UPF1 shRNA was used to inhibit expression of UPF1 as described previously [12]. The shRNA targeted the coding sequence of hUPF1 (5'-GAGAATCGCCTACTTCACT-3'). HEK293 cells stably expressing WT or Q1070X minigenes were stably transfected with plasmids containing the UPF1 or scramble shRNA. The knockdown of the UPF1 protein was analyzed by immunoblot as previously described [12].

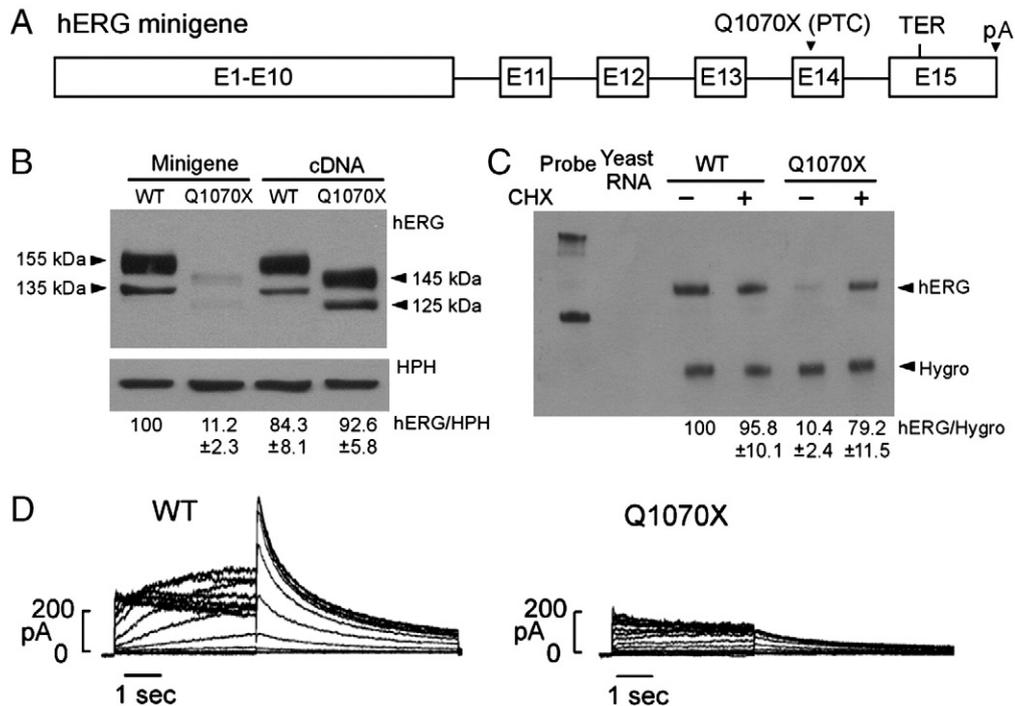
### 2.6. Patch clamp recordings

Membrane currents were recorded in whole cell configuration using suction pipettes as previously described [18]. hERG current was activated by depolarizing steps between -70 and 60 mV from a holding potential of -80 mV. The hERG tail current was recorded following repolarization to -50 mV. All patch clamp experiments were performed at room temperature (22–23 °C). The patch clamp data are presented as mean ± SEM and analyzed by Student's *t*-test. *p* < 0.05 is considered statistically significant.

## 3. Results

### 3.1. Effect of NMD on the Q1070X mutation in minigene experiments

We previously reported that the Q1070X mutation was able to form functional hERG channels when expressed from hERG cDNA [13]. However, the Q1070X mutation led to a marked decrease in mutant mRNA transcripts by the NMD pathway in the context of a minigene system. The minigene construct used in the previous study contained only the hERG genomic sequence from exon 12 to exon 15, which precluded analyses of hERG protein and channel function. In order to study the effect of NMD on the Q1070X mutation at protein and functional levels, we constructed a minigene that contained hERG cDNA from exon 1 to exon 10 and hERG genomic DNA from intron 10 to the poly(A) site (Fig. 1A). When expressed in HEK293 cells, this minigene was properly spliced to produce the full-length hERG protein. As shown in Fig. 1B, the wild-type (WT) minigene expressed two protein bands at 155 and 135 kDa, consistent with the sizes of the hERG protein expressed from the hERG cDNA construct. The 135-kDa band represents the core-glycosylated immature form of the channel protein located in the ER, and the 155-kDa band represents the complex-glycosylated mature form of the channel protein located in the plasma membrane [20]. The Q1070X minigene generated two protein bands at 125 and 145 kDa, reflecting the deletion of 90 amino acids due to the premature termination codon. However, the level of the two protein bands in the Q1070X mutant minigene was markedly decreased compared to that of the WT minigene. This is in contrast to the cDNA experiments in which the protein expression levels of WT and Q1070X were comparable (Fig. 1B). To determine whether the decrease in the protein expression level of the Q1070X mutation is due to the degradation of mutant mRNA by NMD, we carried out RNase protection assay (RPA) analysis using a probe spanning a region of exon 12 and exon 13. The mRNA level of the Q1070X minigene was significantly lower than that of the WT minigene (Fig. 1C). Because degradation of mRNA by NMD is dependent on protein synthesis, we examined whether inhibition of protein synthesis by cycloheximide (CHX) abrogates NMD of the mutant mRNA. Treatment with CHX had no effect on the level of WT mRNA but significantly increased the level of Q1070X mutant mRNA, suggesting that the Q1070X mutant mRNA is degraded by NMD. Functional analysis of hERG channels showed that the degradation of the Q1070X mutant mRNA by NMD resulted

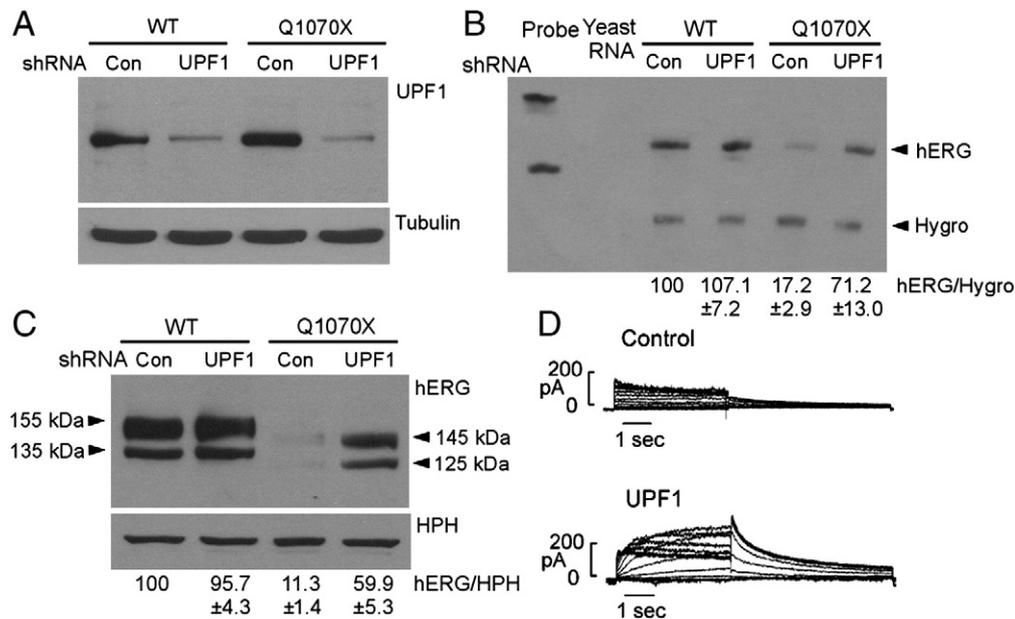


**Fig. 1.** Analysis of the Q1070X mutation in the context of the hERG minigene. (A) Diagram of the hERG minigene structure. The positions of WT termination codon (TER) and the Q1070X mutation-induced PTC are indicated. (B) Immunoblot analysis of WT hERG and Q1070X cDNAs and minigenes. Cell lysates were subjected to SDS-PAGE and probed with anti-hERG and the anti-HPH antibodies. The level of HPH served as a loading control. The level of protein bands was quantified, normalized to HPH, and shown as percentage of WT minigene control (mean  $\pm$  SEM,  $n = 3$ ). (C) Analysis of mRNA in the hERG minigene by RPA. Cells expressing WT and Q1070X minigenes were treated (+) or not treated (-) with 100  $\mu$ g/ml of CHX for 3 h before RNA isolation. The level of hygromycin B resistance gene transcripts (Hygro) served as a loading control. RPA signals were quantified, normalized to hygromycin B resistance gene, and shown as percentage of WT control ( $n = 3$ ). (D) Patch clamp recordings of hERG current in WT and Q1070X minigenes stably expressed in Flp-In HEK293 cells.

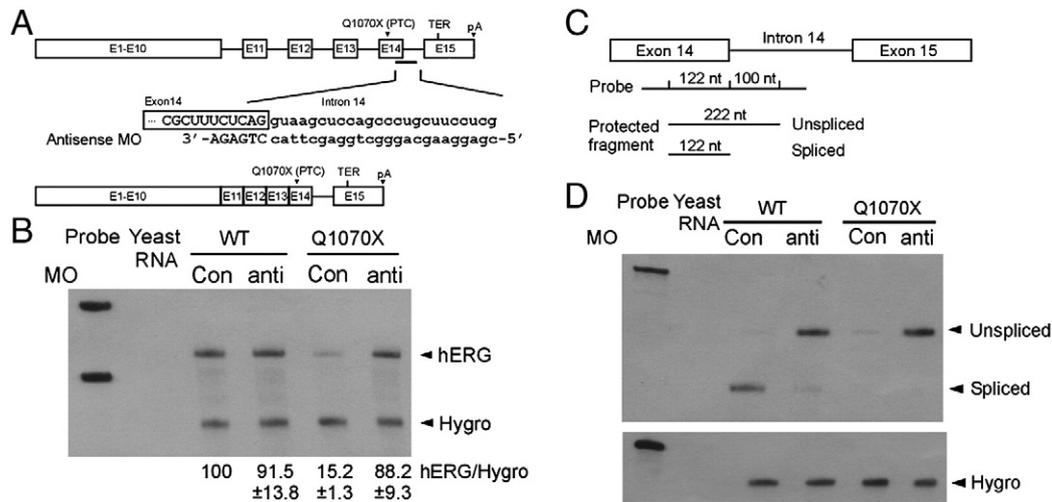
in a significant decrease in hERG current (Fig. 1D). The maximum tail current amplitudes for WT and Q1070X minigenes were  $22.5 \pm 1.7$  pA/pF ( $n = 13$ ) and  $2.6 \pm 0.5$  pA/pF ( $n = 11$ ,  $p < 0.05$ ), respectively. These experiments demonstrate that the full-length hERG minigene is an ideal system to study NMD effects at the mRNA, protein, and functional levels.

### 3.2. Effect of RNAi knockdown of UPF1 on expression of the Q1070X mutation

Since the Q1070X mutation is able to form functional hERG channels when the cDNA construct is used, we hypothesized that inhibition of NMD can restore functional expression of the Q1070X



**Fig. 2.** Suppression of NMD of Q1070X by RNAi knockdown of UPF1. Cells stably expressing the WT and Q1070X minigenes were transfected with UPF1 or scramble shRNA plasmids. (A) Immunoblot analysis of UPF1 protein. (B) RPA analysis of the effect of UPF1 knockdown on the level of mRNA. RPA signals were quantified, normalized to hygromycin B resistance gene, and shown as percentage of WT control ( $n = 3$ ). (C) Immunoblot analysis of the effect of UPF1 RNAi. Protein bands were quantified, normalized to HPH, and shown as percentage of WT minigene control ( $n = 3$ ). (D) patch clamp recordings.



**Fig. 3.** Inhibition of intron 14 splicing by antisense MO prevents NMD degradation of the Q1070X mutant mRNA. Cells stably expressing the WT and Q1070X minigenes were treated with 4  $\mu$ M invert or antisense MO for 48 h. (A) Diagram of the hERG minigene structure and the sequences of antisense MO. (B) RPA analysis of the effect of antisense MO on the level of mRNA. RPA signals were quantified, normalized to hygromycin B resistance gene, and shown as percentage of WT control ( $n = 3$ ). (C) Diagram showing the RPA probe and RNase protected fragments corresponding to spliced and unspliced fragments from intron 14. (D) RPA analysis of the effect of antisense MO on intron 14 splicing.

mutation in the context of the minigene. Although the level of mutant hERG mRNA increases as NMD is inhibited in the presence of CHX, the inhibitory effects of CHX on protein synthesis preclude functional studies of hERG channels. We therefore used the RNAi method to knockdown UPF1, a key effector of the NMD pathway. In these experiments, HEK293 cells stably expressing WT and Q1070X minigenes were transfected with UPF1 or scramble short hairpin RNA (shRNA) plasmids. UPF1 knockdown in the transfected cells was confirmed by immunoblot analysis using an anti-UPF1 antibody (Fig. 2A). RPA analysis showed that the level of Q1070X mutant mRNA was significantly increased in UPF1-shRNA-transfected cells (Fig. 2B). The knockdown of UPF1 also resulted in an increase in the level of the Q1070X mutant protein. As shown in Fig. 2C, the protein level of Q1070X was increased more than fivefold in UPF1-shRNA-transfected cells compared to scramble shRNA-transfected cells. Patch clamp analysis revealed that UPF1 knockdown significantly increased hERG current amplitude of the Q1070X mutant (Fig. 2D). The maximum tail current amplitudes for scramble shRNA and UPF1-shRNA were  $1.9 \pm 0.4$  pA/pF ( $n = 8$ ) and  $10.8 \pm 2.8$  pA/pF ( $n = 9$ ,  $p < 0.05$ ), respectively. These findings indicate that inhibition of NMD by UPF1 knockdown upregulates the level of the Q1070X mutant protein leading to functional expression of the Q1070X mutant channel.

### 3.3. Inhibition of NMD by antisense MO upregulates Q1070X mutant mRNA

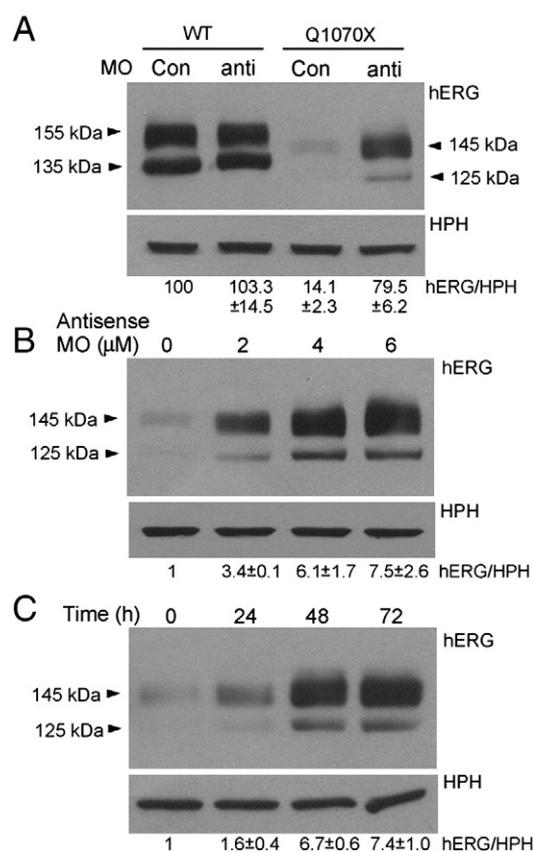
The above findings suggest that inhibition of NMD by UPF1 knockdown might provide a therapeutic strategy for patients carrying the Q1070X mutation. However, downregulation of NMD components potentially has adverse consequences as cellular clearance of PTC-containing mRNAs by NMD is a critical house-keeping function [21]. It is important to develop an approach that can specifically inhibit NMD of the Q1070X mutant mRNA transcripts. Because the downstream intron splicing plays an important role in the degradation of PTC-containing mRNAs by NMD [22], we reasoned that inhibition of downstream intron splicing by antisense MO might prevent NMD of the Q1070X mutant mRNA and lead to the functional rescue of the truncated mutant channels. MOs are nonionic DNA analogs that can form RNA-morpholino hybrids to alter pre-mRNA splicing events by blocking the access of splicing factors to splice sites. The Q1070X mutation is located in exon 14 of the hERG gene with only one downstream intron (intron 14). We designed an antisense MO targeting the 5' splice site of intron 14 to inhibit the splicing of intron

14 (Fig. 3A). In these experiments, HEK293 cells stably expressing WT or Q1070X minigene were treated with antisense or invert MO for 48 h. As shown in Fig. 3B, treatment with antisense MO resulted in a significant increase in the level of the Q1070X mutant mRNA transcripts compared to the control MO. To confirm the inhibition of intron 14 splicing by antisense MO, we carried out RPA analysis using a probe spanning the region from exon 14 to intron 14. This probe generates a protected fragment of 222 nt if the intron 14 is unspliced and a 122-nt fragment if the intron 14 is spliced (Fig. 3C). Treatment with the antisense MO resulted in an efficient block of intron 14 splicing leading to a significant increase in the unspliced form (Fig. 3D). These results suggest that inhibition of intron 14 splicing by antisense MO can prevent NMD of the Q1070X mutant mRNA transcripts.

### 3.4. Increase of hERG mutant proteins in antisense MO treated cells

To determine whether inhibition of NMD by antisense MO also results in the restoration of the Q1070X mutant at the protein level, we analyzed hERG protein expression by immunoblot. HEK293 cells stably expressing WT or Q1070X minigene were treated with antisense or invert MO for 48 h. Treatment with antisense MO significantly increased the level of the Q1070X mutant protein compared with the control invert MO (Fig. 4A). Because the Q1070X mutation introduces a stop codon in exon 14, which is upstream of the exon 14–intron 14 junction, intron 14 retention did not result in any changes in the protein size of the Q1070X mutant. In HEK293 cells expressing WT hERG, the antisense MO treatment allowed the read through of protein synthesis into intron 14 up to a termination codon within intron 14. This resulted in the replacement of the last 47 amino acids of hERG protein by 77 amino acids encoded by the intron 14 sequence. The replacement caused a small increase in molecular mass of WT hERG protein (~3 kDa), which did not cause a significant shift of protein bands on immunoblot analysis. In addition, the level of WT hERG protein was not significantly changed. The effect of antisense MO on the level of the Q1070X protein showed concentration and time dependence. The level of mutant protein was increased with 2  $\mu$ M antisense MO and reached a maximum at 6  $\mu$ M antisense MO (Fig. 4B). The increase of the mutant protein level was observed by 24 h and reached a maximum by 72 h after treatment with antisense MO (Fig. 4C).

To test whether antisense MO treatment can restore protein expression of hERG frameshift mutations, we analyzed two LQT2

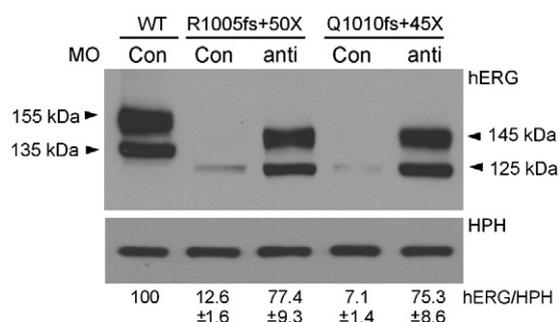


**Fig. 4.** Restoration of Q1070X mutant protein expression by antisense MO. (A) Immunoblot analysis of the effect of antisense MO on the level of hERG protein. Cells expressing the WT and Q1070X minigenes were treated with 4 μM invert (Con) or antisense MO for 48 h. Cell lysates were subjected to SDS-PAGE and immunoblotted with anti-hERG and anti-HPH antibodies. Signals were quantified, normalized to HPH, and shown as percentage of WT control ( $n = 3$ ). (B) Concentration dependence of the effect of antisense MO. Cells expressing Q1070X minigene were treated with antisense MO at indicated concentrations for 48 h. Signals were quantified, normalized to HPH, and shown as fold changes (0 concentration = 1,  $n = 3$ ). (C) Time course of the effect of antisense MO. Cells expressing Q1070X minigene were treated with 4 μM for indicated times. Signals were quantified, normalized to HPH, and shown as fold changes (0 time = 1,  $n = 3$ ).

mutations R1005fs + 50X and Q1010fs + 45X by immunoblot. Both R1005fs + 50X and Q1010fs + 45X are located in exon 13 and introduce a PTC in exon 14 at amino acid 1056 [7,10]. HEK293 cells stably expressing the R1005fs + 50X or Q1010fs + 45X minigene were treated with antisense or invert MO for 48 h. Treatment with antisense MO significantly increased the levels of R1005fs + 50X and Q1010fs + 45X mutant proteins (Fig. 5). These results suggest that the antisense approach is able to restore the expression of truncated proteins generated from LQT2 nonsense and frameshift mutations.

### 3.5. Functional rescue of LQT2 mutant channels by antisense MO

To study functional rescue of the LQT2 mutant channels, we performed patch clamp recordings of hERG current from antisense MO-treated cells. Cells stably expressing WT and LQT2 mutants were treated with 4 μM antisense MO for 48 h. For WT hERG minigene, the current was not affected by the antisense MO treatment (Fig. 6A, B). The maximum tail current amplitudes for WT hERG in the presence of invert and antisense MO were  $24.8 \pm 2.9$  pA/pF ( $n = 8$ ) and  $23.5 \pm 2.5$  pA/pF ( $n = 8$ ,  $p \geq 0.05$ ), respectively. This suggests that the replacement of the last 47 amino acids of hERG protein with 77 amino acids encoded by the intron 14 sequence does not have significant effect on WT hERG channel function. For the Q1070X and R1005fs + 50X



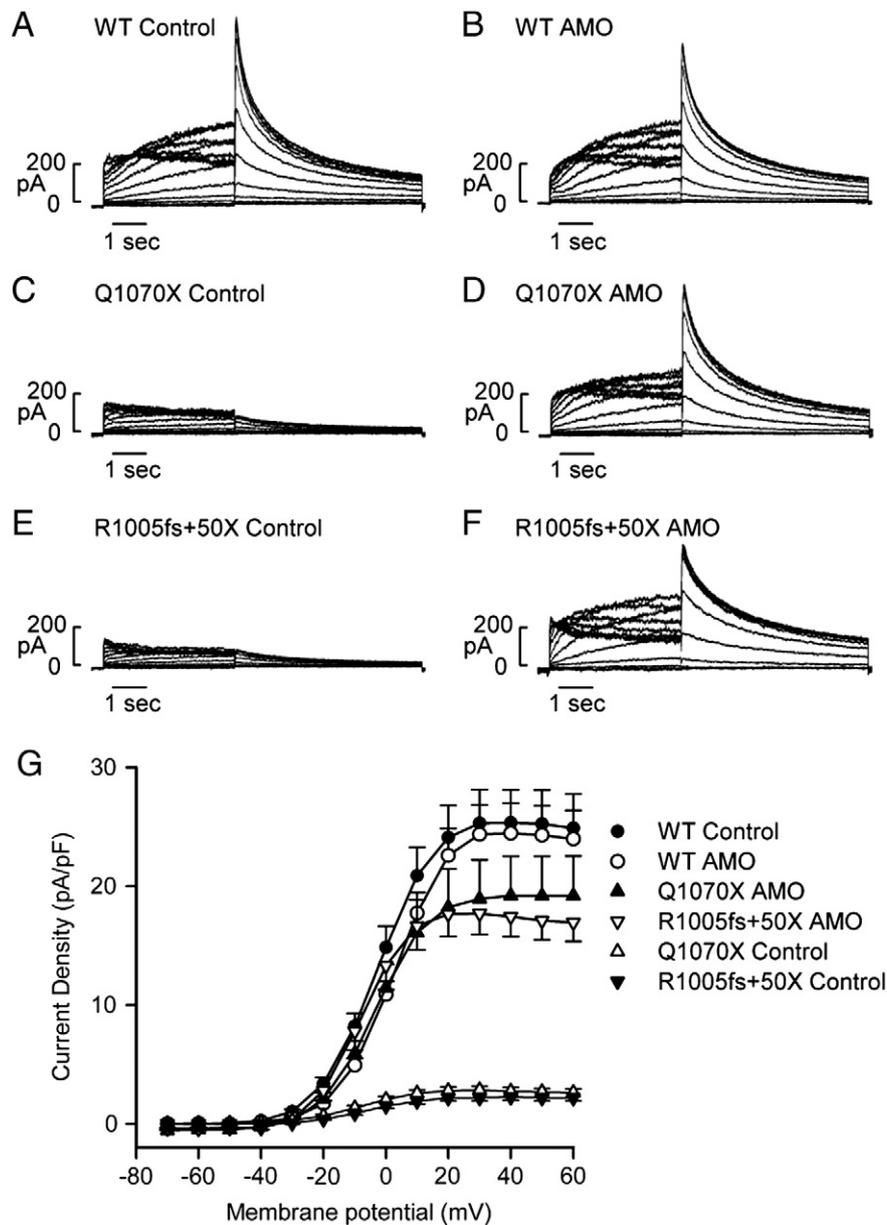
**Fig. 5.** Restoration of R1005fs + 50X and Q1010fs + 45X mutant protein expression by antisense MO. Cells expressing the R1005fs + 50X and Q1010fs + 45X minigenes were treated with 4 μM invert (Con) or antisense MO for 48 h. Cells expressing the WT minigenes treated with invert MO were used as control. Cell lysates were subjected to SDS-PAGE and immunoblotted with anti-hERG and anti-HPH antibodies. Signals were quantified, normalized to HPH, and shown as percentage of WT control ( $n = 3$ ).

mutant minigenes, the hERG current was significantly increased by the antisense MO treatment (Fig. 6C, D, E, F). The maximum tail current amplitudes for the Q1070X mutant treated with invert and antisense MO were  $2.4 \pm 0.3$  pA/pF ( $n = 8$ ) and  $19.2 \pm 3.3$  pA/pF ( $n = 9$ ,  $p < 0.05$ ), respectively. The maximum tail current amplitudes for the R1005fs + 50X mutant treated with invert and antisense MO were  $2.0 \pm 0.2$  pA/pF ( $n = 6$ ) and  $17.7 \pm 1.8$  pA/pF ( $n = 7$ ,  $p \leq 0.05$ ), respectively. Antisense MO also increased the current amplitude of the Q1010fs + 45X mutant (data not shown). To determine the I-V relationship of WT and mutant channels, the peak tail current amplitudes measured at -50 mV were plotted against preceding test voltages in the presence of invert and antisense MO (Fig. 6G). The voltage dependence of hERG channel activation was determined by fitting the normalized tail currents with a Boltzmann function. The half maximal activation voltages ( $V_{1/2}$ ) and slope factor ( $k$ ) for WT hERG, Q1070X, and R1005fs + 50X were shown in Supplemental Table S1. These patch clamp experiments demonstrate that inhibition of NMD by antisense MO can rescue functional expression of the LQT2 nonsense and frameshift mutations.

## 4. Discussion

NMD is an evolutionarily conserved mRNA surveillance pathway that detects and eliminates PTC-containing mRNA transcripts, thereby preventing the synthesis of truncated and potentially harmful proteins [23,24]. NMD as a modifier of phenotypic severity has been reported in many human diseases [14,15,25]. In most cases, NMD is beneficial, preventing the production of C-terminally truncated proteins that often have dominant-negative effects. However, NMD could be detrimental if it prevents the production of truncated proteins that are fully or partially functional [26]. This appears to apply to the hERG Q1070X mutation in which the degradation of mutant mRNA by NMD precludes the formation of functional, truncated channels and causes profound QT prolongation and severe ventricular arrhythmias in homozygous subjects [13,17]. In the present study, we show that specific inhibition of downstream intron splicing by antisense MO effectively suppresses NMD of hERG transcripts containing LQT2 mutations Q1070X, R1005fs + 50X, and Q1010fs + 45X, leading to the restoration of mutant protein expression and rescue of hERG channel current. The functional rescue of hERG mutant channels by antisense MO is efficient, restoring up to about 80% of the WT current level. Our findings demonstrate the feasibility of targeting downstream intron splicing by antisense MO to inhibit NMD as a means to rescue functional expression of mutant hERG channels in LQT2.

Our results indicate that downstream intron splicing is required for efficient degradation of hERG transcripts containing PTCs by the NMD pathway. Pre-mRNA splicing results in deposition of a protein



**Fig. 6.** Functional rescue of hERG current by antisense MO. (A–F) Representative currents recorded from Flp-In HEK293 cells stably expressing WT, Q1070X, and R1005fs + 50X minigenes. Cells were treated with 4  $\mu$ M invert (control) or antisense MO (AMO) for 48 h. (G) I–V plot of hERG tail current amplitude measured at  $-50$  mV following test voltages from  $-70$  to  $60$  mV for WT hERG, Q1070X, and R1005fs + 50X cells in the presence of invert or antisense MO.

complex known as the exon junction complex (EJC) 20–24 nt upstream of the splice junction [27,28]. According to the proposed rule for NMD in mammalian cells, termination codons located  $>50$ – $55$  nt upstream of the 3' most exon–exon junction elicit NMD [29]. The EJC is usually displaced by ribosomes during the pioneer round of translation. If translation terminates at a termination codon that is located 50–55 nt upstream of an exon–exon junction, the presence of a downstream EJC serves as a binding platform for NMD factors and triggers NMD. Recently, it has been shown that some PTC-containing mRNAs undergo NMD in the absence of a downstream EJC. These experimental data support an NMD model in which the competitive interaction between UPF1 and Poly(A) binding protein with the ribosome-bound releasing factors is the key determinant for NMD initiation [30]. Although the EJC is not required for NMD initiation in this model, it may act as an enhancer for NMD [30]. Thus, in both proposed models, EJCs play an important role in mammalian NMD. The LQT2 mutations examined in this study introduce PTCs in exon 14 and are located  $>50$ – $55$  nt upstream of the last exon–exon junction.

Inhibiting splicing of the last intron precludes EJC deposition downstream of these PTCs and prevents NMD of the mutant mRNAs. Nonsense and frameshift mutations that introduce PTCs upstream of exon 14 may require the inhibition of splicing of additional introns to prevent degradation by NMD. Over 20 LQT2 nonsense and frameshift mutations introduce PTCs within exon 14, and these mutant transcripts are potential candidates for rescue by the antisense approach.

Several pharmacological approaches have been used to rescue expression of mutant mRNAs that are targeted by the NMD pathway [24,25]. For example, aminoglycoside antibiotics have been used to suppress recognition of stop codons, leading to the synthesis of full-length proteins from PTC-containing transcripts. This approach has been tested for PTC mutations in several diseases including cystic fibrosis, Duchenne muscular dystrophy, and LQT2 [15,31–33]. Aminoglycoside antibiotics have been shown to increase hERG current of LQT2 nonsense mutations R1014X and W927X [33]. The major limitations of aminoglycoside antibiotic treatment are its low

efficiency and toxic side effects [32]. Recently, a small molecule PTC 124 has been shown to induce read through of PTCs and has less effect on normal termination codons [32]. Another approach to restore the expression of PTC-containing mRNA is the use of RNAi to knockdown NMD factors. Inhibition of NMD by RNAi knockdown of UPF1 and SMG-1 has been shown to restore the expression of mutant collagen VI  $\alpha 2$  protein in Ullrich disease [16]. Our results also show that inhibition of NMD by RNAi-mediated knockdown of UPF1 results in the functional rescue of Q1070X mutant channels. It has been suggested that inhibition of the key factors involved in the NMD pathway may represent a potential therapeutic strategy for some genetic diseases exacerbated by NMD [16]. However, many physiological transcripts are subject to NMD regulation, and systematically inhibiting NMD will likely result in undesirable side effects. Our present findings demonstrate that targeting downstream intron splicing by antisense MO offers an effective approach to specifically prevent NMD of PTC-containing mRNA transcripts without inhibiting the normal NMD pathway.

The antisense approach has been used to modulate mRNA splicing in a variety of disease models such as thalassemia, cystic fibrosis, Duchenne muscular dystrophy [21,34,35]. The primary applications of the antisense approach in these disease models are to restore normal splicing by blocking cryptic splice sites and to rescue the normal reading frame by skipping of exons that contain PTC mutations. In a mouse model of Duchenne muscular dystrophy, antisense oligonucleotide-mediated exon skipping has been shown to efficiently restore expression of functional dystrophin in skeletal and cardiac muscles by systemic delivery of peptide-conjugated MO [36,37]. To our knowledge, our present work is the first reported use of antisense MO to inhibit NMD of PTC-containing mRNA by blocking downstream intron splicing. Since the antisense approach can specifically inhibit NMD of targeted mRNAs without interfering with factors that are necessary for normal NMD functions, it may represent a novel therapeutic approach for some PTC mutations in LQT2 and other NMD-related diseases.

Supplementary materials related to this article can be found online at doi:10.1016/j.yjmcc.2010.10.022.

## Disclosures

None declared.

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