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The use of Bcl-2 over-expression to stabilize hybridomas specific to the HERG potassium channel

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Abstract

We encountered a high degree of clonal hybridoma loss in the course of generating antibodies specific for the hERG potassium channel a protein that is crucial for controlling heart rhythm, is abundant in parts of the brain and is abnormally expressed in some tumors. Intracellular domains of the protein were used for immunogens and generated adequate antibody responses in mice. Subsequent hybridomas created using Ag8 myeloma fusion partner yielded clones that secreted specific antibody but none could be successfully maintained in culture. A variety of mechanisms, including polyploidy inherent to hybridoma development or production of cytotoxic antibodies, may be responsible for eventual loss of cell viability by mechanisms that may include apoptosis. When spleen cells were fused to the NSO myeloma cell line that stably over-expresses the anti-apoptotic protein Bcl-2, hybridoma clones were generated that remained viable in culture with high level of hERG-specific antibody production. When the parental NSO cell line not over-expressing Bcl-2 was used, no stable hybridomas were produced. Antibodies secreted by NSO-Bcl-2 hybridomas were specific for hERG and performed well in immunoblot, immunoprecipitation and immunofluorescence assays. This work demonstrates a feasible option when faced with antigens that seem to be associated with clonal instability in the process of generating monoclonal antibodies.

Introduction

One of the genes that most commonly harbors mutations leading to the hereditary cardiac long QT syndrome (LQTS) is HERG (human *Ether-a-go-go* related gene, also known as Kv11.1 and KCNH2). HERG encodes an intrinsic surface membrane protein that forms a potassium ion-selective channel and is expressed in the heart, brain and several other peripheral tissues. Deleterious mutations of HERG are associated with cardiac rhythm disturbances that may lead to syncope and sudden death at a young age (Moss, 2003, Modell

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and Lehmann, 2006, Sanguinetti et al., 1995)). More than 300 LQTS-associated mutations have been reported and of those that have been studied, more than half show assembly, trafficking and protein stability defects (Anderson et al., 2006). Moreover, acquired LQTS—a much more common entity—is nearly always due to drug interactions with the HERG channel that either block its ion conduction properties or confer trafficking defects upon the wild type channel (Witchel, 2010). Such common drug interactions have led both the U.S. Food and Drug Administration as well as EU's European Medicines Agency to mandate that every newly developed drug be tested for such an adverse effect (1996, 2005, Darpo et al., 2006). Accordingly, high-specificity and affinity reagents such as monoclonal antibodies are desirable to enhance future investigative efforts. We set out to develop HERG monoclonal antibodies that could potentially be used for these applications. Unexpectedly, we encountered repeated difficulty in keeping HERG-specific antibody-producing hybridomas alive in stark contrast to simultaneous creation of monoclonal lines against another potassium channel protein. Here report the nature of the problem we encountered and describe one possible solution that we found successful.

Methods

Antigen preparation

Small antigen peptides were prepared first by performing PCR on HERG cDNA template (see Figure 1 A) using the following primer pairs: F2: cggaattccagagccgtaagttcatcatcgc and cgaagcttttaccatcctcgttcttcacgg, F4: cggaattcgctgtcatcatgttcaccc and cgaagcttttagttgtccatggctgtcac, F6: cggaattcgtgacagccatggacaaccac and cgaagcttttacgaggtggagttgagcaag, F8: cggaattcgactccgacctcgtgcgtac and cgaagcttttagatgggtccagcgggtggatgc, F10: cggaattcaacatggagcagcc and cgaagcttttagatcttctctgagttgggtgttg, F12: cggaattcgcccgctaccacacacagatg and cgaagcttttacaccagtgtgtccctggc, F14: cggaattccatgctgtgggacctgctc and cgaagcttttagccgggatcatgttg, F16: cggaattctccccggcagtaag and cgaagcttttagttgcaagtgtcgtgctc, and F18: cggaattcgctgtgaggagctgccc and cgaagcttttaactgcccgggtccgagccg. The PCR products were digested with EcoRI and HindIII, and ligated into the pMAL-c2 vector (New England BioLabs). These constructs encoding maltose binding protein (MBP)-HERG fragment fusions were expressed in B121(DE3) *E. coli* strain (Invitrogen). Recombinant protein-expressing bacteria were pelleted, resuspended in MBP buffer (20mM Tris-HCl pH~7.4, 0.2M NaCl, 10mM beta-mercaptoethanol, 1mM EDTA) supplemented with complete protease inhibitors (Roche). After cell disruption, protein lysates were applied to an amylose resin (New England BioLabs) affinity column and washed. Elution was accomplished using MBP buffer containing 10mM maltose. Final purification was achieved using FPLC gel filtration.

SDS Page and Immunoblots

Antigen samples (F2-F18) were separated on 4-15% gradient gels (BioRad) and stained with Coomassie brilliant blue dye. HEK293 cell lysates were separated on 7.5% linear gels and transferred onto nitrocellulose membranes via a semi-dry blotting unit (Fischer Scientific). The membranes were blocked with 5% non-fat milk, probed with appropriate primary antibody and then incubated with either IRDye700 donkey-anti-rabbit or IRDye800 donkey-anti-mouse (Rockland) secondary antibodies. Infrared signal was scanned using the Odyssey system (LiCor).

Immunization

6-week old female BALB/c mice were used and all procedures and handling were in accordance with the approved protocol reviewed by the Einstein Animal Institute

Committee. Mice were immunized with the following pools of antigen: pool A: F2, F4, and F8; pool B: F10; pool C: F12, F14, F16, and F18 (Figure 1A). All immunizations were via intraperitoneal injection. Initial immunization on day-1 was done using an emulsion of 100µl of complete Freund's adjuvant (CFA) mixed with 100µl of antigen pool per mouse. Subsequent boosts on week 3 and week 6 were similar, except incomplete Freund's adjuvant (IFA) was used. Anti-HERG antibody titers were monitored from tail-bled sera, using a 28-channel miniblotted cartridge (Immunetics) on nitrocellulose membranes carrying single-lane HEK293-HERG lysate. To further boost initial titers, pool A and B mice were immunized with mariculture keyhole limpet hemocyanin (mcKLH) to generate KLH specific T cell help, and subsequently boosted three more times with mcKLH covalently cross-linked to pool A and pool B antigens, respectively (using the Inject Immunogen EDC Kit, Thermo Scientific). Mice were allowed to rest for at least 1 month before final boosts. Four (day 1) and 3 (day 2) days before the fusion, the mice were injected intraperitoneally with 100µg of pure pooled antigen in PBS (pool C) or with 100µg pure pooled antigen combined with 100µg of mcKLH-linked antigen (pools A and B) and then sacrificed and their spleen cells fused on day 4.

Spleen cell-myeloma fusion

Our hybridoma preparation technique was based on a modification of the protocol of de StGroth and Scheidegger (de StGroth and Scheidegger, 1980). On the day of the fusion, mice were sacrificed and placed into 70% ethanol. Subsequently, their spleens were removed and placed in DMEM (Cellgro) supplemented with penicillin/streptomycin. The spleens were next pierced with a syringe, and their cellular content was slowly washed out with DMEM. Splenocytes were separated from debris by gravity sedimentation (at 4°C) and then centrifuged at 400g for 10min; myeloma cells obtained from logarithmically growing cell cultures were similarly pelleted and washed with DMEM. Next, the spleen cell pellet was resuspended in cold 0.17M NH₄Cl, pH~7.0 and incubated for 10min on ice to lyse contaminating erythrocytes. This process was then quenched by slow addition of ice-cold DMEM followed by centrifugation. Cells were then counted, mixed in 2:1 ratio of spleen cells to myeloma cells and centrifuged once more. Cell fusion was achieved by addition of 0.5ml 50% polyethyleneglycol 4000 (Merck) in saline (137mM NaCl, 5.4mM KCl, 10.0mM Na₂HPO₄, 5mM NaH₂PO₄, and 11.1mM glucose) to the pellet and incubation for exactly 90-seconds at 37°C; this was concluded by slow addition of 20ml saline and a 10min incubation at room temperature. Finally, the fused cells were centrifuged, resuspended in HAT media (DMEM, high glucose (Cellgro), 10% NCTC-109 (Gibco), 20% FBS (Atlanta), 1% non-essential amino acids, 2% of 50× HAT solution (composed of 0.68g/l hypoxanthine, 0.20g/l thymidine, and 8.8mg/l aminopterin stock), penicillin/streptomycin), distributed onto 96-well plates at 30,000 cells/100µl/well and then propagated at 37°C under 10% CO₂ atmosphere.

Hybridoma culture and screens

After 1 week, the hybridoma lines were fed with 100µl of HAT media. Following another week of incubation, the 96-well plates were examined for ~20% confluent colonies 3 times per week. Supernatants from these mature colonies were collected and screened by ELISA against MBP-HERG fragment fusion pools as well as against MBP alone. Screens using a 28-channel miniblotted cartridge (Immunetics) on nitrocellulose membranes carrying single-lane HEK293-HERG lysate were also done in parallel. All hybridoma cells were kept in HAT media for a total of 3 weeks, followed by 2 week incubation in HT media (HAT media deficient without aminopterin), and eventually moved to growth media devoid of all selective compounds. Clones identified as positive were expanded and subcloned using soft agarose or by limiting dilution (French et al., 1987). Media were either harvested directly

from dense hybridoma populations, or the cells were cultured in CELLline bioreactors (Integra) and media containing high concentrations of monoclonal antibodies were collected.

Immunofluorescence

HEK293 cells and HEK293-HERG cells were fixed with 4% paraformaldehyde and stained with the produced monoclonal antibodies. Alexa Fluor 488 donkey-anti-mouse antibodies (Invitrogen) were used for detection. Images were collected on Leica AOBS SP5 laser confocal scanning microscope.

Small-scale immunoprecipitation

Two 10cm dishes confluent with HEK293-HERG were lysed using 2ml NDET buffer (150mM NaCl, 5mM EDTA, 1% NP-40, 0.4% deoxycholate, 25mM Tris pH~7.5, Roche's Complete protease inhibitors). After a 10min 13,000rpm centrifugation, the supernatant was pre-cleared with 20µl protein G UltraLink beads (Thermo Scientific). Half of the supernatant was then incubated with 20µl non-specific mouse IgG (Santa Cruz), while the other half was immunoprecipitated with 20µl of CELLline bioreactor-concentrated 25A2 antibody at the same concentration as the control. After 2 hours of vigorous mixing, 20µl of protein G beads were added, and the mixture was shaken overnight at 4°C. The beads were then washed three times with NDET, eluted with SDS-PAGE loading buffer for 30min, separated on a 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane and detected using rabbit-anti-HERG (H175) antibody (Santa Cruz).

Large-scale immunoprecipitation

First, pooled monoclonal antibodies were covalently cross-linked to protein A/G agarose beads (Santa Cruz) following the procedure published by AbCam (AbCam, 2010). Briefly, 200µl (bed volume) protein A/G beads were washed with PBS-B (PBS with 0.1% BSA) and incubated with pooled monoclonal antibodies (200µl 25A2, 200µl 10H12, 200µl 1H4, all CELLline bioreactor-concentrated supernatants plus 200µl spent media from 23A7-secreting cells) for ~2hrs. The beads were washed 1× with PBS-B, 1× with PBS, and 1× with wash buffer (0.2M triethanolamine in PBS). Cross-linking was achieved with a set of three 30-minute incubations with 1ml of fresh-prepared 13mg/ml dimethyl pimelimidate (DMP, Pierce) in wash buffer, each followed by a brief rinse in plain wash buffer. Two 5min incubations in quench buffer (50mM ethanolamine in PBS) terminated the reaction. After a quick rinse in plain PBS, the beads were treated with elution buffer (1M glycine, pH~3.0) twice for 10min to remove all unlinked antibody. A single wash in plain PBS and two washes in NDET concluded the preparation of the resulting HERG affinity column.

Six 15cm plates confluent with HEK293 cells and an equal amount of HEK293-HERG cells were lysed in 1.5ml NDET per plate. Post-nuclear supernatants were pre-cleared with 50µl protein A/G beads for ~1hr and then shaken with 80µl of antibody-cross-linked beads overnight at 4°C. Next, the beads were washed 5× with NDET and eluted 2× with 50µl elution buffer (10min each). The thus obtained samples were immediately mixed with SDS-PAGE loading buffer (containing DTT, 25mM final concentration) boiled for 5min, and loaded on a large 6-15% polyacrylamide gel. After separation, the gel was silver-stained using the Dodeca silver stain kit (BioRad).

Results

Expression of HERG antigens

Though the full-length HERG protein would be the optimal antigen, large-scale expression of entire mammalian ion channel proteins in heterologous systems has, in our experience, met with limited success. Even expression of GST-fusions of medium-length segments of

the HERG channel, such as those encompassing the entire N- and C-termini (Fig 1A) led to low-level protein production in bacterial and baculoviral expression systems. Accordingly, we decided to produce short (~100 amino acids long) HERG segments selected for their high immunogenicity index as well as low hydrophobicity as predicted by Lasergene software (DNASar). Our best results occurred when we expressed them as N-terminal maltose binding protein (MBP) fusions (Fig 1B). Most, but not all, of these constructs were detected in their full predicted length of ~48kDa to ~55kDa, although extensive degradation/truncation bands were also visible. The exception was the N-terminal fragment F6, which resulted in an apparently MBP-only product and was thus excluded from subsequent experiments.

Immunization

Three pools of antigens were established: pool A (F2, F4, and F8), pool B (F10), and pool C (F12, F14, F16, and F18) to be injected into three sets of mice. Based on Western-blot analysis using lysates from HEK293 cells stably expressing full length HERG (HEK293-HERG), the antibody titers in mice immunized with pool C mice were greater than 1:50,000, while sera from the other ten animals had lower titers (1:25,000 or less). Consequently, we immunized the mice that had received pools A and B with one injection mCKLH (mariculture keyhole limpet hemocyanin, Thermo Scientific) and then boosted three times with pools A or B covalently cross-linked to mCKLH. This resulted in immunoblot-based titers of close to 1:50,000. After resting the mice for at least one month, mice were selected (based on immunoblot titers of 1:50,000 or higher) and boosted on day 1 and 2 with the respective antigen pools in saline and sacrificed 4-days later. Splenic cell populations were fused with a variety of mouse myeloma cell lines (see below). There were two spontaneous animal deaths: one animal in pool C expired during the immunization period, while another previously healthy pool C mouse died after being boosted 3 days prior to the intended splenic fusion procedure.

Anti-HERG hybridoma line production

The first fusion was with the spleen from animal C1 immunized with the pool C antigens. Spleen cells were fused with the Ag8 myeloma line and distributed into 1920 wells at a concentration that typically results in 2-3 healthy hybridoma colonies/well (see methods). Compared to other fusions done exactly the same way and at the same time but with other antigens (KCNQ1 ion channel), hybridoma cells grew to colonies much more slowly and even after 4 weeks post-fusion, only 224 wells contained a viable, dividing cell-line (~25-times less than expected). Supernatants from the healthy colonies were screened by ELISA against a pool C-MBP and MBP protein alone. Twenty supernatants were identified as positive (i.e. positive for MBP-antigen and negative for MBP alone), but only 4 of these were able to detect HERG on an immunoblot. Furthermore, antibody production in these 4 clones declined rapidly and became undetectable within 1 week of the initial screen. Attempts to isolate producing clones by limiting dilution or soft agar cloning proved unsuccessful. Furthermore, the surviving subclones did not produce any immunoglobulin, suggesting that the production of the antibody was lethal to the cells. Since we had high titer anti-HERG polyclonal antibodies from mouse that was used for the fusion, we grew the parental fusion partner in the presence of various dilutions of the antisera. We did not detect any inhibition of growth of Ag8 myeloma cell line by this polyclonal antiserum suggesting that the monoclonal antibodies were not killing the hybridoma cells by reacting with some form of the HERG protein expressed on the surface of the cells.

Our inability to isolate stable secreting hybridomas led us to hypothesize long term production of the antibodies is somehow internally toxic to the cells, possibly triggering programmed cell death. As a result, we decided to fuse the splenic cells from the next pool C

mouse (C2) with NSO myeloma line stably over-expressing the Bcl-2 anti-apoptotic gene. These cells were constructed and characterized by Ray and Diamond (Ray and Diamond, 1994), who used them to develop stable anti-DNA producing hybridomas that were the product of autoantibody producing B cells that were pre-programmed to undergo apoptosis. The use of the Bcl-2 expressing fusion partner thus allowed the rescue of hybridomas that could not be obtained using the usual fusion partners. We plated 3072 wells with NSO-Bcl-2 fusions along with 480 wells with NSO (wild-type) and 480 wells with Ag8 fusions. At week-4 post-fusion, we screened 1246 NSO-Bcl-2, 69 NSO, and 125 Ag8 confluent hybridoma populations, and detected 7 that reacted with HERG but not MBP by ELISA. All 7 were from the NSO-Bcl-2 fusion partner. Of these 7, 4 were also positively reactive on HERG immunoblots. We further screened 215 NSO-Bcl-2, 5 NSO, and 34 Ag8 ELISA-negative populations (chosen at random) on Western blots to look for monoclonal antibodies that might not react HERG by ELISA but would on Westerns and found another 35 positives that were also all from the NSO-Bcl-2 fusions. However, all of these ELISA-negative, Western blot-positive populations lost anti-HERG antibody production within 2 weeks of the initial screen. Conversely, the 4 ELISA-positive, Western blot-positive hybridomas were sub-cloned twice and maintained the same levels of antibody expression and reactivity for over 3 months. We have also been able to recover these antibody producing hybridomas from the freezer and three of them retained reactivity and production in bioreactors (see below).

Further attempts to produce HERG-specific monoclonal antibodies did not result in antibodies that reacted with HERG by Western analysis. Mice from pool A (A1) as well as pool B (B1) were boosted with their respective antigens, and their spleen cells were fused solely with NSO-Bcl-2 cells. In each case, 1920 wells were plated; ELISA screens of 864 and 576 confluent populations, respectively, yielded several positives, none of which produced immunoblot-detectable antibodies. Consequently, as summarized in table I, the 4 immunization/fusion experiments led to the production of 4 anti-HERG hybridomas, all resulting from NSO-Bcl-2 fusions.

The hybridoma clones that reacted with HERG by Western (1H4, 10H12, 23A7 and 25A2) were expanded, subcloned again and tested by Western to confirm that they were stable and then grown in CELLline bioreactors (Integra) to produce highly concentrated antibody supernatants (all app. 1mg/ml). Because the clone 23A7 displayed very slow growth, direct tissue culture supernatants rather than bioreactor harvests were used for further experiments.

Antibody characterization

Isotyping by ELISA showed that all 4 antibodies were IgG₁. Clones 10H12, 23A7, and 25A2 all produced antibodies specific to HERG fragment F18, while the 1H4 clone was specific for F12 (see Figure 1). Serial antibody dilutions of 25A2 showed that specific HERG signal could be detected (by SuperSignal-WestDURA, Pierce) at titers as low as 1:75,000 (Supplementary figure 1).

Analysis by immunoblot of cell lysates from HEK293-HERG cells showed that the F18-specific antibodies (10H12 and 25A2 diluted 1:1000, 23A7 diluted 1:200) detected HERG as two intense bands (along with a faint high molecular weight doublet possibly representing HERG multimers), while not binding significantly to any epitope from the HEK293 paternal cell line (Fig 2). Conversely, the 1H4 antibody (diluted 1:200) resulted in fainter HERG signal detection.

To reveal whether the HERG monoclonal antibodies are also reactive and specific for immunofluorescence applications, we used them as probes on paraformaldehyde-fixed HEK293 as well as HEK293-HERG cells. 10H12 (1:1000), 23A7 (1:100), and 25A2

(1:1000) intensely stained HERG-expressing cells, while there is not staining of the untransfected HEK293 cells, even when scanned with the microscopes photomultiplier detectors (PMTs) set to maximum gain (Fig 3). 1H4 (1:100) was also unreactive with fixed HEK293 cells, but produced only faint staining of HERG; also, in some cells, it resulted in a granular pattern, suggesting the F12 epitope is poorly exposed/folded in stained cells. HERG immunofluorescence imaging done with other antibodies (Chen et al., 2009, Um and McDonald, 2007) generally resembles immunostaining patterns seen with the 10H12, 23A7, and 25A2 antibodies rather than 1H4.

We further tested whether the 25A2 antibody is able to immunoprecipitate (IP) the HERG channel. As seen in figure 4, the antibody was successful in pulling down a significant portion of the HERG protein from lysed HEK293-HERG cells, though certain portion of the highly abundant immature form of HERG was not immunoprecipitated.

Finally, we wished to find out whether the now abundant and concentrated monoclonal antibodies could be used to produce large scale affinity columns able to purify copious amounts of HERG for further downstream applications. To this end, we pooled the 1H4, 10H12, 23A7, and 25A2 antibodies (200 μ l each, equal to app. 600 μ g of total antibody) and covalently cross-linked them to protein A/G agarose beads (200 μ l packed volume, predicted to have a binding capacity of up to 3.8mg of mouse IgG). The resulting affinity column was then used to pull-down HERG from large-scale HEK293-HERG cell lysate (along with HEK293 cell lysate as a control). Large-gel silver stain revealed very strong HERG doublet, absent in the control lane. Moreover, a large amount of other HEK-HERG-specific bands were also detected, possibly representing HERG-degradation products along with HERG-interacting partners. Two of these bands (~30 and ~70kDa) were excised and submitted for LC-MS/MS analysis (W.M. Keck Foundation, Yale University); the bands were identified as 14-3-3 ϵ / θ and Heat shock protein 70-2 (Hsp70-2), respectively. Both are previously discovered interacting proteins (Kagan et al., 2002, Ficker et al., 2003).

Discussion

In order to obtain monoclonal antibodies that could be used in Western analysis of HERG and for studies of its intracellular distribution and interacting proteins, we initially employed standard techniques. While we succeeded in generating a set of 4 monoclonal antibodies that are described here, we encountered technical problems at each step. HERG is a medically and physiologically important protein and consistently reliable monoclonal antibodies have not been forthcoming. If future efforts to generate specific antibodies against this or other important human protein are met with similar difficulty the solutions we present here may be useful.

The first problem was our inability to express the whole HERG protein or even fragments of it much larger than ~100 amino acids in *E. coli* or insect cells. In fact, substituting 6 $\times$ His peptide for the MBP tag abolished bacterial expression even of these short peptides. This is consistent with a scenario in which the HERG protein has very stringent requirements for conditions regulating its synthesis, folding and/or degradation, but that under certain circumstances, protein tags capable of chaperone function (such as MBP) may remedy this (Kapust and Waugh, 1999, Wang et al., 1999).

We were able to obtain reasonable titers of antibodies to HERG in ELISA assays by immunizing mice with pools of multiple short peptides taken from the HERG sequences. We pooled the peptides so that fewer mice would have to be immunized. The highest titers of antibody that were reactive on Western blot were achieved with a pool of peptides from the C-terminal part of the protein and in the end we were only able to obtain monoclonal

antibodies to C-terminal epitopes including 3 to the most C-terminal peptide (F-18, see figure 1) and one that reacted with a more proximal C-terminal segment (F-12) of the channel. We tried additional immunization protocols to obtain a strong antibody response to the N-terminal peptides and even though we eventually were able to obtain antibody responses, the titers of antibodies that reacted by Western were never as high as those obtained to the C-terminal pool.

The next major problem was that the hybridomas that were generated using the usual fusion partners grew very slowly. Even though we identified hybridomas that were producing antibodies that reacted well on Westerns to HERG, these quickly lost the ability to secrete immunoglobulin. Even though we cloned these hybridomas very early in their propagation and examined hundreds of subclones for ones that retained production, we were unable to obtain stable antibody producing clones. This suggested to us that the antibody was toxic for the hybridomas. The deaths of two out of five mice immunized with the pool C peptides could have also been a consequence of toxicity of the antigen or the humoral response against it.

Furthermore, although a total of 39 C-terminus-specific hybridomas were identified, only 4 that reacted with F12 and F18 (Table 1) maintained stable immunoglobulin production. This again suggested that most pool C antibodies were toxic. This was consistent with the observation that while none of the hybridomas that were generated with the standard fusion partners continued to secrete immunoglobulin, the stable hybridomas that we did obtain were from the fusion with the NSO-Bcl-2 fusion partner. While this observation is anecdotal because it would require repeating many more fusions to obtain statistically significant data, it is likely that the presence of the anti-apoptotic protein made it possible to rescue the hybridomas cells from the toxicity of the antibody.

It is not clear exactly why hybridoma cells producing antibodies to HERG were unable to maintain stable production of the those antibodies. The fact that the antisera from the immunized mice did not inhibit the growth of the standard fusion partners when it was added to the media suggests that the antibody was not killing the cells by reacting with a surface antigen. Furthermore, supplementation of the 1H4, 10H12, and 25A2 antibodies into growth media did not visibly affect plain NSO cell growth or morphology (data not shown). This suggests that the antibodies were causing toxicity intracellularly. Moreover, no N-terminal or pore-loop-specific antibodies could be isolated, even when Bcl-2-expressing fusion partner was employed, and despite relatively high pre-fusion serum titers. This is all consistent with findings that HERG is very sensitive to perturbations of its early assembly and trafficking so the intracellular presence of antibody could block the function of HERG or an HERG like protein in the hybridoma cells. To date, no study has reported the expression of ERG protein in mouse multiple myeloma lines, and our own anti-HERG immunoblots on NSO and Ag8 cell lysates show no reactivity (data not shown). However, several studies have revealed functional expression of HERG in immune cell tumors, particularly leukemias, where its levels correlate with malignant potency (Pillozzi et al., 2007, Guasti et al., 2008). It may be that even low, antibody-undetectable expression of functional ERG is necessary for mouse hybridoma survival; this can be blocked intracellularly by the expressed antibodies directed against a C-terminal (intracellular) epitope. Further, extensive studies will be needed to explore this as well as other possibilities.

Potential applications of these monoclonal antibodies are many. Here, we show that large-scale immunoprecipitation of HERG from mammalian cells is now possible. This opens the door to further studies, including proteomic analysis of low abundance co-

immunoprecipitated proteins or purification of larger amounts of the channel to be used for structural studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Antigen design and expression

A) Diagram of HERG antigen peptides. Longer antigens (BAC-N and BAC-C) were expressed in baculoviral system as GST fusions. Shorter antigens (F2-14) were expressed in *E. coli* as MBP fusions.

B) Coomassie brilliant blue stain of purified HERG antigens expressed in *E. coli*. Baculoviral constructs achieved very poor expression and are not shown. Putative MBP-only degradation/truncation product is marked by an arrow.

Figure 2. Immunoblots using HERG monoclonal antibodies

The newly produced HERG monoclonal antibodies were used to probe HEK293-HERG and plain HEK293 cell lysates. The 25A2 and 10H12 antibodies were diluted 1:1,000. The 1H4 and 23A7 antibodies were diluted 1:200. Secondary antibody, IRDye800 donkey-anti-mouse (Rockland), was used at a 1:10,000 dilution.

Figure 3. Immunofluorescence images using HERG monoclonal antibodies

The newly produced HERG monoclonal antibodies were used as laser confocal scanning microscopy probes in HEK293-HERG and plain HEK293 cells. The images show the 25A2 (A), 23A7 (B), 10H12 (C), and 1H4 (D) clones.

Figure 4. Immunoprecipitation using the 25A2 clone

HEK-HERG cell lysate was divided into two equal aliquots, and immunoprecipitated with either 20 μ l non-specific mouse IgG ("pre-immune") or with 20 μ l of the 25A2 antibody; each reaction was precipitated with 20 μ l of Protein G beads. Immunoblots were detected using a commercial anti-HERG antibody (H175). Pre- and post-immunoprecipitation lysate was also analyzed ("Input" and "Post IP" respectively).

Figure 5. Immunoprecipitation using pooled monoclonal antibodies allows for large-scale preparations of the HERG protein

HEK293 and HEK293-HERG-cell lysates (six confluent 15cm plates lysed in a total of 9ml NDET for each cell type) were immunoprecipitated using pooled monoclonal antibodies covalently cross-linked to base beads (80μl beads were used per lysate). Silver stain of large 6-15% acrylamide gel reveals intense band doublet corresponding to HERG MW (filled arrows). Two co-immunoprecipitating bands identified as Hsp70-2 and 14-3-3ε/θ (open arrows).

Table 1**Hybridoma summary**

Mouse	Fusion partner	Wells plated	Screened by ELISA	Positive by ELISA	Screened by WB	Positive by WB	Maintained secretion
C1	Ag8	1920	224	20	88	4	0
C2	Ag8	480	125	0	34	0	0
C2	NSO	480	69	0	5	0	0
C2	NSO-Bcl2	3072	1246	7	222	39	4
B1	NSO-Bcl2	1920	864	42	91	0	0
A1	NSO-Bcl2	1920	576	21	140	0	0