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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Received January 8, 2014; accepted May 14, 2014

ABSTRACT

Both human ether-à-go-go–related gene (hERG1) and the closely related human ether-à-go-go (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. An important finding of this work is that this transformation could be reversed by chronic incubation (for 2–3 weeks) with the hERG channel blocker dofetilide (100 nM), whereas more acute applications (for 1–2 days) were ineffective. The hERG1 expression resulted in a profound loss of cell contact inhibition, multiple layers of overgrowing 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 fibers, 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. The most important conclusion of this study is that 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 (Pardo et al., 2005; Schonherr, 2005; Fraser and Pardo, 2008; Arcangeli et al., 2009). One of these is the voltage-gated K⁺ channel, human ether-à-go-go related gene 1 (hERG1, K₁.11.1).

hERG1 channels are members of the ether-à-go-go (K₁.10–12) 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) (Lees-Miller et al., 1997; London et al., 1997; Crociani et al., 2003). 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 that this apparent upregulation is not due simply to altered gene expression with adaptation to in vitro culture conditions. Thus, hERG1 channels are overexpressed in endometrial adenocarcinoma (Cherubini et al., 2000), colorectal cancer (Lastraïoli et al., 2004; Dolderer et al., 2010), gastric cancer (Shao et al., 2008), glioblastoma multiforme, myeloid leukemias (Pilolzi et al., 2002), and acute lymphoblastic leukemias (Pilolzi et al., 2002; Smith et al., 2002); but expression is below detectable limits in noncancerous tissues. Interestingly, hERG1 expression in tumors correlates with metastatic cancers and a poorer prognosis (Lastraïoli et al., 2004; Masi et al., 2005; Ding et al., 2008).

hERG1 channels appear to regulate an array of cell behaviors, including cell proliferation (Pilolzi et al., 2002; Suzuki and Takimoto, 2004; Glassmeier et al., 2012), apoptosis (Wang et al., 2002), secretion of proangiogenic molecules such as vascular endothelial growth factor-A (Masi et al., 2005), and invasiveness and metastasis (Pilolzi et al., 2007). These activities are reported to be modified by hERG channel-selective blockers. Although 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 to 1000 times the pharmacologically determined IC50 values for inhibition of hERG1 currents (Pilolzi et al., 2002; Crociani et al., 2003; Afsarzadeh et al., 2010), 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+ channels and ion conduction in cancer cell biology remains controversial. Although it has been proposed for many years that K+ 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+ channel superfamily can support these roles. Only a select group of K+ channels ([K+]1,3, [K+]9,1, human ether-à-go-go (hEAG1), hEAG2, hERG1) influence proliferation and have been linked to cancer (Bianchi et al., 2003; Tajima et al., 2006; Huang et al., 2012), but expression is below detectable limits in noncancerous tissues. Interestingly, hERG1 expression in tumors correlates with metastatic cancers and a poorer prognosis (Lastraïoli et al., 2004; Masi et al., 2005; Ding et al., 2008).

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Materials and Methods

Cell Culture, Stable Transfection, and Clone Selection by [3H]Dofetilide Binding Assay. hERG1 was subcloned into pcDNA3 (Invitrogen, Paisley, UK) between EcoRI 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 RasV12 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 U of penicillin and streptomycin and 500 µg ml–1 G418 for a maximum of 10 passages. The [3H] 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 and 1 mM EDTA (pH 7.4) and centrifuged at 40,000g for 20 minutes at 4°C. The homogenization was repeated in fresh buffer and the membranes resuspended in assay buffer containing 71.5 mM NaCl, 60 mM KCl, 1 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, pH 7.4. Membranes (75 µg total protein) were incubated with 10 nM [3H]dofetilide at room temperature for 120 minutes, filtered through glass-fiber disks (Whatman; GE Healthcare, Little Chalfont, UK) presoaked in 0.25% polyethyleneimine, washed three times, and bound ligand quantified by scintillation counting. Nonspecific 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) 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 minutes. Measurements were taken from groups of three to seven cells illuminated with excitation light (460–500 nm, 100- to 640-nm wavelengths (Hardy et al., 2006).

Cell Proliferation Assays. A number of assays were performed to assess the 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 Wahlr, 1992); 1 × 105 cells per well were plated on a 24-well plate. After 45 hours, cells were incubated for 3 hours with 0.5 µCi [3H]thymidine. Cells were transferred to G/F/B filters (GE Healthcare) washed, lysed, and DNA-precipitated by incubation in 5% (w/v) trichloroacetic acid for 5 minutes, followed by wash steps in 2 ml of 10% potassium acetate in ethanol and 1 ml of ethanol: diethylther (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, Ann Arbor, MI). Images were taken of four fields per well at intervals of 2 hours over a 48-hour period. Cell confluence was calculated from the percentage surface area covered by cells (Green et al., 2009), and the percentage of change in confluency per
hour was calculated from the gradient of the linear portion of the growth curve. Adhesion-independent proliferation was assessed by colony formation in soft agar; 1 × 10^5 cells were suspended in 0.35% agar plated between two 0.7% agar layers. After 21 days, visible colonies were stained for 24 hours with 20 mg ml^-1 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldiazoliumbromide (MTT; Sigma-Aldrich, Gillingham, UK). Loss of contact inhibition of growth was tested by the ability to form foci of densely overgrowing 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 International, Radnor, PA) 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.** Individual mice were s.c. injected with 4–7 × 10^6 cells in phosphate-buffered saline (PBS) into the right flank of athymic nude mice (Harlan Laboratories, Indianapolis, IN). 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 cm^2 for maximum length × maximum breadth or if moderate adverse effects became evident. Postmortem examinations were performed, and tumors were excised, fixed in 4% paraformaldehyde, paraffin-embedded, sectioned, and stained with 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-minute intervals for 10 hours using the Incucyte system (Essen Instruments). Wounds were made in cells cultured in 24-well ImageLock plates (Essen Instruments) using the Woundmaker apparatus (Essen Instruments). Four wells of a 24-well plate were used per experimental condition. In some experiments, wound width was measured at five premarked points by taking photographs of wounds at 60-minute 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) for 15 minutes, permeabilized for 2 minutes in 0.2% Triton X-100 in PBS, and blocked for 20 minutes in 5% goat serum in PBS. Focal adhesion complexes were labeled for 1 hour with a 1:100 dilution of mouse monoclonal anti-vinculin (V9131; Sigma-Aldrich) in 1% goat serum, followed by incubation with 1:100 anti-mouse secondary antibody conjugated to Alexafluor 488 (Molecular Probes; Invitrogen, UK) in 1% goat serum. Filamentous actin was labeled with a 1:500 dilution of Texas–Red–conjugated phalloidin (Molecular Probes) in 0.1% bovine serum albumin in PBS for 30 minutes. Coverslips were washed, mounted using ProLong Gold antifade reagent (Molecular Probes), and visualized on an Olympus FV500 confocal microscope.

**Single-Cell Migration.** The 5 × 10^4 cells were plated on 35-mm dishes in a 37°C incubator for 5–8 hours. 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-minute intervals for 1 hour by OpenLab modular imaging software (Improvision, Coventry, UK). 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 µm^2) was assessed by outlining the periphery of single cells using ImageJ Software. Cell polarization was determined from the proportion of cells with one lamellum protrusion localized to the leading edge, resulting in an asymmetric cell shape (Nobes and Hall, 1999). Twelve values (three experiments, four 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 are provided in the relevant figure legends. Figures and statistical analysis were prepared using Prism software (GraphPad, San Diego, CA). Unless stated otherwise, statistical significance was analyzed using a one-way analysis of variance 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 it has been extensively used 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. An important characteristic of this cell line is that it 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 pharmacologic inhibitors.

The hERG1-transfected NIH-3T3 clones were screened for stable hERG1 expression using a 1H(dofetilide binding assay (Fig. 1A). Two clones (NIH-16 and NIH-50) were selected with specific binding (total binding minus nonspecific binding) that was comparable to levels in guinea pig myocardium. No specific binding was detected in the vector control (NIH-VC) and RasV12–transformed (NIH-Ras) clones. These findings were also confirmed by Western blotting (Supplemental Fig. 3D). NIH-16 and NIH-50 are distinct clones as determined from the relative expression of hERG1 relative to β-actin by quantitative realtime polymerase chain reaction (Supplemental Fig. 1). NIH-16 and NIH-50 cell lines expressed functional channels at the plasma membrane as illustrated by the characteristic currents shown in Fig. 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 used 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 toward 0 mV, produced a robust and reversible increase in the fluorescence ratio, which was largest for the hERG1 expressing clones (Fig. 1, C and 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 but 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 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 overgrowing cells surrounding regions where the cells grew in a more uniform monolayer (Fig. 2A).

To quantify the amount of overgrowth, saturation densities were measured 4 days after confluence. 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 ($P < 0.05, n = 5$) (Fig. 2, B and C). Time-course experiments showed that cell numbers initially increased at similar rates while cultures were still subconfluent (Fig. 2D; see also thymidine incorporation results in Fig. 5B), but by day 6, while NIH-VC cells had reached confluence and stopped dividing, hERG1-expressing cells and NIH-Ras cells continued proliferating at the same rate, independent of confluence. Phase-contrast images of cells before and after reaching confluence showed that the increase in cell number was not due simply to a reduction in the area of the cellular footprint but was due to cells overgrowing one another (Fig. 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 (Fig. 2E).

**hERG1 Expression Increases the Rate of Cell Migration into a Wound.** The changes in NIH-16 and NIH-50 cell morphology postconfluence 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 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 (Hofmann et al., 2001; Cherubini et al., 2002, 2005; Arcangeli et al., 2004). NIH-VC cells migrated into scratch wounds in an ordered fashion, maintaining cell-cell contacts and appearing to move as a “sheet” of cells (Fig. 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 (Fig. 3A). Observed changes in the pattern of migration in hERG1-expressing clones coincided with NIH-16 and NIH-50 migrating $45 \% \pm 2 \%$ ($P < 0.01, n = 15$) and $35 \% \pm 4 \%$ ($P < 0.01, n = 13$) more quickly, respectively, than NIH-VC (Fig. 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 (Lampugnani, 1999; Herren et al., 2001; Huang et al., 2003). To gain 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 proteins. The results were highly extracellular matrix-dependent. The transformed morphology of hERG1-expressing cells was more pronounced on substrata pretreated with fibronectin $(2 \ \mu g \ \text{ml}^{-1})$ than on untreated plastic, laminin, or collagen.
with cell bodies being more spindle-shaped and smaller (Fig. 3, C and D). True-speed migration was significantly ($P < 0.01$) faster for hERG1-expressing cells and also often had long, thin cellular processes on the trailing edges (Fig. 3C).

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

**hERG1-Expressing Cells Exhibit Substratum-Independent Proliferation and Xenograft Tumor Formation.** The overexpression of hEAG1 channels confers a robust proliferative advantage in Chinese hamster ovary 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 (Fig. 5A) or by quantification of DNA synthesis by [3H]thymidine incorporation (Fig. 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 clones generated on average $16 \pm 6 \text{(n}=8\text{)}$ and $16 \pm 7 \text{(n}=8\text{)}$ macroscopic and metabolically active colonies, respectively. NIH-Ras cultures produced $306 \pm 55$ colonies (n = 8) (Fig. 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 of 12 mice injected with hERG1-expressing cells developed subcutaneous tumors (Fig. 6), and most developed earlier than in control mice (Table 1). The subcutaneous tumors were slow to initiate compared with 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 ongoing rapid cell division observed in high-magnification images (Fig. 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 nonexcitable 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., 2010; Crociani et al., 2003; Pillozzi et al., 2002). We found that long-term (i.e., 2 to 3 weeks) 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 hours before experiments were performed failed to block the effects of hERG1 expression on cellular transformation (Fig. 7, A and B). Small reductions in single-cell migration were observed (Fig. 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-hour cell cultures was able to inhibit hERG1 currents in voltage-clamp experiments (Supplemental Fig. 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, whereas 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 nonselective cell toxicity. However, this is in contrast to results obtained when hERG-expressing NIH-16 cells were maintained in culture medium containing dofetilide (100 nM) for up to 3 weeks (Fig. 8). Initially (day 0), the NIH-16 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

24-well ImageLock plates and left overnight. A wound was made in the monolayer using the Woundmaker apparatus, and the size of the wound was measured over 10 hours. Data shown are means ± S.E.M. from >32 wells. (Representative images of NIH-VC, hERG1-expressing NIH-16, and NIH-Ras cells plated on fibronectin during single-cell migration studies. (D) Quantification of mean single-cell migration rates, cell size, and cell polarization (see Materials and 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 analysis of variance with Dunnett’s post-test with NIH-VC as control (**P < 0.01; ***P < 0.001).
medium containing dofetilide, the cells become less polarized, exhibit lamellipodia, and have morphology indistinguishable from NIH-VC and wild-type NIH-3T3 cells (Fig. 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 dofetilide. The changes in NIH-16 cell morphology with dofetilide correlated with changes in single-cell migration rates (Fig. 8B). NIH-16 cells migrated faster than vector-control cells at early time points, but migration then slowed progressively over the next 3 weeks and was not significantly different from NIH-VC after 14 and 21 days (Fig. 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.

As an alternative to pharmacologic inhibition, we also investigated whether inhibiting hERG 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 Fig. 3). Unlike the wild-type hERG1-expressing clones, the nonfunctional 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 (Fig. 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 they were distinct from RasV12-transformed NIH-3T3 cells. Allogeneic subcutaneous transplant of hERG1-expressing cells into nude mice resulted in an increased incidence of subcutaneous tumors. An important result of our studies is demonstration 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., 2012), acute

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<th>Clone Injected</th>
<th>No. of Mice with Subcutaneous Tumors</th>
<th>No. of Weeks</th>
<th>No. of Mice (of 6) with Tumors at Distant Sites</th>
<th>No. of Mice (of 6) with Tumors of Either Description</th>
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NIH, National Institutes of Health; VC, vector control.
myeloid leukemias (AMLs) (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). These findings are consistent with hERG1 expression supporting metastasis and invasion. Although we do not currently have direct evidence for metastasis in vivo, we observed a variety of tumors in nude mice that were in tissues remote from transplantation sites. We also found that 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 focal adhesion kinase and phosphoinositide-3-kinase (Hofmann et al., 2001; Cherubini et al., 2002, 2005; Lastraioli et al., 2004; Pillozzi and Arcangeli, 2010). In leukemia cells, β1-integrin and hERG1 complexes also recruit and activate the vascular endothelial growth factor receptor-1, Fk1, resulting in a strong promigratory 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 with 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 (Hegle et al., 2006; Downie et al., 2008). 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 an 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 small interfering RNA–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 confers only 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 (Hegle et al., 2006; Downie et al., 2008; Becchetti, 2011). 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 the regulation of transcription factors and altered gene expression that regulate promigratory and prosurvival 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 used as a biomarker for cancer detection and malignancy grading (e.g., Lastraioli et al., 2006, 2012). 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 pharmacologic 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.

Acknowledgments

The authors thank Dr. Matt Hardy for assistance with optical measurements of membrane potential.

Authorship Contributions

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

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

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

References


