Genomic amplification and oncogenic properties of the *KCNK9* potassium channel gene

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Summary

Representational difference analysis (RDA) of human breast cancer was used to discover a novel amplicon located at chromosomal region 8q24.3. We examined a series of breast cancer samples harboring amplification of this region and determined that *KCNK9* is the sole overexpressed gene within the amplification epicenter. *KCNK9* encodes a potassium channel that is amplified from 3-fold to 10-fold in 10% of breast tumors and overexpressed from 5-fold to over 100-fold in 44% of breast tumors. Overexpression of *KCNK9* in cell lines promotes tumor formation and confers resistance to both hypoxia and serum deprivation, suggesting that its amplification and overexpression plays a physiologically important role in human breast cancer.

Introduction

Amplification of oncogenes such as *HER2* and *CCND1* is one of the key genetic mechanisms underlying the development of human breast cancer. The size of the genomic region surrounding oncogenes that is amplified in tumors can vary considerably, from 700 kb to 4.5 Mb for *CCND1* and from 350 kb to over 1 Mb for *NMYC* (Tanigami et al., 1992; Hiemstra et al., 1994). Genes that are coamplified along with the "driver" gene underlying selective amplification are often overexpressed (Wimmer et al., 1999; Pollack et al., 2002). Consequently, overexpression of an amplified gene in and of itself is not convincing evidence for a role in tumor formation. More compelling genetic evidence for a gene's candidacy as a "driver" gene comes from high-resolution mapping of amplicon boundaries, which to date remains labor intensive and has been carried out in only a few cases of reported candidate breast cancer-amplified oncogenes, including *ZNF217*, *CYP24*, and *PPM1D* (Collins et al., 1998; Albertson et al., 2000; Li et al., 2002).

In this report, RDA (Lisitsyn et al., 1995) was used to isolate four DNA probes that all originated from a 550 kb region of a novel breast cancer amplicon. High-resolution mapping led to the identification of a single overexpressed gene, *KCNK9*, that was shown in functional studies to convey properties highly consistent with a role in tumor formation.

Results

We applied RDA to a series of breast tumor samples and tested the resultant cloned sequences for their ability to detect genomic amplification by DNA hybridization. From one particular cancer sample, we isolated six RDA-derived sequences (RDA probes) that detected amplification, two of which were mapped by analysis of radiation-hybrid panels to the *HER2* locus, and the other four of which were mapped to chromosomal region 8q24.3, approximately 10 Mb telomeric to the *MYC* oncogene. An initial survey of breast tumors by DNA hybridization showed that the 8q24.3 RDA probes detected amplification (4-fold to 10-fold) in 4 out of 70 primary breast tumors. Two of these four tumors harboring amplification also showed 3-fold amplification

SIGNIFICANCE

Many of the genetic alterations that drive the development and progression of sporadic breast cancer remain poorly characterized. Whole-genome approaches that can pinpoint relatively small genomic aberrations at high resolution allow identification of potentially causative cancer genes. Here, RDA was used to find a 550 kb amplicon containing only one overexpressed gene, *KCNK9*. Functional analysis of *KCNK9* has established its ability to act as a dominant oncogene, making KCNK9 a potential target in treatment of tumors presenting amplification and overexpression of *KCNK9*. Additionally, our finding that *KCNK9* overexpression confers resistance to hypoxia, together with its reported role in mediating other oxygen-sensitive physiological responses, opens up a potential new line of investigation into the development of hypoxia tolerance in tumor cells.

of *MYC*, whereas the other two tumors were not amplified for *MYC*, indicating that amplification of this region can occur independently. We performed a more extensive survey of primary invasive breast cancers with quantitative PCR and determined that 24 out of 247 breast tumors (approximately 10%) were amplified for this locus (3-fold to 10-fold, data not shown).

As a first step to find the gene underlying the selective amplification of this region, the four 8q24.3 RDA probes and nearby sequence-tagged sites (STSs) were used to isolate human genomic DNA clones. These clones were sequenced and the resultant genomic sequence was used to construct a map of the amplified locus. To identify the amplification epicenter, the genomic amplification status of STSs distributed across this locus was determined in a set of five amplified tumors and cancer cell lines. Two samples displayed equal copy number increase (3-fold to 5-fold) throughout the 1.3 Mb region analyzed, suggesting that other genes outside of this region may provide selective pressure for increased genomic copy number in these tumors (data not shown). However, we identified a clearly delineated 550 kb amplicon in the primary tumor from which the RDA-derived sequences were obtained and an overlapping 700 kb amplicon in the breast cancer cell line ZR-75-30 (Figure 1). This 550 kb amplified region was also present in the broader amplicon of an additional primary tumor (Figure 1). These results suggest that the common 550 kb amplified region harbors a gene that when amplified provided a selective advantage to the tumor cells.

Two genes were identified within the 550 kb amplicon. One of the genes, termed T1, was identified by exon trapping and subsequent cDNA cloning and is identical to the subsequently published gene encoding the putative protein KIAA1882 (Nagase et al., 2001). T1 is only partially contained within the amplicon since the 5′ region of its coding sequence was found to lie outside of the region (Figure 1). We failed to detect by Northern hybridization a smaller mRNA than the normal sized 4 kb message in the highly amplified breast cancer cell line ZR-75-30, ruling out the possibility that a truncated version of the gene was activated in the tumors (data not shown). Furthermore, T1 was not overexpressed in a number of other tumor samples (data not shown), strongly suggesting that it was not the gene providing the selective pressure for genomic amplification.

The other gene was identified with the gene prediction program GeneMachine (Makalowska et al., 2001) based on sequence homology to the two-pore potassium channel gene *KCNK3*. A full-length cDNA for this gene, which we termed *KCNB,* was cloned and we determined that its entire sequence is located within the 550 kb amplicon (Figure 1). Subsequently, other groups studying two-pore potassium channels cloned this same gene they termed *TASK-3* (Kim et al., 2000; Rajan et al., 2000; Chapman et al., 2000). Hereafter we adopt the officially sanctioned gene symbol KCNK9 (http://www.gene.ucl.ac.uk/). *KCNK9* was overexpressed at least 5-fold and up to over 100-fold in 28 out of 64 breast cancers (44%), including all breast tumors that were amplified for this region as well as tumors in which the genomic DNA was not highly amplified (Table 1). In addition to breast cancers, *KCNK9* was overexpressed in 35% of lung cancers (Table 1).

To test whether there was a correlation of amplification and/or low-level DNA copy number increase with increased gene expression, we classified tumors as harboring DNA copy number increase if the relative value was greater than 1.5-fold, and as exhibiting overexpression if the relative value was greater than 5-fold. Using Fisher's exact test, there was a highly significant correlation between DNA copy number and RNA overexpression ($p = 0.0001$). Thus, like the *HER2* oncogene (Pauletti et al., 1996), the majority of cases in which *KCNK9* is overexpressed in breast and lung cancers correlates with amplification or more modest gain of genomic DNA.

Antipeptide antibodies directed against the *KCNK9* gene product were employed to determine the frequency of overexpression in breast cancers by immunohistochemical analysis of normal and tumor tissue microarrays (Kononen et al., 1998). Twenty-nine out of 71 breast tumor samples surveyed showed KCNK9 protein overexpression, whereas none of the 14 normal breast samples showed overexpression (Figure 2). These numbers are in close agreement to the frequency of overexpression determined by quantitative reverse transcriptase (RT)-directed

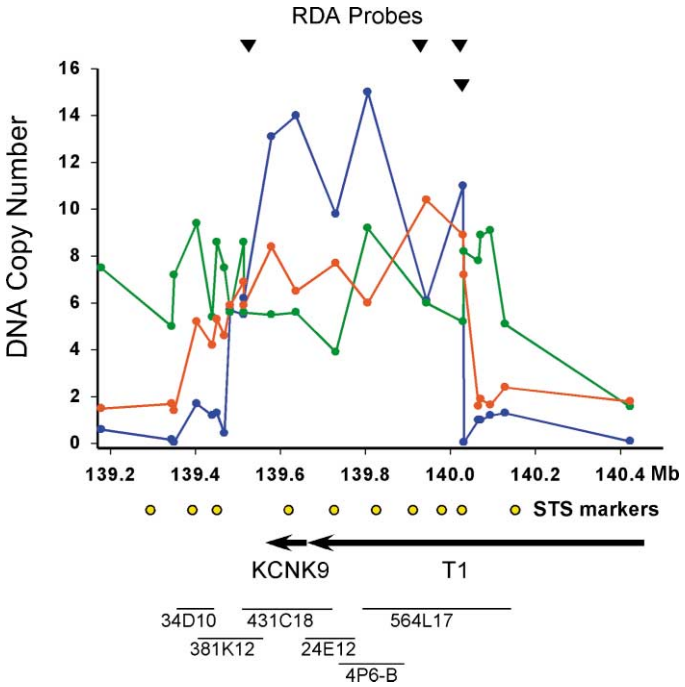

Figure 1. A 550 kb amplicon harboring *KCNK9* at human chromosomal region 8q24.3

DNA copy number for two primary tumors (CHTN9 in blue and CHTN159 in green) and one cell line (ZR-75-30 in red) were determined by Q-PCR and plotted against their chromosomal 8 nucleotide position in megabases (http://genome.ucsc.edu/, June 2002 freeze). Original RDA probes are indicated by the four arrows on top. Ten STS markers are designated per positional information of the June 2002 freeze data set of UCSC Genome Browser (filled circles from left to right): SHGC-143082, SHGC-149465, SHGC-106957, SHGC-85030, D8S452, D8S345, SHGC-102646, SHGC-102946, SGHC-142205, and RH36402. The position of BAC clones are indicated at the bottom. Although the data shown is from single DNA TaqMan assays (each with triplicate measurements with coefficient of variation [CV] of less than 3%), each assay was performed 2 to 3 times with an average CV of 9% and range from less than 1% to 17%. However, the variability seen in DNA copy number from one probe to the next probe along the genome is sometimes greater than 17% and does not always appear to reflect true changes in DNA copy number. Based on unpublished results, this variability is due in part to susceptibility of individual TaqMan probe efficiencies to currently unknown differences in the composition of the tumor sample DNA. Based on this variability, several adjacent TaqMan probes were designed to delineate amplicon boundaries with greater confidence.

Table 1. Amplification and expression status of *KCNK9* in human breast and lung tumors

Tumor type	Sample ID	Relative DNA copy number	Relative RNA expression level
Breast	CHTN159	6.0	14
Breast	90-197	5.0	66
Breast	87-634	3.9	69
Breast	88-468	2.0	27
Breast	90-794	3.0	>100
Breast	94-797	1.9	16
Breast	96-342	1.5	6
Breast	94-847	1.0	3
Breast	95-523	1.0	1
Breast	96-16	1.0	0.7
Lung[a]	LU-1	2.4	>100
Lung[b]	LU-55	2.1	>100
Lung[c]	LU-29	2.0	45
Lung[d]	LU-21	1.8	35
Lung[b]	LU-51	1.5	12
Lung[d]	LU-17	1.2	2
Lung[e]	LU-9	1.2	1
Lung[f]	LU-7	1.0	1
Lung[d]	LU-18	1.0	6
Lung[e]	LU-22	0.9	0.4

Relative DNA copy number and relative RNA expression levels were determined by real-time quantitative PCR assays, and the data shown are the mean values of duplicate or triplicate assays. Relative values above 100 are shown as >100. The coefficient of variation for relative DNA copy number measurements was ≤15%, and that of relative RNA expression levels was ≤27%.

Lung tumor samples are subdivided as indicated: [a]not classified, [b]small-cell lung cancer, [c]carcinoid, [d]squamous cell carcinoma, [e]adenocarcinoma, and [f]bronchioepithelial,.

PCR measurements (44%). In contrast, the human protein that has the closest sequence match to KCNK9, KCNK3, was not preferentially expressed in tumor tissue as judged by immunohistochemistry, nor was the *KCNK3* gene amplified or overexpressed in breast or lung cancers, based on quantitative PCR analysis (data not shown).

We utilized immunohistochemistry to ascertain whether there was a correlation of overexpression of *KCNK9* with either *HER2* overexpression or expression of estrogen receptors. In a set of 50 tumors, we did not detect a statistically significant correlation of *KCNK9* overexpression with either estrogen receptor expression or *HER2* overexpression ($p = 0.1781$ and 0.3978, respectively). Thus, there does not appear to be a specific requirement for *KCNK9* overexpression to collaborate with these pathways.

As the next step to ascertain whether *KCNK9* is a target oncogene whose amplification and overexpression could provide a selective advantage to tumor cells, *KCNK9* was deliberately overexpressed in different cell lines, and phenotypes associated with tumorigenesis were assessed. *KCNK9* overexpression did not transform NIH-3T3 fibroblasts or NMuMG, a cell line derived from mammary epithelial tissue (Hall et al., 1982), as judged by growth in soft agar, growth to a higher saturation density, and growth in low serum. Nor did *KCNK9* overexpression in NIH-3T3 cells induce tumor formation following implanting the cells into athymic mice. However, three out of five mice injected with *KCNK9*-overexpressing NMuMG cells formed tumors within 3 months, whereas none of the five mice that had been injected with control NMuMG cells formed tumors (Figure 3A).

KCNK9 overexpression also strongly enhanced tumorigenicity of C8 cells, which were derived from primary mouse embryo fibroblasts that had been transformed with *RAS* and *E1A* oncogenes (Figure 3B). C8 cells contain a wild-type *TP53* tumor suppressor gene and as such are prone to undergo apoptosis, particularly in response to serum starvation or hypoxia (Lanni et al., 1997; Graeber et al., 1996). Furthermore, in this model, tumors form only after a lag period that allows for the outgrowth of variants that have inactivated the p53 pathway. Overexpression of *KCNK9* shortened the time of tumor formation of these *TP53*-containing cells by several weeks (Figure 3B). In cell culture, *KCNK9* overexpression in C8 cells conferred increased cell viability in low-serum conditions (Figure 4A), although the protective effects were not as strong as that provided by *TP53* disruption, which protects cell almost completely (90%–100%) from loss of viability induced by low-serum concentrations of 0.1% to 0.5% (data not shown). A more dramatic effect of *KCNK9* overexpression was observed when C8 cells were subjected to hypoxia (Figure 4B). *KCNK9* overexpression allowed C8 cells to survive hypoxic conditions as well as *TP53* disruption.

Discussion

The functional studies presented here, taken together with the location of *KCNK9* within an amplification epicenter, strongly suggest that amplification and overexpression of *KCNK9* provides a selective advantage to breast cancer cells. Although it has been known for years that potassium or other ion channels can be activated by mitogens or oncogenes (Thomas and Brugge, 1997), our results are the first indication that genetic alteration of an ion channel gene can play a direct role in cancer.

There is a high incidence of copy number gains that affect the 8q chromosome arm in multiple cancers, including breast carcinomas where 8q gains occur in 40% or more of all tumors (Struski et al., 2002). The recurrent selection for this large region of the genome implies that multiple genes in addition to *MYC* are being selected as "driver" genes to promote tumorigenesis. The data presented here identifies *KCNK9* as a new protooncogene that may provide selective pressure for copy number increases at chromosome 8q, and in particular 8q24.3.

Our studies indicate that one potential mechanism by which *KCNK9* overexpression may contribute to tumorigenesis is by promoting cancer cell survival in the poorly oxygenated areas of solid tumors. In view of a recent report implicating KCNK9 as a key mediator of physiological effects of hypoxia in a human lung cancer cell line (Hartness et al., 2001), it is tempting to speculate that the protection from hypoxia we observed is related to an ability of the KCNK9 channel to respond to oxygen levels. The ability to suppress tumorigenicity by interfering with the ability to respond to lowered oxygen levels has previously been demonstrated (Kung et al., 2000). It will be important to determine whether inhibiting KCNK9 function will disrupt responses to hypoxia in tumor cells, and whether this will affect tumorigenicity.

Experimental procedures

Tumor samples and cell lines

Breast and lung human tumor samples were obtained from the Cooperative Human Tissue Network and Duke University. NMuMG cells were from ATCC. NIH-3T3, ZR-74-30, C8, and A9 cells were obtained from Cold Spring Harbor Laboratory. C8 and A9 are isogenic transformed cells that express both

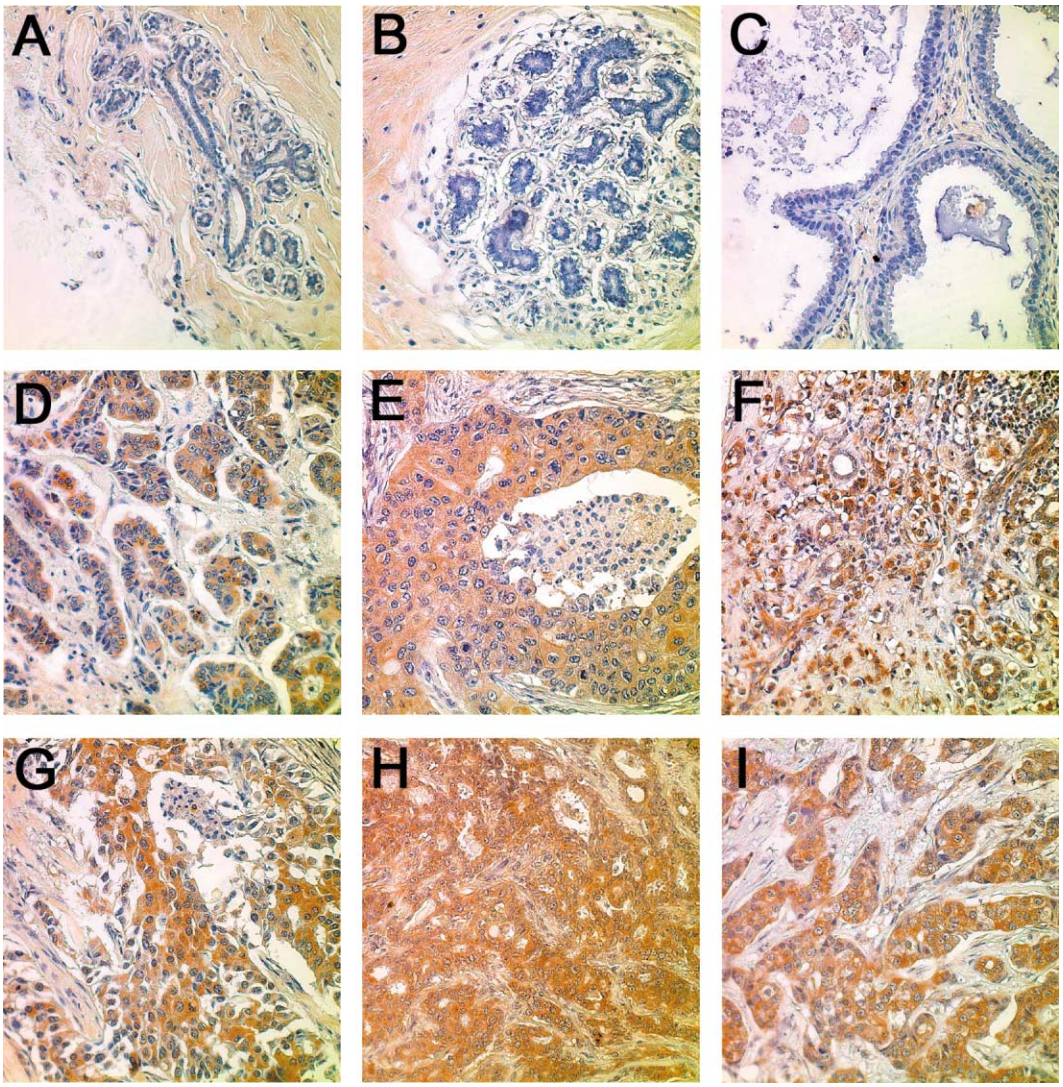

Figure 2. Confirmation that *KCNK9* is expressed preferentially in cancerous breast tissue

Representative elements of a tissue microarray stained with anti-KCNK9 antibody. Immunohistochemical stains demonstrate absent or weak staining of normal breast (**A–C**) and strong staining in breast tumor samples (**D–I**). Shown in **D–I** are infiltrating ductal carcinoma, with the exception of **F**, which is invasive lobular carcinoma. Magnification 200×.

activated *HRAS* and *E1A* oncogenes; C8 cells contain wild-type *TP53*, while A9 cells contain a homozygous deletion of *TP53* (Lanni et al., 1997). Retroviral transfection was conducted as described (Serrano et al., 1997). Genomic DNA was prepared using the proteinase K method, and total RNA was extracted using the Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Genomic methods

RDA and DNA hybridization using genomic representations was performed as described (Lisitsyn et al., 1995; Lucito et al., 1998). Radiation hybrid mapping of RDA probes was performed with the GeneBridge4 panel from Research Genetics (Invitrogen). The CITB human BAC library, release 4, was purchased from Research Genetics (Invitrogen) and screened as recommended by the supplier with PCR primers to the RDA sequences. Both termini of positive BAC clones were sequenced and PCR primers were designed to allow identification of overlapping BAC clones from the same library. Two of the six BACs (431C18 and 564L17 of accession AC007869 and AC007871, respectively) of the contig shown in Figure 1 were completely sequenced (Lander et al., 2001), and their sequence is part of the current human genome sequence (http://www.ncbi.nlm.nih.gov/genome/seq/). The other four BACs (34D10, 381K12, 24E21, and 4P6-B) were sequenced at low-pass coverage (3× to 6×) without assembly, and their alignment in the contig shown in Figure 1 was performed by BLASTN analysis to the draft genome assembly of twenty 400 to 700 bp reads picked from each BAC at random in addition to the BAC end sequences. The DNA sequences of these reads is available from the authors. One of these BACs, which we designated 4P6-B, is a subclone of the original stab received from Research Genetics and differs in its sequence from the GenBank sequence for 4P6 (AC007870). Exon trapping was performed with a kit from GIBCO-BRL (Invitrogen).

Quantitative PCR

Fluorogenic Taqman Q-PCR probes were designed using the PrimerExpress software (Applied Biosystems, Foster City, CA) and synthesized by Operon Technologies. DNA copy number was quantified using the ABI 7700 or ABI 7900 Sequence Detection Systems (Applied Biosystems, Foster City, CA). mRNA levels of *KCNK9*, *KCNK3*, and *T1* in tumors and in corresponding normal tissues (Clontech, Palo Alto, CA) were determined by performing quantitative PCR with fluorogenic TaqMan probes directly following reverse

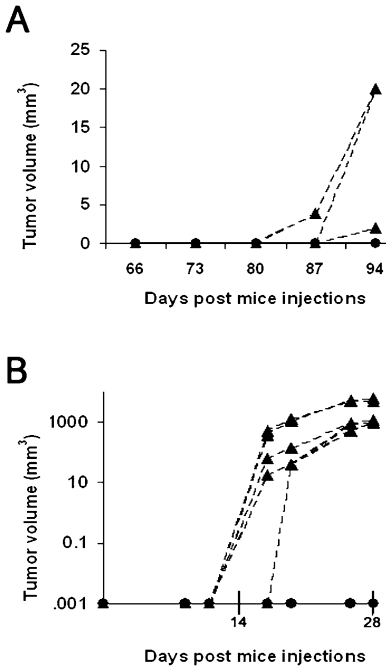

Figure 3. *KCNK9* overexpression promotes tumorigenicity

Normal murine mammary gland epithelial cells (NMuMG) (**A**) and C8 mouse embryonic fibroblast cells (**B**) retrovirally transfected with a *KCNK9* expression vector (triangles) or empty expression vector (circles) were injected into athymic nude mice subcutaneously for observation of tumor formation.

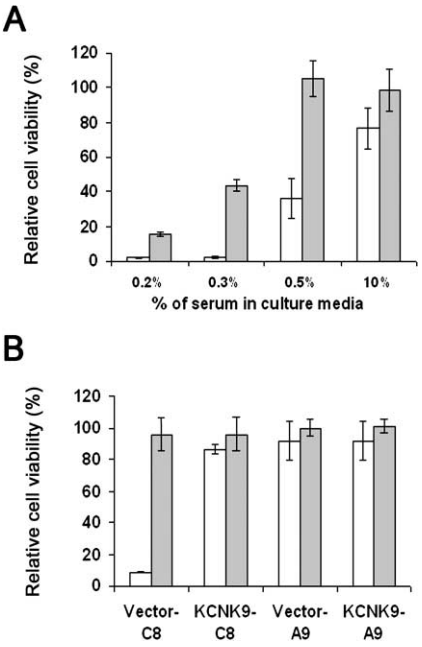

Figure 4. Functional analysis of *KCNK9*

A: *KCNK9* overexpression promotes survival in low-serum conditions. Cell viability was measured after 2 days in medium containing different serum concentrations. The gray-shaded bars represent viability measurements of C8 cells retrovirally transfected with a *KCNK9* expression vector, and the white bars are from cells transfected with empty vector.

B: *KCNK9* overexpression protects $p53^+$ cells against hypoxia. Cell viability was measured after 3 days in medium containing different oxygen concentrations. The gray-shaded bars represent viability measurements of cells maintained with normal oxygen levels, and the white bars are from cells maintained with low oxygen levels as described in Experimental Procedures. C8 ($p53^+$) and A9 ($p53^-$) are isogenic and are only different in their *p53* status.

transcriptase reactions. Absolute mRNA levels for *KCNK9* were within 50% for three different samples of normal breast tissue and three different samples of normal lung tissues. β-actin was used as a reference probe.

cDNA cloning

KCNK9 was PCR amplified with *Pfu* Turbo polymerase (Stratagene) from ZR-75-30 cDNA using primers designed from the coding sequence predicted by GeneMachine analysis of the DNA sequence of BAC 431C18: 5′-TCATG GATCCACCATGAAGAGGCAGAACGTGCGGACTCTG and 5′-TTCCGAAT TCTAAACGGACTTCCGGCGTTTCATCAGC. Subsequently it was cloned into BamHI/EcoRI sites of a retroviral vector, pLPC, and sequenced. Our *KCNK9* cDNA sequence contains a silent single base transition (T→C) at the codon of amino acid Gly212 compared to our predicted *KCNK9* cDNA sequence. For cloning T1 cDNA, PCR primers to an exon trapping product of BAC 431C18 (5′-CGAGGTAGAAGGTGCTGGAG and 5′-ACGGACAGC CATGTGACC) were used to screen arrayed human cDNA clones purchased from Origene (Rockville, MD) following the manufacturer's protocol. The resulting cDNA clone was sequenced by constructing a series of nested-deletion mutants. The GenBank accession number for the *KCNK9* cDNA clone is AY190605 and for the T1 cDNA clone is AY190606.

Tumorigenicity, low-serum, and hypoxia assays

All functional assays were repeated at least twice with independently derived retroviral transfectants. For tumorigenicity assays, 2×10^5 of C8 cells and 5×10^6 of NMuMG cells were injected s.c. into athymic mice (Crl:CD-1-nuBR from Charles River, Wilmington, MA), with five mice for each transfectant per individual experiment. Mice were observed weekly for tumor formation at the injection sites and tumor sizes were determined. For survival under low-serum conditions, 5×10^5 cells were seeded in triplicate in 12-well culture plates and allowed to attach overnight in DMEM/F-12 with 10% fetal bovine serum. The next day, cells were washed with phosphate-buffered saline and replenished with DMEM/F-12 containing various amounts of fetal bovine serum. After 2 days, cell viability was scored by measuring absorbance at 490 nm using Celltiter 96 Aqueous One Solution (Promega, Madison, WI). To calculate relative cell viability, all 490 nm absorbance readings were normalized to the highest of the three readings of *KCNK9*-C8 cells in 10% serum. To subject cells to hypoxia, Modular Incubator Chambers were purchased from Billups-Rothenberg (Del Mar, CA). 12-well culture plates bearing 100,000 cells/well in triplicate were placed in the chambers and flushed with low-oxygen air (0.02% oxygen, 5% carbon dioxide, 94.98% nitrogen) at 20–25 l/min for 5 min. The chambers were flushed with low-oxygen air twice daily and incubated at 37°C. After 3 days, viable cells were quantified using Celltiter 96 Aqueous One Solution. Relative cell viability is expressed by normalizing all readings to highest of the three readings of *KCNK9*-A9 cells exposed to normal air. Low-oxygen air tank was purchased from General Welding (Westbury, NY).

Other methods

Tissue microarray slides bearing normal breast and breast tumor sections were purchased from Imgenex (http://www.imgenex.com). Anti-*TASK1* antibodies were purchased from Chemicon (http://www.chemicon.com). Polyclonal rabbit antibodies raised against KCNK9 peptide (REEEKLKAEEIRIKGKY NISSEDYRQ) and secondary anti-rabbit antibodies purchased from Biogenex (http://www.biogenex.com) were used to probe tissue slides following the manufacturer's protocol.

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