Long-term channel block is required to inhibit cellular transformation by human ether-à-go-go-related gene (hERG1) potassium channels

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Non-standard abbreviations

hERG1, human ether-a-go-go related gene; hEAG1, human ether-à-go-go; di-8-ANEPPS, 4-(2-[6-(dioctylamino)-2-naphthalenyl]ethenyl)-1-(3-sulfopropyl)pyridinium inner salt; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide; ECM, extracellular matrix; VEGF-A, vascular
endothelial growth factor-A; AML, acute myeloid leukemias; FAK, focal adhesion kinase; PI3K, phosphoinositide-3-kinase; siRNA, small interfering RNA;
Abstract

hERG1 and the closely related hEAG1 channel are aberrantly expressed in a large proportion of human cancers. In the present study we demonstrate that transfection of hERG1 into mouse fibroblasts is sufficient to induce many features characteristic of malignant transformation. Importantly, this transformation could be reversed by chronic incubation (for 2-3 weeks) with the hERG channel blocker dofetilide (100nM), while more acute applications (for 1-2 days) were ineffective. hERG1 expression resulted in a profound loss of cell contact inhibition, multiple layers of over-growing cells and high saturation densities. Cells also changed from fibroblast-like to a more spindle-shaped morphology, which was associated with a smaller cell size, a dramatic increase in cell polarization, a reduction in the number of actin stress fibres and less punctate labeling of focal adhesions. Analysis of single cell migration and scratch wound closure clearly demonstrated that hERG1-expressing cells migrated more rapidly than vector-transfected control cells. In contrast to previous studies on hEAG1, there were no increases in rates of proliferation, or loss of growth factor-dependency, however, hERG1-expressing cells were capable of substrate-independent growth. Allogeneic transplantation of hERG1-expressing cells into nude mice resulted in an increased incidence of tumors. In contrast to hEAG1, the mechanism of cellular transformation is dependent on ion conduction. Trafficking deficient and conduction deficient hERG1 mutants also prevented cellular transformation. These results provide evidence that hERG1 expression is sufficient to induce cellular transformation by a mechanism distinct from hEAG1. Importantly, selective hERG1 channel blockers have therapeutic potential in the treatment of hERG1-expressing cancers.
Introduction

Potassium-selective (K⁺) channels are the largest and most diverse subset of the ion channel superfamily. In addition to having vital roles in electrical signaling in excitable cells, it is becoming increasingly clear that K⁺ channels are also involved in other cellular functions, such as cell volume homeostasis, electrolyte transport, proliferation, cell cycle progression and apoptosis. In addition to these physiological processes, there is growing evidence for the involvement of a small number of potassium channels in the pathophysiology of cancer (Arcangeli et al., 2009; Fraser and Pardo, 2008; Pardo et al., 2005; Schonherr, 2005). One of these is the voltage-gated K⁺ channel, hERG1 (Kv11.1).

hERG1 (human ether-à-go-go-related gene 1) channels are members of the ether-à-go-go (Kv10-12) family of voltage-gated K⁺ channels. The function of hERG1 is best understood in the heart, where it has a critical role in action potential repolarization. hERG1 channels are an important target for treating cardiac arrhythmia and a large number of selective hERG channel blockers are available. hERG1 exists as two isoforms, the full length gene (sometimes referred to as hERG1a) and a version with a much shorter N-terminus (hERG1b) (Croci et al., 2003; Lees-Miller et al., 1997; London et al., 1997). Aberrant hERG1 expression has been documented in many cancer cell-lines derived from a variety of tissues, including epithelial, neuronal, leukemic, connective and soft tissues (reviewed in Jehle et al., 2011). More importantly, expression of hERG1 isoforms is elevated in primary human cancers, suggesting this apparent up-regulation is not simply due to altered gene expression with adaptation to in vitro culture conditions. Thus, hERG1 channels are over-expressed in endometrial adenocarcinoma (Cherubini et al., 2000), colorectal cancer (Dolderer et al., 2010; Lastraioli et al., 2004), gastric cancer (Shao et al., 2008), glioblastoma multiforme, myeloid leukemias (Pillozzi et al., 2002) and acute lymphoblastic leukemias (Pillozzi et al., 2002; Smith et al., 2002), while expression is below detection limits in non-cancerous tissues. Interestingly, hERG1 expression in tumors correlates with metastatic cancers and a poorer prognosis (Ding et al., 2008; Lastraioli et al.; Masi et al., 2005).

hERG1 channels appear to regulate an array of cell behaviors, including cell proliferation (Glassmeier et al., 2012; Pillozzi et al., 2002; Suzuki and Takimoto, 2004), apoptosis (Wang et al., 2002), secretion of...
pro-angiogenic molecules, such as vascular endothelial growth factor-A (Masi et al., 2005), and invasiveness and metastasis (Pillozzi et al., 2007). These activities are reported to be modified by hERG channel-selective blockers. While such reports provide some evidence that therapeutic interventions targeting hERG1 channels could be suitable for oncology therapies, the concentrations of blockers required were often 100-1000 times the pharmacologically determined IC\textsubscript{50} values for inhibition of hERG1 currents (Afrasiabi et al.; Crociani et al., 2003; Pillozzi et al., 2002), raising questions about the importance of hERG1 channel conduction in cancer development and the specificity of action of hERG1 blockers at these concentrations.

The role of K\textsuperscript{+} channels and ion conduction in cancer cell biology remains controversial. While it has been proposed for many years that K\textsuperscript{+} conduction is important for changes in membrane potential during cell cycle progression, or for regulation of cell volume in proliferating cells, it is clear that not all members of the K\textsuperscript{+} channel superfamily can support these roles. Only a select group of K\textsuperscript{+} channels (K\textsubscript{v}1.3, K\textsubscript{2P}9.1, hEAG1, hEAG2, hERG1) influence proliferation and have been linked to cancer (Bianchi et al., 1998; Fraser et al., 2003; Pardo et al., 1999; Pei et al., 2003; Preussat et al., 2003; Tajima et al., 2006, Huang et al., 2012), suggesting that channel properties other than K\textsuperscript{+} selectivity are important. Intriguingly, of this small group hERG1, hEAG1 and hEAG2 belong to the same subfamily and are closely related. Eliminating the K\textsuperscript{+} permeation of hEAG1 channels by mutations of the selectivity filter does not modify the enhanced rate of proliferation of hEAG1-expressing cells (Hegle et al., 2006), or xenograft tumor formation by hEAG1-transfected cells (Downie et al., 2008). In the current study we investigated the oncogenic potential of hERG1 channels and the requirement for K\textsuperscript{+} conduction. We report that hERG1 channel expression is sufficient on its own to produce a transformed phenotype in mouse fibroblast cells and, unlike with hEAG1, pore conduction is required. Dominant negative hERG1 mutants that block conduction at the selectivity filter or are trafficking deficient do not induce cellular transformation. Interestingly, while acute applications of channels blockers failed to prevent transformation, chronic application of therapeutically-relevant concentrations (close to the IC\textsubscript{50} for current inhibition) did reverse the transformed phenotype of hERG1-expressing cells and indicate that
pharmacological inhibitors of hERG may have therapeutic potential for treating some hERG expressing cancers.

Materials and Methods

Cell culture, stable transfection and clone selection by $[^3\text{H}]$dofetilide binding assay. hERG1 was subcloned into pcDNA3 (invitrogen) between EcoR1 and HindIII. Cells stably expressing hERG1 were obtained by transfection with pcDNA3 hERG1 in a selection medium containing 500 µg mL$^{-1}$ G418. After 3 weeks, cells were serially diluted and plated onto 96 well plates. Clones were grown up from single cells. Vector control clones (NIH-VC) were generated from the same stock of NIH-3T3 cells by transfection with the empty pcDNA3 vector and maintained in the same selection media. NIH-3T3 cells stably expressing the oncogene V12-Ras were a kind gift from Dr J Downward (MRC Cancer Research Institute, London, UK). Cells were maintained in low glucose (1 mg mL$^{-1}$) DMEM culture medium, with 10% fetal bovine serum, 50,000 units penicillin and streptomycin and 500 µg mL$^{-1}$ G418 for a maximum of 10 passages. The $[^3\text{H}]$dofetilide binding assay was used as an initial screening approach to identify clones expressing hERG channels. Cell pellets were homogenized in 50 mM Tris/HCl, 1 mM EDTA (pH 7.4) and centrifuged at 40,000 g for 20 min, at 4°C. The homogenization was repeated in fresh buffer and the membranes re-suspended in assay buffer containing 71.5 mM NaCl, 60 mM KCl, 1 mM CaCl$_2$, 2 mM MgCl$_2$, 10 mM HEPES, pH 7.4. Membranes (75 µg total protein) were incubated with 10 nM $[^3\text{H}]$dofetilide at room temperature for 120 min, filtered through glass-fiber discs (Whatman, UK) pre-soaked in 0.25% polyethyleneimine, washed three times and bound ligand quantified by scintillation counting. Non-specific binding was determined by incubating samples with 10 µM unlabeled dofetilide.

Measurements of hERG1 channel currents and optical measurements of membrane potential. hERG1 currents in NIH-3T3 cells were measured at room temperature using the whole-cell configuration of the patch clamp technique as described previously (Cockerill et al., 2007). A561V and G628S hERG1 properties and the stability and efficacy of dofetilide and terfenadine in cell culture conditions were
investigated on hERG1 currents expressed in *Xenopus* oocytes by two-electrode voltage clamp (Perry et al., 2004).

The contribution of hERG1 channels to the membrane potential of NIH-3T3 cells was investigated using the potentiometric fluorescent indicator di-8-ANEPPS (Invitrogen, Paisley, UK) using methods adapted from Hardy *et al.* (2006). Cells were grown overnight on poly-L-lysine-treated coverslips and loaded with 5 μM di-8-ANEPPS for 20 min. Measurements were taken from groups of 3-7 cells illuminated with excitation light (460-500 nm, 100 ms pulses at 0.1 Hz) and membrane potential detected from the ratio of emitted light at 540-580 nm and 600-640 nm wavelengths (Hardy *et al.*, 2006).

*Cell proliferation assays.* A number of assays were performed to assess rates of cell proliferation, adhesion-independent proliferation and loss of contact inhibition of cell growth. Rates of proliferation of each clone were measured by [3H]thymidine incorporation (Nilius and Wohlrab, 1992). 1 x 10⁵ cells per well were plated on a 24-well plate. After 45 h, cells were incubated for 3 h with 0.5 μCi [3H]thymidine. Cells were transferred to GF/B filters (Whatman, UK) washed, lysed and DNA precipitated by incubation in 5% (w/v) trichloroacetic acid for 5 min, followed by wash steps in 2 mL 10% potassium acetate in ethanol and 1 mL ethanol : diethylether (3:1 volume). [3H]thymidine incorporation was quantified by scintillation counting. In some experiments, cell proliferation was determined by calculating cell monolayer confluence changes with time using an automated imaging platform (IncuCyte™, Essen Instruments, Michigan, USA). Images were taken of 4 fields per well at intervals of 2 h over a 48 h period. Cell confluency was calculated from the percentage surface area covered by cells (Green *et al.*, 2009) and percentage change in confluency per hour calculated from the gradient of the linear portion of the growth curve. Adhesion-independent proliferation was assessed by colony formation in soft agar. 1 x 10⁵ cells were suspended in 0.35% agar plated between two 0.7% agar layers. After 21 days, viable colonies were stained for 24 h with 20 mg mL⁻¹ 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT; Sigma-Aldrich, UK). Loss of contact inhibition of growth was tested by the ability to form foci of densely over-growing cells, or from saturation-density assays. Cells were grown in 6 cm plates until confluent monolayers had formed. Foci formation was assessed by Leishman’s
stain (VWR, UK) after 21 days of culture. Saturation density was measured after 5 days of culture. Cells were lifted from the plate, stained with trypan blue and viable cells counted using a hemocytometer.

*Tumor formation in Nude mice.* 4 - 7 x 10⁶ cells in PBS were injected subcutaneously into the right flank of Athymic Nude mice (Harlan Laboratories). Three mice of each gender were inoculated for each cell clone. All animal experiments were performed under UK Home Office authority. Mice were monitored daily and culled before subcutaneous tumors reached a value of 1.44 cm² for maximum length x maximum breadth or if moderate adverse effects became evident. Post-mortem examinations were performed and tumors were excised, fixed in 4 % paraformaldehyde, paraffin-embedded, sectioned and stained with haematoxylin and eosin (H&E) using standard procedures.

*Scratch-wound cell migration assay.* The rate of cell migration into a scratch wound in a confluent monolayer of cells was determined from average wound widths measured at 60 min time intervals for 10 h using the Incucyte system (Essen Instruments, Michigan, USA). Wounds were made in cells cultured in 24-well ImageLock plates (Essen Instruments, Michigan) using the Woundmaker apparatus (Essen Instruments, Michigan). Four wells of a 24 well plate were used per experimental condition. In some experiments wound width was measured at 5 pre-marked points by taking photographs of wounds at 60 min intervals under a calibrated microscope. Average wound widths were plotted against time and migration rate measured from the gradient of line of best fit.
**Immunocytochemistry.** Cells on coverslips were washed twice with PBS, fixed in 2% paraformaldehyde (Sigma-Aldrich, UK) for 15 min, permeabilized for 2 min in 0.2% Triton X-100 in PBS and blocked for 20 min in 5% goat serum in PBS. Focal adhesion complexes were labeled for 1 h with a 1:100 dilution of mouse monoclonal anti-vinculin (V9131, Sigma-Aldrich, UK) in 1% goat serum, followed by incubation with 1:100 anti-mouse secondary antibody conjugated to Alexafluor 488 (Molecular Probes, UK) in 1% goat serum. Filamentous actin was labeled with a 1:500 dilution of Texas-Red-conjugated phalloidin (Molecular Probes, UK) in 0.1% BSA in PBS for 30 min. Cover slips were washed, mounted using ProLong Gold antifade reagent (Molecular Probes, UK) and visualized on an Olympus FV500 confocal microscope.

**Single cell migration.** 5 x 10⁴ cells were plated on 35 mm dishes in a 37°C incubator for between 5 to 8 h. Dishes were placed on a 37°C temperature-controlled stage of an inverted microscope (Eclipse TI-S, Nikon, Japan). Automated image acquisition of a selected field of 20-30 cells was performed at 10 min intervals for 1 h by OpenLab modular imaging software (Improvision, USA). Speed of migration was determined by tracking the position of the centroid of each individual cell in consecutive images. The true migration speed (µm min⁻¹) was calculated from total cell displacement divided by recording time. Cell spreading (in µm²) was assessed by outlining the periphery of single cells using ImageJ Software. Cell polarization was determined from the proportion of cells with one lamellar protrusion localized to the leading edge, resulting in an asymmetric cell shape (Nobes and Hall, 1999). Twelve values (3 experiments, 4 time points) were obtained for each clone.
Data analysis. Results are presented as mean (± S.E.M.) where appropriate and ‘n’ numbers and replicate information provided in the relevant figure legends. Figures and statistical analysis were prepared using Prism software (GraphPad, San Diego, USA). Unless stated otherwise, statistical significance was analyzed using a one-way ANOVA multiple comparison with Dunnett’s post-hoc test, using the NIH-VC as the reference group.

Results

Generation and characterization of hERG1-expressing stable cell-line. hERG1 expression has been reported in many different cancer tissues and tumor-derived cell-lines. However, it is not yet known whether hERG1 expression per se is sufficient for cell transformation. To test the transforming potential of hERG1 it was important to be able to compare the effects of hERG1 expression relative to a vector-transfected control in a well-characterized cell background. We chose the NIH-3T3 cell-line as this has been extensively utilized for this type of study and exhibits a distinct morphology, a well-defined contact-inhibition of growth, and a clear dependence on growth factors and adhesion to extracellular matrices. Importantly this cell-line does not endogenously express hERG1 or hEAG1. We first examined the effect of hERG1 expression on morphology, proliferation, and migration under baseline conditions before investigating changes in response to pharmacological inhibitors.

hERG1-transfected NIH-3T3 clones were screened for stable hERG1 expression using a [³H]dofetilide binding assay (Figure 1A). Two clones (NIH-16 and NIH-50) were selected with specific binding (total binding minus non-specific binding) that was comparable to levels in guinea-pig myocardium. No specific binding was detected in the vector-control (NIH-VC) and V12Ras-transformed (NIH-Ras) clones. These findings were also confirmed by Western Blotting (Supplemental Figure 3D). NIH-16 and NIH-50 are distinct clones as determined from the relative expression of hERG1 relative to β-actin by quantitative real-time PCR (Supplemental Figure 1). NIH-16 and NIH-50 cell-lines expressed functional channels at the plasma membrane as illustrated by the characteristic currents shown in Figure 1B. This current was
blocked by cisapride, a potent inhibitor of hERG1 currents, and was not detected in NIH-VC or NIH-Ras cells (data not shown).

To investigate the contribution of hERG1 current to resting membrane potential, an optical recording method (Hardy et al., 2006) was utilized to measure membrane potential changes in response to hERG1 channel block. Switching the solution from 4 to 140 mM K⁺ Tyrode, to depolarize the membrane potential towards 0 mV, produced a robust and reversible increase in the fluorescence ratio, which was largest for the hERG1 expressing clones (Figure 1C, D). Dofetilide (10 μM) application to selectively block hERG1 channels caused a rapid increase in the fluorescence ratio in both NIH-16 and NIH-50 cells and not in the NIH-VC cells demonstrating that hERG1 channels are functionally expressed and contribute to a more hyperpolarized membrane potential than is observed in NIH-VC cells.

hERG1 expression causes loss of contact inhibition. A very clear phenotype of the hERG expressing cell lines was a loss of contact inhibition and an ability of these cells to overgrow one another. Loss of contact inhibition of growth is commonly associated with transformation and plays an important role in the development of a malignant phenotype. NIH-VC cells maintained a uniform monolayer even when cultured for 21 days after reaching confluency, which is a well-defined characteristic of this cell-line. In contrast, NIH-16 and NIH-50 clones showed a significantly different pattern of overgrowth, characterized by a network of interconnecting ridges of densely packed and over-growing cells surrounding regions where the cells grew in a more uniform monolayer (Figure 2A).

To quantify the amount of overgrowth, saturation densities were measured 4 days after confluency. hERG1-expressing cells reached significantly (p<0.01) greater saturation densities than NIH-VC cells and total protein levels per plate were also significantly higher (p<0.05, n=5) (Figure 2B,C). Time course experiments showed that cell numbers initially increased at similar rates while cultures were still sub-confluent (Figure 2D, see also thymidine incorporation results in Figure 5B), but by day 6 while NIH-VC cells had reached confluency and stopped dividing, hERG1-expressing cells and NIH-Ras cells continued proliferating at the same rate, independent of confluency. Phase contrast images of cells before and after reaching confluency showed the increase in cell number was not simply due to a reduction in the area of
the cellular footprint, but due to cells overgrowing one another (Figure 2E). When NIH-16 and NIH-50 clones became confluent the cells switched to a more transformed phenotype that shared morphological features with the NIH-Ras cells (Figure 2E).

*hERG1 expression increases the rate of cell migration into a wound.* The changes in NIH-16 and NIH-50 cell morphology post-confluency led us to investigate what impact hERG1 expression had on cell migration. The rate of migration of cells is limited by factors such as adhesion and availability of unpolymerized actin. Both of these factors are altered when a cell is transformed leading to a characteristic increase in mobility (Yamazaki et al., 2005). In line with this, confluent hERG1-expressing cells appear to have reduced contact with the culture surface. This effect may be mediated by the documented functional interaction between integrins and the hERG1 channel (Arcangeli et al., 2004; Cherubini et al., 2005; Cherubini et al., 2002; Hofmann et al., 2001). NIH-VC cells migrated into scratch wounds in an ordered fashion, maintaining cell-cell contacts and appearing to move as a ‘sheet’ of cells (Figure 3A, see Supplemental movie 1). NIH-16 and NIH-50 clones showed quite different patterns of migration to this (Supplemental movies 2 and 3 respectively). Cells at the edge of the wound quickly changed from an initially compact form to a longer, more extended morphology as the leading edges rapidly moved into the void. Cells behind the leading edge adopted the same extended morphology, migration was not coordinated and gaps appeared in the cell layer (Figure 3A). Observed changes in the pattern of migration in hERG1-expressing clones coincided with NIH-16 and NIH-50 migrating 45 ± 2% ($p<0.01$, $n=15$) and 35 ± 4% ($p<0.01$, $n=13$) quicker, respectively, than NIH-VC (Figure 3B).

Migration of cells in a scratch-wound assay is a complex process that can be affected by a variety of different factors, including cell-to-cell contact, secretion of signaling molecules, extracellular matrix proteins and cell density (Herren et al., 2001; Huang et al., 2003; Lampugnani, 1999). To get more detailed insight into the effects of hERG1 channels on cell migration and substrate dependent behavior we characterized single-cell true-speed migration and morphology at low cell densities and on different extracellular matrix (ECM) proteins. The results were highly ECM-dependent. The transformed morphology of hERG1-expressing cells was more pronounced on substrata pre-treated with fibronectin (2
μg mL⁻¹) than on untreated plastic, laminin or collagen, with cell bodies being more spindle-shaped and smaller in size (Figure 3C,D). True-speed migration was significantly (p<0.01) faster for hERG1-expressing cells; which also often had long thin cellular processes on the trailing edges (Figure 3C).

Phalloidin staining of filamentous actin and immunolabeling of vinculin revealed hERG1 dependent changes in the organization of the cell cytoskeleton (Figure 4). Whereas single NIH-50 and NIH-16 cells exhibited similar cytoskeletal organization to NIH-VC cells in non-confluent cultures, in over-confluent cultures cytoplasmic stress fibers were no longer observable, F-actin staining was more peripheral and vinculin staining was diffuse with fewer punctate regions (Figure 4). The NIH-16 and NIH-50 clones therefore appear to switch to a transformed morphology analogous to NIH-Ras cells on becoming over-confluent.

**hERG1-expressing cells exhibit substratum-independent proliferation and xenograft tumor formation.**

hEAG1 channel over-expression confers a robust proliferative advantage in CHO and NIH-3T3 cells (Hegle et al., 2006; Pardo et al., 1999). In contrast, we found hERG1 expression had little impact on cell proliferation when measured either as rates of change of monolayer confluence (Figure 5A) or by quantification of DNA synthesis by [³H]thymidine incorporation (Figure 5B).

Anchorage-independent cell survival and proliferation are essential to the development of metastatic cancers, thus, we tested whether hERG1 expression could induce this characteristic. NIH-VC cells were unable to proliferate in soft agar. In contrast, NIH-16 and NIH-50 cells generated on average 16 ± 6 (n=8) and 16 ± 7 (n=8) macroscopic and metabolically-active colonies, respectively. NIH-Ras cultures produced 306 ± 55 colonies (n=8) (Figure 5C). We noticed that amphotericin B and nystatin, ionophores commonly used to inhibit fungal contamination in long term cultures, had a substantial impact on colony formation, reducing it by 52%, 23% and 89% for NIH-Ras, NIH-16, and NIH-50 cultures, respectively. Since these ionophores increase the membrane permeability to monovalent cations we suggest that the observed toxicity may reflect changes to membrane potential that inhibit proliferation under these culture conditions. The ability of hERG1-expressing cells to proliferate in an anchorage-independent manner suggests that hERG1-expressing cells have the capacity to form tumors. We tested this directly by
implanting cell suspensions into nude mice, which were carefully monitored for up to 60 weeks. Five out of twelve mice injected with hERG1-expressing cells developed subcutaneous tumors (Figure 6), and most developed earlier than in control mice (Table 1). The subcutaneous tumors were slow to initiate compared to similar xenograft experiments of hEAG1-expressing cells (Pardo et al., 1999). The tumors from mice injected with hERG1-expressing cells were well vascularized, with many mitoses characteristic of on-going rapid cell division observed in high magnification images (Figure 6C). The one tumor from an NIH-VC injected mouse contained regions of necrosis that were absent in all other subcutaneous tumors.

*Long term exposure to selective blockers inhibits hERG mediated cellular transformation*

K⁺ channel function has been implicated in cell proliferation, volume regulation, chemotaxis and migration. Much of the evidence for these functions in non-excitable cells has relied on the use of K⁺ channel blockers. However, in many cases K⁺ channel blockers are used at comparatively high concentrations relative to the concentration required to block channel conductance (Afrasiabi et al., 2009; Crociani et al., 2003; Pillozzi et al., 2002). We found that long term (2-3 week) exposure of cultures to selective hERG channel inhibitors was effective in reversing cellular transformation, but short-term applications were ineffective. Thus, application of hERG1 channel blockers up to 24 h before experiments were performed, failed to block the effects of hERG1 expression on cellular transformation (Figure 7A, B). Small reductions in single cell migration were observed (Figure 7B), but these changes were not restricted to hERG1-expressing cells. We considered the possibility that dofetilide might lose efficacy in our culture conditions. However, this was not the case since dofetilide (100 nM)-containing medium taken from 24 h cell cultures was able to inhibit hERG1 currents in voltage clamp experiments (supplemental Figure 2). Short-term application of two other hERG channel blockers terfenadine (1 μM) or fluoxetine (3 and 10 μM) also had no impact on cell proliferation, while higher concentrations of terfenadine resulted in cell death of all clones (data not shown). Thus, acute application of hERG channel blockers either had no effect, or at high concentrations resulted in non-selective cell toxicity. However, this is in contrast to results obtained when hERG expressing NIH-16 cells and NIH-VC cells were
maintained in culture medium containing dofetilide (100 nM) for up to three weeks (Figure 8). Initially (Day 0), the cells have the transformed phenotype characterized by a spindle-shaped, polarized morphology with long membranous processes on the trailing edges of cells. However, with time in medium containing dofetilide the cells become less polarized, exhibit lamellipodia and have a morphology indistinguishable from NIH-VC and wild-type NIH-3T3 cells (Figure 8A). This switch in NIH-16 phenotype was unique to dofetilide treated cells and was never observed in other long term cultures of NIH-16 or NIH-50 cells, such as routine cultures used to maintain cell stocks. No changes in morphology were observed in control cells treated with 100 nM dofeltilide. The changes in NIH-16 cell morphology with dofetilide, correlated with changes in single-cell migration rates (Figure 8B). NIH-16 cells migrated faster than vector-control cells at early time-points, but migration then slowed progressively over the next three weeks and was not significantly different from NIH-VC after 14 and 21 days (Figure 8B). The responses to dofetilide were specific to the hERG expressing cells. Dofetilide had no significant effect on mean true-speed properties of control (NIH-VC) cells. These results strongly suggest that chronic application of therapeutically relevant concentrations of dofetilide, but not acute application of hERG channel blockers, can reverse the effects of hERG1 channel function on cellular transformation.

As an alternative to pharmacological inhibition we also investigated if inhibiting hERG1 channel function using dominant-negative channel mutations had the same effect as long-term inhibition with dofetilide. G628S hERG1 is a mutation of the selectivity filter that abolishes ion permeation, whereas A561V hERG1 is a mutation that eliminates trafficking of hERG1 channels to the plasma membrane (see supplemental Figure 3). Unlike the wild-type hERG1-expressing clones, the non-functional mutants failed to induce cell transformation. There was no loss of contact inhibition in saturation density assays, and cell morphology, true-speed migration and scratch wound closure rates were not significantly different from vector-control cells (Figure 9). Similar results were seen in two other A561V and G628S hERG1-expressing clones (data not shown). Thus perturbation of hERG1 channel function, either by long-term inhibition or loss of function mutations inhibits the transformative activity of these channels.
Discussion

hERG1 channels are expressed in a variety of cancer cells. The current study adds to the growing evidence that this is not coincidence and that the expression of functional hERG1 channels promotes cellular transformation and tumor progression. The effects of hERG1 expression were similar in the two hERG1-expressing clones studied, but distinct from V12 Ras-transformed NIH-3T3 cells. Allogeneic subcutaneous transplant of hERG1-expressing cells into nude mice resulted in an increased incidence of subcutaneous tumors. Importantly, our studies also demonstrated that the oncogenic effect could be reversed by pharmacologically inhibiting channel conduction.

hERG1 expression has pronounced effects on cell morphology and migration properties. Reports in the literature indicate that the effects of hERG1 expression appear to be cell-type and cancer type-dependent. For example, hERG1 channel function can modulate cell proliferation in lung cancer cells (Glassmeier et al.), acute myeloid leukemias (AML) (Pillozzi et al., 2002) and neuroblastoma cells (Crociani et al., 2003); migration and invasiveness in colorectal and AML cells (Lastraioli et al., 2004); angiogenesis through secretion of VEGF-A in high-grade astrocytomas (Masi et al., 2005) and AML cells (Pillozzi et al., 2002), and escape from apoptosis in acute lymphoblastic leukemias (Pillozzi et al., 2011). The effects of hERG1 expression that we observed in mouse fibroblast cells appear to be primarily on cell migration, loss of contact inhibition of growth and the ability to evade apoptosis and survive without attachment to solid surfaces (e.g. in soft agar cultures). This is consistent with hERG1 expression supporting metastasis and invasion. Although we do not currently have direct evidence for metastasis in vivo, interestingly we observed a variety of tumors in nude mice that were in tissues remote from transplantation sites. We also found cell morphology and migration properties varied when cells were cultured on different extracellular matrices, probably as a result of specific interactions with adhesion receptors. There is growing evidence that there are functional interactions between hERG1 channels and integrin adhesion receptors that may mediate the migration and invasiveness of hERG1-expressing tumor cells by recruiting signaling proteins such as FAK and PI3K (Cherubini et al., 2005; Cherubini et al., 2002; Hofmann et al., 2001; Lastraioli et
In leukemia cells, β1-integrin and hERG1 complexes also recruit and activate the VEGF receptor-1, Flt1, resulting in a strong pro-migratory phenotype that results in hERG1-expressing cells efficiently migrating out of bone marrow and invading the peripheral circulation (Pillozzi et al., 2007).

**Ion conduction is required for hERG1-dependent transformation.** One of the most important questions we wished to address was the importance of channel conduction for the oncogenic properties of hERG1 channels. On the one hand, several studies have suggested that channel blockers are effective in reducing cell proliferation and migration of hERG1-expressing cell-lines and primary cancer cells. On the other hand, concentrations required are often very high compared to the concentration required to inhibit hERG1 currents and the potential for off-target actions of these chemicals cannot be overlooked. Furthermore, several hEAG1 channel studies have now shown that abolishing channel conduction by mutation of the selectivity filter has only a limited impact on proliferation *in vitro* and tumor development *in vivo* (Downie et al., 2008; Hegle et al., 2006). Thus, the importance of K⁺ channel conductance for cellular transformation has become confused by conflicting results. In this study, conduction-deficient mutants completely failed to induce signs of transformation and long-term application of therapeutically-relevant concentrations of hERG blockers reversed the effect of hERG1 expression on cell migration and morphology. We did not observe any hERG1-specific effects of short-term application of a hERG channel blocker and do not have an explanation for this disparity, although another group has recently shown that application of E-4031 for 3 days also had minimal effects on proliferation of small cell lung cancer cells, whereas siRNA-mediated knockdown of hERG1 had a pronounced effect over the same time-period (Glassmeier et al., 2012).

The mechanism by which channel blockers reduce the transformative effect of hERG1 remains unclear. By binding within the pore and blocking conduction (Kamiya et al., 2006; Perry et al., 2010), blockers like dofetilide act like the G628S hERG1 mutation and cause depolarization of resting membrane potential. However, it seems unlikely that hERG1 channel expression only confers a selective advantage to cancer cells by simply influencing membrane potential dynamics since other K⁺ channels could
perform this function equally well and yet only a small proportion of K⁺ channels exhibit this oncogenic potential. Nevertheless, since hERG1 channels are sensing the membrane electric field via their voltage-sensing domains, membrane depolarization may be sufficient to induce conformational changes that switch off the intracellular signaling pathways that mediate its oncogenic functions. This type of mechanism, in which voltage-sensor conformation, rather than ion conduction is crucial, has been proposed for hEAG1 effects on cell proliferation and tumor formation (Becchetti, 2011; Downie et al., 2008; Hegle et al., 2006). The fact that blockers have to be applied for quite long durations before being effective also implicates signaling pathways with slow time-courses, possibly involving regulation of transcription factors and altered gene expression that regulate pro-migratory and pro-survival pathways. Regardless of the precise molecular mechanisms for transformation, the clinical implications of our study are striking. Several studies already suggest that hERG1 could be utilized as a biomarker for cancer detection and malignancy grading (e.g. (Lastraioli et al., 2012; Lastraioli et al., 2006)). Most importantly, our study provides further evidence that hERG1 channel blockers have therapeutic potential for treating hERG1-expressing cancers (see also (Pillozzi et al., 2011). The pharmaceutical industry has been reluctant to consider hERG1 as a target for pharmacological interventions, because of the cardiovascular side-effects of many of these compounds. Our study suggests that low concentrations of these compounds may be more effective than previously reported, particularly when used for extended periods of time.

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**Authorship contributions**

Participated in research design; Mitcheson, Challiss, Pritchard, Tresize, Pullar.

Conducted experiments; Pier, Shehatou, Giblett, Mitcheson.

Performed data analysis; Pier, Shehatou, Mitcheson, Pullar.

Wrote or contributed to the writing of the manuscript; Mitcheson, Pier, Challiss, Pritchard.
References


Footnotes

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4 George Shehatou, Department of Pharmacology and Toxicology, University of Mansoura, Egypt
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6 David Pier, School of Clinical Sciences, University of Edinburgh, UK
Figure Legends

FIGURE 1. hERG1 channels contribute to resting membrane potential in stably transfected NIH-3T3 cells. A: hERG1 channel expression was quantified by $[3^H]$dofetilide binding. Membrane preparations of NIH-3T3 clones were incubated with $[3^H]$dofetilide for 120 min before separation of bound and free components. hERG1-bound ligand was determined by subtracting non-specific binding (NSB) determined in the presence of unlabeled dofetilide (10 µM) from total binding. Specific binding was detected in NIH-16 and NIH-50 cells and guinea-pig (G/P) cardiac tissue, but not in vector control (NIH-VC) or Ras-transformed (NIH-Ras) cells. Graph presents values measured in triplicate from a single assay (repeated on at least 2 further occasions with similar results). Data are means ± S.E.M. B: Representative trace of hERG1 tail currents recorded from a hERG1-expressing cell (NIH-16) using conventional whole cell patch clamp. hERG1 currents were elicited by a voltage protocol shown in inset. Shown is a magnified view of hERG1 tail currents, corresponding to the boxed region of the voltage protocol. The ‘hook-shaped’ tail currents on repolarization are due to the distinctive gating properties of hERG characterized by rapid recovery from inactivation to the open state (causing large negative current), followed by a slower decrease in amplitude as the channels close (deactivate). C, D: Effect of selective hERG1 channel blocker, dofetilide, on resting membrane potential. Change in fluorescence ratio recorded from vector-control (NIH-VC; C) and hERG1-expressing (NIH-50; D) clones using the membrane-sensitive dye, Di-8 ANEPPS (see Methods). Cells were initially superfused with 4 mM K+ Tyrode solution before inducing membrane depolarization by application of 140 mM K+ Tyrode. After washing off the 140 mM K+ solution, dofetilide (10 µM) was added. Dofetilide had no effect on NIH-VC cells, but caused a depolarization in hERG1-expressing cells, indicating that hERG1 channels contribute to the resting membrane potential. Vertical arrows indicate when 140 mM K+ solution was applied and washed off.
FIGURE 2. **hERG1 expression alters the saturation density and growth properties of NIH-3T3 cells.**

**A:** NIH-3T3 cells were plated at a density to quickly achieve confluency and were cultured for 21 days with regular medium changes. Representative images of NIH-VC, NIH-Ras, and hERG1-expressing NIH-16, NIH-50 cells at 21 days post-confluency stained with Leischman’s reagent. **B:** Saturation densities. Wells were seeded to be confluent with $1 \times 10^6$ cells in 6 well plates. After 4 days post-confluency viable cell numbers were counted following staining with trypan blue. $n = 7 – 11$ experiments per clone. Bars represent means ± S.E.M. from 5 experiments (*p<0.05). **C:** Total protein measurements from proliferating (pre-confluent) cultures (~40% confluency) harvested after 24 h and post-confluent cultures (4 days after achieving confluency). Cells were solubilized with RIPA buffer and total protein determined using the method of Lowry *et al.* (1951). (**p<0.01, n = 5). **D:** Time-courses of cell proliferation and saturation densities. Cells were plated at low density ($1 \times 10^5$ cells per well in 6-well plates) and allowed to proliferate for up to 9 days. Medium was changed every 4 days. At days 2, 4, 6, and 9 viable cell numbers were quantified following staining with trypan blue. The number of viable cells was significantly larger for NIH-16, NIH-50 and NIH-Ras at days 6 (p<0.01) and 9 (p<0.01) than for NIH-VC. **B-D,** Statistical significance was assessed using a one-way ANOVA analysis with Dunnett’s post-test with NIH-VC as control. **E:** Images of NIH-3T3 cell cultures after reaching confluency (Day 0), and from the same cultures after a further 2 days (Day 2). Prior to confluency, the two hERG1-expressing clones had a similar morphology to NIH-VC cells, although cells have a more spindle-like shape. However, when NIH-16 and NIH-50 clones became confluent the cells switched to a more transformed phenotype. Cell bodies were smaller and more rounded and long cellular processes were formed that passed over neighboring cells. Boundaries between cells became less visible as cells overgrow one another. These changes in morphology are more clearly evident in time-lapse movies (Supplemental movies 4 - 6). Scale bars represent 100 μm.

FIGURE 3. **hERG1 expression profoundly influences cell migration behavior and morphology.**

**A:** NIH-3T3 clones were plated at confluency overnight in untreated 24-well plates. NIH-VC and
hERG1-expressing NIH-16 cell monolayers are shown immediately following scratch-wound formation (0 h) and after 16 h (16 h). B: Quantification of scratch-wound closure rates using IncuCyte imaging system. Cells were plated at confluency in 24-well ImageLock plates and left overnight. A wound was made in the monolayer using the Woundmaker apparatus and the size of the wound measured over 10 h. Data shown are means ± S.E.M. from >32 wells. C: Representative images of NIH-VC, hERG1-expressing NIH-16 and NIH-V12Ras cells plated on fibronectin during single-cell migration studies. D: Quantification of mean single-cell migration rates, cell size and cell polarization (see Methods). Data are shown as means ± S.E.M. from a minimum of 12 experiments in which migration of 20-25 cells were analyzed. Statistical significance was assessed using a one-way ANOVA analysis with Dunnett’s post-test with NIH-VC as control (**p<0.01, ***p<0.001).

FIGURE 4. **hERG expression alters cytoskeletal organization.** Cells were imunofluorescently labelled for vinculin (green) and phalloidin stained for filamentous actin (red). Left column: sparsely plated, non-confluent, cells. Middle column: post-confluent cells. Right hand column: vinculin staining only. Images on left and middle were taken with a confocal microscope. Images on right taken with conventional epifluorescence microscope so that multiple layers of cells can be visualized. Scale bar represents 25 μm.

FIGURE 5. **hERG1 channel expression has no effect on cell proliferation rates, but permits attachment-independent growth.** A: Confluency was assessed using the IncuCyte microscope system and was measured under control (+ 10% FCS) conditions over a period of 24-48 h (***p<0.001, n = 7). B: Cell proliferation rates were measured by [3H]thymidine incorporation into newly synthesized DNA. Stable NIH-3T3 clones were grown in medium containing 10, 5, 1 or 0% FCS for 48 h. [3H]thymidine incorporated into DNA was measured as described in the Methods section. Data represent means ± S.E.M. from three experiments performed in triplicate. NIH-50 and NIH-16 [3H]thymidine incorporation was not significantly different from NIH-VC, at any FBS concentration, whereas NIH-Ras was significantly different from NIH-VC at all FBS concentrations (p <0.01) except 1 % FBS. NIH-Ras
proliferation did not vary significantly with FBS concentration, and was significantly higher than NIH-VC \( (p<0.01) \) at 0 % FBS. 

**C:** Attachment-independent growth. Each bar represents the mean number of macroscopic and viable colonies that were able to grow in soft agar over 21 days. Data represent means ± S.E.M. from 4 experiments performed in duplicate \( (**p<0.01) \). **A &C** Statistical significance was assessed using a one-way ANOVA analysis with Dunnett’s post-test with NIH-VC as control. **B** Statistical significance was assessed using a two-way ANOVA, clone and FBS concentration co-varied, with Bonferroni’s post-test.

**FIGURE 6.** Mice implanted with hERG1-expressing cells develop large subcutaneous tumours. NIH-3T3 clones were subcutaneously grafted into the flanks of nude mice. **A:** Representative example of tumor at site of implantation of NIH-50 cells. **B:** Representative image illustrating well vascularised tumor from NIH-16 implanted mouse. Tumors were excised, fixed in 4 % paraformaldehyde and sectioned for histological analysis. **C:** Hematoxylin and eosin stained section (x40 magnification) from subcutaneous tumour in (A).

**FIGURE 7.** Short-term (<24 h) pharmacological channel blockade by dofetilide does not inhibit oncogenic effects of hERG1-expression. **A, B:** Short-term incubation of cells with dofetilide. **A:** Mean rates \( (n = 4) \) of scratch-wound closure over 10 h measured by IncuCyte imaging system. Where indicated dofetilide \( (10 \mu M) \) was added to the medium immediately prior to the scratch-wound being made. **B:** Single-cell true-speed migration rates on fibronectin-coated plastic was measured by time-lapse microscopy: dofetilide \( (1 \mu M) \) was added to the culture medium 1 h prior to starting experiments. Each bar represents means ± S.E.M. from 3 experiments performed in duplicate.

**FIGURE 8.** The oncogenic effects of hERG1 expression are inhibited by long term channel blockade with dofetilide. Effects of long-term incubation (up to 3 weeks) of cells with dofetilide \( (100 nM) \). Cells were maintained in the presence of dofetilide for 7, 14 or 21 days with dofetilide-containing...
medium changed every 3 days, or when cells reached 70% confluency and were passaged. Day 0 represents results from cells before dofetilide application. **A**: Representative images of the morphology of hERG1-expressing NIH-16 cells cultured in control medium or dofetilide-supplemented medium for up to 21 days. **B**: Single-cell true-speed migration rates for NIH-VC and NIH-16 cells at indicated time-points after dofetilide treatment. Following chronic dofetilide treatment for indicated time cells were plated at low densities on to fibronectin, allowed to attach and cell migration measured 5-8 h later. Results are pooled from 3 experiments (4 time points in each). Statistical significance was assessed using a two-way ANOVA, clone and day co-varied, with Bonferroni’s post-test (**p<0.001). After 14 and 21 days migration rates from NIH-16 cells are not significantly different from NIH-VC cells.

**FIGURE 9. Oncogenic effects of hERG1 expression are abolished in conduction-deficient hERG1 channel mutants.** NIH-3T3 clones were made stably expressing G628S hERG1 (NIH-C3) a non-conducting mutant or A561VhERG1 (NIH-C12) that is retained in the endoplasmic reticulum and not trafficked to the cell-surface. **A**: [3H]dofetilide binding assays revealed that channel expression in C3 and C12 clones was similar to (or greater than) hERG1 expression in NIH-16. These clones were initially identified by qRT-PCR and validated by Western blotting (Supplemental Figure 2). **B**: Cell viability and saturation density. 4 x10⁴ cells were plated per well of a 24-well plate and viability determined 4-8 days after plating by MTT colorimetry. Data points represent means ± S.E.M. from 3 experiments each with 4 replicate samples (**p<0.01). **C-E**: Images of NIH-VC, NIH-C3 and NIH-C12 cells 5 h after plating at low density on fibronectin-coated substrata. **F**, **G**: Migration properties on fibronectin-coated plates measured by time-lapse microscopy (single-cell true-speed migration, **F**) or by measuring scratch-wound closure (**G**). Statistical significance was assessed using a one-way ANOVA analysis with Dunnett’s post-test with NIH-VC as control (**p<0.01).
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**TABLE 1.** Tumor formation in allografted nude mice. Adult nude mice (three of each gender per clone) were subcutaneously implanted in the right flank with $4 \times 10^6$ to $7 \times 10^6$ cells in PBS. The mice were monitored for tumor development up to a maximum of 60 weeks. Number of weeks in third column indicates time after implantation when each mouse with a subcutaneous tumor was culled. In brackets is time of first observation of subcutaneous tumor. Post-mortem inspections were conducted on all mice and some were identified to have tumors at sites away from the site of implantation.
Figure 2

A. Microscope images of NIH-VC, NIH-Ras, NIH-16, and NIH-50 cells.

B. Graph showing cell number (×10⁵) for NIH-VC, NIH-Ras, NIH-16, and NIH-50 before and after confluence. The bars indicate significant differences (*p < 0.05, **p < 0.01).

C. Bar graph illustrating total protein (mg) for NIH-VC, NIH-Ras, NIH-16, and NIH-50 before and after confluence. The bars indicate significant differences (*p < 0.05, **p < 0.01).

D. Line graph depicting viable cell count (×10⁴) over time from day 0 to day 10 for NIH-VC and NIH-16, with a trend line for NIH-Ras. The graph shows a significant increase in viable cells over time for NIH-16.

E. Photographs of cell cultures at day 0 and day 2 for NIH-VC, NIH-Ras, NIH-16, and NIH-50, demonstrating cell growth and morphology changes.
Figure 4
Figure 7

(A) Rate of wound closure (μm hr⁻¹) vs [Dofetilide] (μM)

(B) Mean true speed (mm min⁻¹) vs [Dofetilide] (μM)