A novel transgenic rabbit model of a long QT syndrome caused by a dominant-negative mutation of KCNE1 gene for investigating experimental especially non ischaemic model of cardiac arrhythmias and heart failure

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Introduction and Aim

In cardiac myocytes, the I_{Ks} channel is composed of a pore-forming α (KCNQ1) and the modulatory β subunits (KCNE1), also KCNQ1 alone assembles to form voltage-gated potassium channel, the presence of KCNE1 is required to reproduce the kinetic properties of the native I_{Ks} channel (Sanguinetti et al. 1996). I_{Ks} the slowly activating cardiac potassium current is an important determinant of myocardial repolarization. KCNE1 also known as Mink was the first among the Kv channel accessory subunits, which was cloned from human heart (Murai et al. 1989). Mink encoded by the KCNE1 gene on human chromosome 21 is a small 129 amino acid protein, with a single transmembrane spanning domain (Nerbonne and Kass 2005). Mutations in either KCNQ1 or KCNE1 can alter the biophysical properties of I_{Ks} and mutations of KCNE1 underlie congenital long QT syndrome type 5 (LQT5). Long QT syndrome is characterized by a prolongation of the QTc interval on the electrocardiogram. LQTs cause sudden death in affected individuals due to the development of a characteristic ventricular tachycardia known as Torsades des Pointes (TdP) and subsequent fatal ventricular fibrillation. LQTs can be acquired due to drugs or occur as part of an inherited syndrome (Harmer et al. 2010). Mutations in the cardiac Na^+ channel and two K^+ channels and their related proteins, that form the rapid I_{Kr} and slow I_{Ks} currents have been shown to be the commonest cause of hereditary LQTs (Moss and Kass 2005; Nerbonne and Kass 2005). A complete understanding of the mechanisms how individual mutations may lead to arrhythmias and sudden death requires investigations in experimental animal models. Transgenic mouse models of LQT syndromes were reviewed and besides their advantages, the limitations were emphasized (Salama and London 2007). Mice have heart rates ~ 10 times higher than humans and have different repolarizing currents, which are carried by different channels (Nerbonne and Kass 2005). The KCNE1 knockout (Drici et al. 1998; Kupershmidt et al. 1998) knock-in (Nishio et al. 2009; Rizzi et al. 2008) or dominant negative loss-of-function mutations (Demolombe et al. 2001) in transgenic mouse models were able to only partly mimic the human LQT phenotypes (Salama and London 2007). The rabbit as experimental model has significant advantages over the mouse in this respect. For example, the contractile cycle is significantly longer than in mice and the size of the rabbit heart makes it possible to use tools developed for the clinical evaluation of human cardiac function. In spite of this, only two transgenic LQT rabbit models, overexpressing dominant-negative pore mutants: the human KvLQT1 (LQT1 loss of I_{Ks}) or the HERG channels (LQT2, loss of I_{Kr}) were published to date (Brunner et al. 2008). Mutations of the KCNE1 gene are rare and account for ~3% of all LQT

mutations (Splawski et al. 2000). A novel missense mutation, G to A at position 154 in the KCNE1 gene, which leads to an amino acid substitution of arginine (R) for glycine (G) at position 52 was described in a Chinese family (Ma et al. 2003). Expression studies in Xenopus oocytes revealed the critical role of glycine 52 in the transmembrane domain. The mutant G52R-KCNE1 had dominant negative effect, leading to 50 % reduction in I_{Ks} current amplitude and prolongation of the cardiac action potential (Ma et al. 2003). Recent data obtained *in vitro* in mammalian cell lines revealed that the G52R mutation does not alter subunit assembly or trafficking to the cell membrane, but unable to modulate the gating properties of KCNQ1 (Harmer et al. 2010).

The aim of our development was to create a G52R-KCNE1 transgenic rabbit and evaluate *in vivo* the phenotypic consequences of expressing a dominant-negative mutant modulatory protein, through recording the basic electrophysiological data. This transgenic conscious animal method has the advantage to be a experimental tool for investigating experimental non ischaemic model of cardiac arrhythmias and heart failure

Methods and Results

Rabbit transgenesis

New Zealand White rabbits were obtained from S & K Kft. (Hungary). Collection of rabbit zygotes and laparoscopic transfer of injected embryos to recipient does was performed as described earlier (Besenfelder and Brem 1993). All experiments were carried out in compliance with the *Guide for the Care and Use of Laboratory Animals* (USA NIH publication NO 85-23, revised 1996) and conformed to the Directive 2010/63/EU of the European Parliament. The protocols have been approved by the Animal Care and Ethics Committee of the Agricultural Biotechnology Center, and the Ethical Committee for the Protection of Animals in Research of the University of Szeged, (approval number: I-74-5-2012) and by the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development (authority approval numbers 22.1/433/003/2010 and XIII/1211/2012).

Transgene construct

The 4533 bp long rabbit β-myosin heavy chain gene promoter (r-MYH7) including two non-translated introns were amplified with the primers 5'- ACA AAG CCC AGC TCC CTA AT-

3'(nt:2509-2529) and 5'-GGC TGT ACC TGT AGT GAG CG-3'(nt: 7022 – 7042, Genbank Ac. No.: AF192306.1). PCR was performed with TaKaRa LA Taq polymerase, which is a mixture of *Taq* Polymerase with a proofreading polymerase optimized for amplification of long DNA templates (Clontech), conditions were 95C for 1 min, 58C for 45 sec and 72C for 5 min for 35 cycles, using New Zealand White genomi DNA as template. The PCR product was cloned into the TOPO site of the pCR®-Blunt II-TOPO vector with the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen™). The 410 bp human KCNE1 cDNA was isolated from a Human Cardiac myocyte cDNA library (Cat no:SC6204; 3H Biomedical AB, Uppsala, Sweden). The amplified KCNE1 cDNA was cloned into the PCR product insertion site of the pSC-A- amp/kan vector with the Strata Clone PCR Cloning Kit (Stratagene, an Agilent Technologies company, USA). At position 154 a guanine was changed to adenine with the Quick Change® XL Site Directed Mutagenesis Kit. This base change resulted a substitution of arginine for glycine at amino acid 52 (G52R-KCNE1). The mutated human KCNE1 cDNA was sequenced before subloning (ABI PRISM 310 DNA sequencer, Applied Biosystems). The mutated KCNE1 cDNA was isolated with Eco RV and Sma I enzymes and blunt end cloned behind the r-β-MHC promoter at the EcoRV site of the pCR®-Blunt II-TOPO vector. The insert orientation was controlled by restriction enzyme digest. The ~5900 bp long microinjected fragment, which included the mutated human KCNE cDNA under the 4533 bp long rabbit β-MHC promoter with three non-translated exons and the bovine GH polyA tail (MYH7-G52R-KCNE1-BGHpolyA) was isolated with NheI-SexAI digestion and purified with QIAquick gel extraction kit (Qiagen, Cat no. 28704) according to the manufacturer instructions, with the modification that we eluted with the Millipore EmbryoMax® Injection Buffer (Millipore, Cat no. MR-095-10F). The DNA concentration was set at 4ng/ul.

Results

Creation of transgenic LQT5 rabbits

We used the rabbit β-myosin heavy chain gene (r-MYH7) promoter to drive heart ventricular specific expression in transgenic rabbit. Rabbit heart atria express α-myosin heavy chain at all developmental stages, whereas the ventricles express both α- and β-MHC isoforms, with the β-MHC being the predominant adult isoform (Sinha et al. 1982). Earlier data showed that the rabbit r-MYH7 promoter directs ventricle-specific expression in transgenic rabbit heart

(Brunner et al. 2008; Sanbe et al. 2005). The KCNE1 coding area of the transgene was amplified and analyzed by automated sequencing (**Figure 1**).

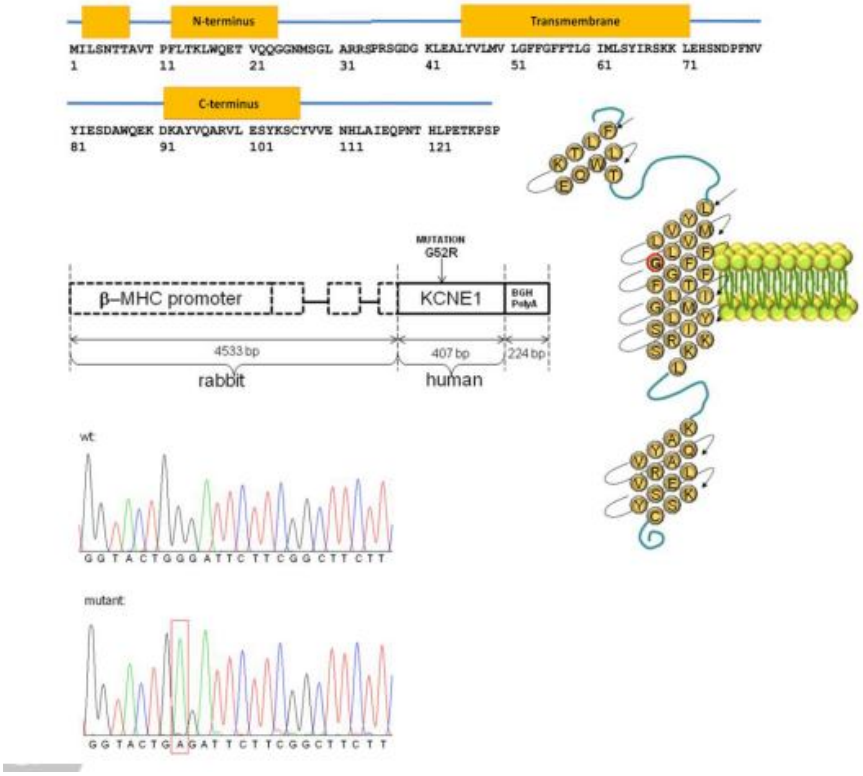
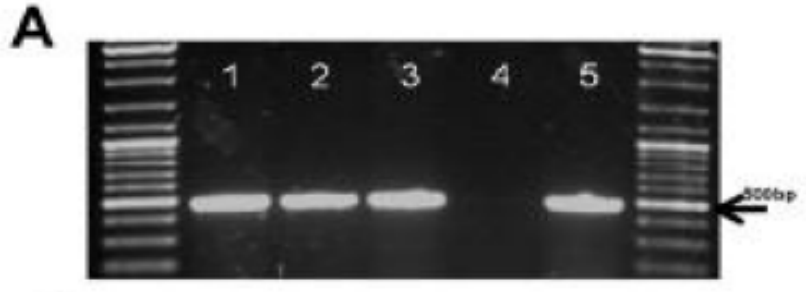


Figure 1. The G52R mutation and the transgene construct. Schematic drawing of the mutation in KCNE1 polypeptide (top panel) and the transgene construct (middle panel). Mutation G to A at position 154 of human KCNE1 cDNA and wild-type sequence. Sense-strand sequences are shown (bottom panel).

The MYH7-G52R-KCNE1-bGHpolyA insert was isolated and used to microinject rabbit embryos. Out of 497 injected embryos, 466 were transferred into 21 pseudopregnant recipients through laparoscopy. 38 offspring (8%) were born alive, out of which four (10%) were found to be transgenic by genomic PCR (**Figure 2A**).



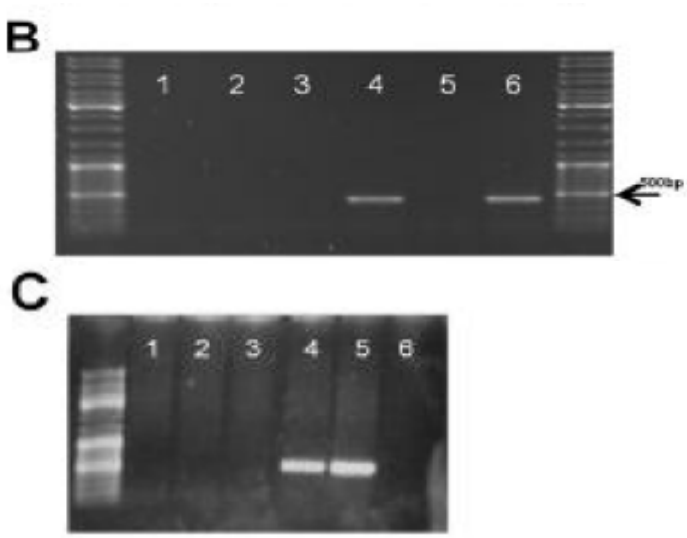


Figure 2. Identification of G52R founders and tissue specific transgene expression. (A) Transgene specific PCR of genomic DNA of three founders. Line 1: G52R-JTJKK; line 2: female founder without transgenic offspring; line 3: G52R-14-JKK; line 4: negative control; and line 5: positive control (injected construct). (B) Expression profile of hKCNE1 specific mRNAs detected by RT-PCR in ventricular myocardium and diverse tissues from transgenic (tr) rabbit #G52R-JTJKK. Beta-actin specific PCR reaction was carried out to control the quality of RNA and the RT step in all sets of samples (data not shown). Molecular weight marker: Fermentas Gene Ruler DNA Ladder Mix (Cat.No.: SM0331); Line 1: tr smooth muscle; 2: tr striated muscle; 3 tr atrial myocardium; 4: tr ventricular myocardium; 5: tr brain; 6: human ventricle. (C) Expression profile of hKCNE1 specific mRNAs detected by RT-PCR in ventricular myocardium and diverse tissues of transgenic (tr) rabbit #G52R-JKK. Beta-actin specific PCR reaction was carried out to control the quality of RNA and the RT step in all sets of samples (data not shown). Molecular weight marker: Fermentas Gene Ruler DNA Ladder Mix (Cat.No.: SM0331). Line 1: tr smooth muscle; line 2: tr striated muscle; line 3: tr brain; line 4: tr right ventricle ; line 5: tr left ventricle; line 6: tr atrium .

ECG parameters and incidence of arrhythmias in wild type and transgenic rabbits

There were no significant differences in heart rate, QT, QTc, PQ and QRS intervals between wild type and transgenic animals at baseline (**Table 1**). The short-term variability of the RR interval (STV_{RR}) was also similar in the two groups both at baseline and following dofetilide administration (**Fig. 3**).

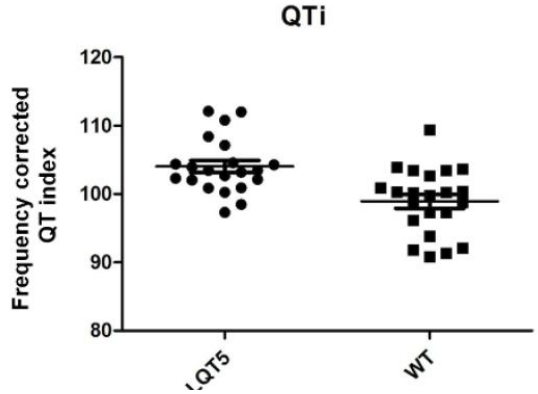
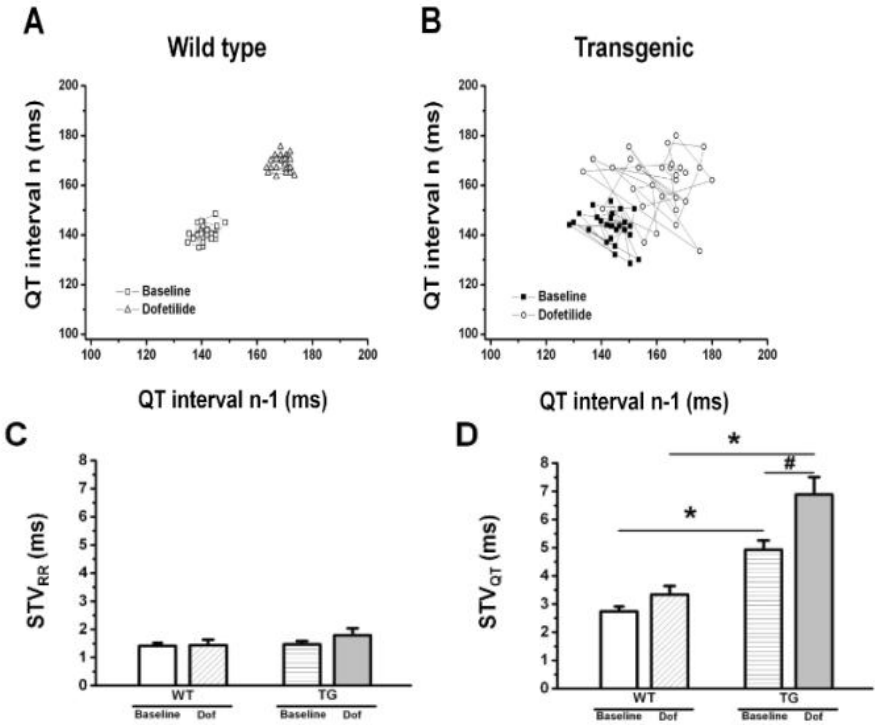


Figure 3. Heart rate corrected QT index in anesthetized rabbits. Plots indicating the heart-rate corrected QT-indices (QTi) in LQT5 transgenic (n=21) and wild-type littermate (WT; n=22) rabbits under anaesthesia with ketamine/xylazine. Mean±S.E.M. are indicated as horizontal lines. $p<0.001$.

Figure 4A shows Poincaré plots constructed from QT intervals of representative individual wild type and transgenic rabbits before and following the application of the I_{Kr} blocker dofetilide. Importantly, as representative plots (**Fig.4**) and grouped data show the short-term variability of the QT interval (STV_{QT}) was significantly higher in transgenic rabbits already at baseline, suggesting that transgenic animals exhibited increased temporal repolarization instability before any repolarization challenge, represented by the administration of the I_{Kr} blocker dofetilide in our experiments. As expected, administration of dofetilide (20 μg/kg, i.v.) significantly prolonged the QT and QTc intervals in both groups (**Table 1**), however, dofetilide significantly decreased heart rate only in transgenic animals (**Table 1**). Dofetilide caused a further and significant increase in STV_{QT} in transgenic animals (**Fig3**). While dofetilide led to similar QTc prolongation in the two groups, its STV_{QT} elevating effect was more pronounced and was accompanied by higher incidence of arrhythmias in transgenic animals. Dofetilide provoked Torsades des Pointes arrhythmias in 3 of 11 animals in wild type animals, while it induced TdP with significantly higher incidence, in 12 of 15 rabbits, in the transgenic group (**Fig. 3**). In transgenic animals, the durations of TdP episodes were also significantly longer compared to wild type rabbits (**Fig.3D**).



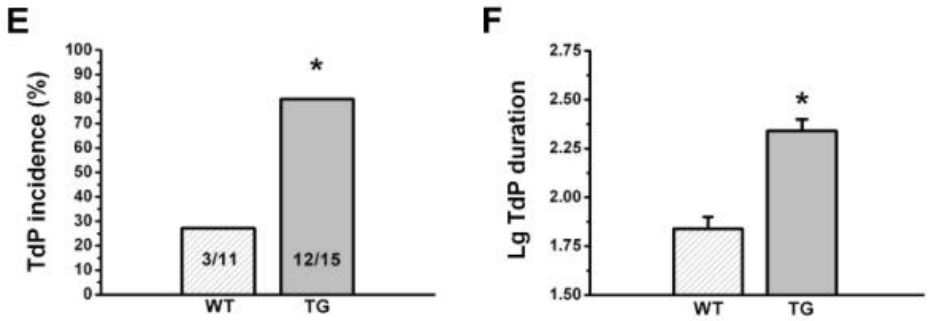


Figure 4. Short-term variability of the RR and QT intervals and Torsade-de-Pointes in thiopental anesthetized rabbits. (A-B) Representative Poincaré plots demonstrate higher short-term beat-to-beat variability of the QT interval (STV QT) in anesthetized transgenic rabbits at baseline conditions compared to wild-type animals. Following the administration of the IKr blocker dofetilide, STV QT further and markedly increased only in transgenic animals. (C) There were no differences in short-term variability of the RR interval (STV RR) between wild-type (WT) and transgenic (TG) rabbits either at baseline conditions or following the administration of the IKr blocker dofetilide. (D) Short-term variability of the QT interval (STV QT) was higher in TG animals at baseline conditions and following dofetilide infusion, indicating increased temporal instability of repolarization in transgenic rabbits. (E). Accordingly, transgenic animals exhibited Torsade-de-Pointes (TdP) with significantly higher incidence. (F) The duration of TdP episodes was significantly longer in TG rabbits, expressed as the log10 of duration in seconds (to allow statistical comparison of data with normal distribution). Dof: dofetilide (20 μg kg-1, i.v.); n = 11 and 15 animals in WT and TG groups, respectively on panels (C) to (E); n = 3 and 12 animals on panel (F); *p<0.05 vs. wild-type; #p<0.05 vs. baseline in the same group.

Table 1. Different ECG parameters in wild type (WT) and transgenic (TG) rabbits at baseline and following the administration of the I_{Kr} blocker dofetilide. HR: heart rate; DOF: measured at 10 min after the end of dofetilide infusion.

	WT baseline	WT prior to arrhythmia	WT DOF	TG baseline	TG prior to arrhythmia	TG DOF
PQ (ms)	69.6 ± 2.83	70.8 ± 2.49	71.9 ± 3.04	67.9 ± 1.67	69.1 ± 1.48	71.6 ± 3.86
QRS (ms)	36.2 ± 1.28	39.2 ± 2.13	40.6 ± 3.26	34.6 ± 1.10	35.4 ± 1.71	40.1± 2.79
RR (ms)	226.6 ± 7.93	222.5 ± 9.61	228.2 ± 8.96	230.1 ± 5.44	235.8 ± 6.45	255.1 ± 9.78#
HR (1/min)	267.9 ± 9.11	268.9 ± 8.88	267.3 ± 10.1	262.9 ± 5.47	255.8 ± 6.12	244 ± 9.04#
QT (ms)	139.3 ± 4.54	145.5 ± 4.77	159.9 ± 5.97#	140.2 ± 2.49	146.7 ± 5.22	172.9 ± 5.24#
QTc (ms)	155.8 ± 4.09	162.1 ± 2.81	175.4 ± 3.70#	154.5 ± 2.86	156.3 ± 2.07	175.5 ± 3.63#

HR: heart rate; DOF: measured at 10 min after the end of dofetilide infusion. #p<0.05 vs. baseline in the same group

Discussion

It is essential to assess the proarrhythmic potential of candidate compounds during drug development to minimize the risk of potentially life-threatening drug-induced arrhythmias, such as Torsades de Pointes (TdP) chaotic ventricular tachycardia that can degenerate into ventricular fibrillation (Farkas and Nattel 2010). However, reliable prediction of drug-induced (TdP) still remains elusive.

The rabbit is a commonly used species for the *in vivo* assessment of TdP liability, and *in vivo* models for the investigation of drug induced TdP include the α_1-adrenoceptor-stimulated anaesthetized rabbit (Carlsson et al. 1990), the chronic atrioventricular block canine model (Vos et al. 1995) and a novel anesthetized rabbit model (Lengyel et al. 2007). Both of the latter two experimental models are based on the impairment of repolarization reserve due to reduced I_{Ks} density or function, either by downregulation of the channel in dogs with chronic AV block (Volders et al. 1999) or by pharmacological block of I_{Ks} in anesthetized rabbits (Lengyel et al. 2007).

Repolarization reserve refers to the phenomenon where impairment of the function of one type of repolarizing transmembrane ion channel due to pharmacological (e.g. potassium channel blocking drugs), congenital (e.g. long QT syndromes) or acquired (e.g. diseases reducing repolarizing function) causes does not necessarily result in excessive repolarization changes because other repolarizing currents can take over and compensate for lost repolarizing function (Roden 1998; Varró and Baczkó 2011).

The identification of genetic mutations responsible for two frequent congenital long QT syndromes, which predisposes human carriers to increased risk of ventricular tachycardia and sudden death led to the generation of the first transgenic rabbit models of LQT1 and LQT2 (Brunner et al. 2008). The LQT1 rabbits showed QT prolongation but did not exhibit spontaneous arrhythmia development nor increase in the incidence of sudden death, while LQT2 animals developed spontaneous arrhythmias resulting in sudden cardiac death in some of the rabbits (Brunner et al. 2008; Odening et al. 2010). Subsequent publications on LQT1 and LQT2 rabbits underlined the usefulness of creating non-mouse models to study "channelopathies" and sudden cardiac death (for a recent review see Duranthon et al. (2012).

Despite the *in vivo* animal models described above, there is still an unmet need for the development of novel models that have better predictive value for the identification of proarrhythmic risk associated with candidate compounds under development.

Mutations within the KCNE1 gene encoding a transmembrane protein, which coassembles with KCNQ1 to form the β and α subunits of K^+ channels that mediate I_{Ks} currents, are implicated in cardiac action potential prolongation and ventricular arrhythmogenicity of LQT5 (Splawski et al. 2000; Splawski et al. 1997). Here we report the creation and primary characterization of the first transgenic LQT5 syndrome rabbit model.

Our novel and previously unpublished results in our new transgenic rabbit model can be summarized as follows:

1. We have successfully created and primarily characterized the first LQT5 syndrome transgenic rabbit model. The LQT5 rabbits were created by expressing human KCNE1, carrying a missense mutation identified in a Chinese LQT syndrome family (Ma et al. 2003). The mutation leads to an amino acid substitution of arginine for glicine at position 52. The G52R-KCNE1 cDNA was placed under the control of rabbit β-myosin heavy chain promoter, which included three non-translated exons and two introns.

2. Two rabbit lines were established and the heart ventricle specific expression of the human G52R-KCNE1 mRNA was proven in both lines **(Figs. 2B and C)**. Immunohistochemistry revealed that the mutant human KCNE1 is associated in the membrane of freshly isolated cardiomyocytes **(Fig. 3B)**, which is in line with earlier *in vitro* data and confirms the hypothesis that this mutation does not affect correct trafficking to the plasma membrane (Harmer et al. 2010).

3. The conventional ECG parameters characterizing repolarization duration, the QT and frequency corrected QT intervals (QTc), were not different in the two groups at baseline **(Table 1)**. Following the administration of the I_{Kr} blocker dofetilide, QT and QTc intervals were significantly prolonged in both groups to a similar extent **(Table 1)**.

4. The short-term variability of the QT interval (STV_{QT}), a novel ECG parameter suggested for the better estimation of temporal instability of cardiac ventricular repolarization and proarrhythmic risk, was significantly higher in G52R-KCNE1 transgenic rabbits compared to wild type already at baseline conditions (i.e. before the administration of the I_{Kr} blocker dofetilide) **(Figs. 4A and B)**.

5. Following the administration of the I_{Kr} blocker dofetilide, the incidence of TdP arrhythmia was significantly higher in G52R-KCNE1 transgenic rabbits compared to wild type (**Fig. 4B bottom left panel)**. Further analysis revealed that the duration of TdP episodes was significantly longer in G52R-KCNE1 transgenic animals compared to wild type **(Fig. 4B bottom right panel)**. It is important to note that a challenge on ventricular repolarization by the administration of the I_{Kr} blocker dofetilide led to similar QTc prolongation in control and

G52R-KCNE1 transgenic animals, however, the increase in STV_{QT} was more pronounced and was accompanied by a higher incidence of TdP arrhythmias in G52R-KCNE1 LQT5 transgenic rabbits compared to wild type controls.

In summary, these results prove that we have successfully created a G52R-KCNE1 transgenic LQT rabbit model and strongly suggest that our LQT5 transgenic rabbits are highly susceptible to arrhythmia development and may represent a useful model capable of testing the proarrhythmic potential of new drugs under development especially in non-ischaemic model of cardiac arrhythmias and heart failure.

The transgenic rabbit model is available for those interested. The possible forms for this purpose are academic collaborations and/or commercially transactions.

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