Isoforms of Ionotropic Glutamate Receptor GRIK2 Induce Senescence of Carcinoma Cells

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Abstract. *Background: The aberrant regulation of growth and proliferation is a key feature of carcinoma cells. In order to use molecular strategies to correct these defects toward therapeutic purposes, it is important to characterize the entire spectrum of causative molecules. Materials and Methods: By using gene transfer technique, SKOV3 ovarian carcinoma cells were transduced with an expression construct of glutamate receptor 6 (glutamate ionotropic receptor kainate type subunit 2, GRIK2) in retroviral vector PQCXIP. The senescence of transduced cells was subsequently characterized. Results: Our results demonstrated that retroviral transduction occurs with high frequency and transduced cells continue to proliferate, albeit at a significantly reduced rate, up to 39 days. Some transduced colonies stopped proliferating after 12 days, and none of the clones proliferated beyond 37 days. The doubling time for these transduced cells increased progressively until they reached a complete cell-cycle arrest. The proliferating cells were distinguished by bromodeoxyuridine incorporation and 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay. The growth and cell cycle arrest in transduced cells accompanied activation of senescence-associated β-galactosidase. Furthermore, we have demonstrated a decrease in the levels of active protein kinase B and increase in the abundance of inactive cyclin-dependent kinase 1. Conclusion: These results indicate involvement of GRIK2 in senescence and suggests GRIK2 as a potential target for therapeutic intervention of cancer cells.*

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Chromosomal transfer has been successfully used to characterize growth-suppressive genes encoded in specific human chromosomes (1). It has been previously shown that transfer of normal human chromosome 6 induces senescence in ovarian cancer cells (2). The senescence phenotype is characterized by the activation of senescence-associated β-galactosidase (3). The transfer of DNA segments corresponding to various regions of chromosome 6 led to the mapping of the senescence causing activity in a 900 kb region on chromosome 6q16.3, and ribonuclease T2 (*RNASET2*) and glutamate ionotropic receptor kainite type subunit 2 (*GRIK2*) were considered, as potential candidate genes involved (4). Although *RNASET2* was suggested to have senescence-inducing activity (5, 6), our results demonstrated that it was primarily responsible for the suppression of growth and proliferation (7). The exclusion of *RNASET2* from consideration has led to the emergence of *GRIK2* as a strong candidate gene involved in senescence.

GRIK2, previously designated as GLUR6, belongs to the ionotropic class of glutamate receptors (8). This receptor is selectively activated by binding to kainate agonist. GRIK2 controls the flux of sodium and calcium ions across the membrane in neuronal cells (9). The different isoforms of *GRIK2* are transcribed in neuronal and non-neuronal cells by two tissue-specific promoters (10). GRIK2A is exclusively expressed in the brain, and isoforms B, C, D and E are known to be expressed in fibroblasts. We previously showed that the GRIK2A isoform is not expressed in non-neuronal cells. The aims of this study were to achieve greater efficiency of gene transfer by using a retroviral construct, and to investigate growth characteristics of ovarian carcinoma cells transduced with non-neuronal GRIK2 isoforms.

Materials and Methods

Cloning of enhanced green fluorescence protein (EGFP)-tagged GRIK2 isoforms into a retroviral vector. The coding sequence (methionine start codon ATG to lysine AAG codon 239) of EGFP was cloned in frame after threonine codon 33 of *GRIK2* cDNA

reverse transcribed from total RNA from either human brain or fibroblast cells. The first 31 amino acids of GRIK2 correspond to its signal peptide. A synthetic DNA sequence containing *Age*I and *Bsr*GI restriction sites was added after nucleotide 99. EGFP was excised from pBOS-H2GFP (Pharmingen, San Diego, CA, USA) construct by digesting with *Age*I and *Bsr*GI restriction enzymes and cloned into the *GRIK2* construct described above. The alternative *GRIK2* transcripts representing isoforms A, B, C, D and E were similarly tagged with EGFP and cloned in a retroviral vector PQCXIP (BD Biosciences, San Diego, CA, USA). EGFP was also cloned without *GRIK2* in the same vector.

Transduction of EGFP-tagged GRIK2 isoforms into SKOV3 ovarian carcinoma cells (American Type Culture Collection, Manassas, VA, USA). The purified plasmid constructs corresponding to each *GRIK2* isoform were transfected into phoenix-amphotrophic producer HEK293T cell line by using Lipofectamine (Thermo Fisher, Waltham, MA, USA) as per the vendor's protocol. After 48 hours of transfection, the cells were grown for 4-5 days in a selection medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10 mg/ml streptomycin and 10 U penicillin) containing puromycin (3 μg/ml). SKOV3 cells were seeded at a density of 10^5 cells per plate 1 day before transduction, and similarly puromycin-selected HEK293T producer phoenix cells were grown at a density of 10^6/100 mm plate one day before transduction. After 24 hours, the viral particles were harvested by passing the viral supernatant through a 0.45 μm filter. The viral particles were added to plates containing SKOV3 cells with a multiplicity of infection of 5.0-10.0. The selection medium containing puromycin (3 μg/ml) was added to plates after 48 h of transduction, and the transduced cells were grown for 2-3 weeks at 37˚C.

Ectopic expression of GRIK2 isoforms. The expression of exogenous GRIK2 isoforms in *GRIK2*-transduced SKOV3 clones was visualized and pictured after 72 hours of transduction by a digital camera attached to a fluorescent microscope driven by SPOT software (SPOT Imaging, Sterling Heights, MI, USA). The relative levels of expression were compared between EGFP-PQCXIP vector control and clones of different *GRIK2* isoforms. The abundance of *GRIK2* isoforms was confirmed by western blot using anti-GFP (Santa Cruz Biotechnology, Dallas, TX, USA) and anti-GAPDH antibodies (Ambion, Thermo Fisher).

Transduction efficiency of SKOV3 cells. The transduction efficiency was determined by plating 50,000 cells in a 60 mm petri dish. The cells were subsequently transduced with either a specific EGFP-tagged isoform of *GRIK2* or the vector containing EGFP. The transduced cells were selected in puromycin for 2 to 3 weeks, and then stained with 1% crystal violet in methanol. The stained colonies were counted, and the numbers of colonies produced by vector and gene transfected cells were compared.

Growth and proliferation of cells expressing exogenous GRIK2. The clones were isolated and grown for 4 hours and then processed for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 1,000 cells from each clone at zero, 1, 3, 5 and 7 days were plated in quadruplicates in a 96-well plate, and 10 μl of MTT reagent was added to each well. The plate was incubated for 2-4 h, which was followed by an additional incubation of 2-4 hours in the dark in the presence of 100 μl of 10% sodium docecyl sulfate. The

color in each well was quantified by measuring the absorbance at 580 nm in a microplate reader. For zero day stage, the plate was processed 6 hours after seeding, and the absorbance was assigned an arbitrary value of 100. The readings after 1, 3, 5 or 7 days were calculated relative to the MTT reading at day zero.

The clones of *GRIK2*-expressing cells were dissociated and subsequently grown for up to 39 days. The number of cells were counted periodically by using a hemocytometer to determine their proliferation rate.

Staining of cells by 5-bromo-deoxy-uridine (BrdU) incorporation and β-galactosidase assay. Approximately 200-300 transduced SKOV3 cells were grown in glass-bottom culture dishes for 12 hours, and then incubated in the presence of 10 μM BrdU (Sigma, St. Louis, MO, USA) for 24 hours. The cells were fixed in FixDenat reagent (Sigma) for 30 minutes, blocked for nonspecific binding sites of antibody and treated overnight with mouse anti-BrdU (Thermo Fisher). The presence of BrdU was detected by adding Texas Red-labeled anti-mouse-IgG (Jackson Immunolabs, West Grove, PA, USA), and the nuclei were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI). The slides were photographed with a digital camera attached to a fluorescence microscope. The numbers of BrdU-stained cells were determined in triplicate samples and compared to the numbers of DAPI-stained nuclei.

β-Galactosidase activity was measured to determine the number of cells undergoing senescence. A total of 20,000 SKOV3 cells were grown for 12 hours and then fixed in 2% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline. The fixed cells were incubated with 1 mg/ml X-Gal stain (Sigma) in 40 mM sodium citrate buffer (pH 6.0) containing 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 μM NaCl and 2 mM $MgCl_2$ for 14 hours at 37˚C in the dark. The cells were pictured under bright field using a phase-contrast microscope attached to digital camera, and the stained cells were recorded as a percentage of the total cells on the slide.

Western blot. The parental cells, *GRIK2* construct-transduced clones and vector-transduced control SKOV3 clones were lysed with radioimmunoprecipitation/phenylmethylsulfonyl fluoride buffer and the protein mixture was electrophoresed in polyacrylamide gel. The separated proteins were blotted onto polyvinylidene difluoride membrane. The membrane blot was probed with antibodies raised against important cell cycle proteins, namely cyclin B, cyclin D3, cyclin-dependent kinase 1(CDK1), CDK1-Tyr15-P, protein kinase B (AKT), AKT-Ser473-P and retinoblastoma (RB). The amount of protein used for each lane was confirmed by probing the blot with anti-GAPDH. The levels of specific proteins were assessed relative to GAPDH abundance. GAPDH antibodies were purchased from Ambion (Thermo Fisher), and the other antibodies were procured from Cell Signaling (Danvers, MA, USA).

Results

Expression of GRIK2-EGFP isoforms in SKOV3 cells. We addressed the role of non-neuronal isoforms of GRIK2 in ovarian carcinoma cell line SKOV3. We introduced expression constructs of *GRIK2* isoforms tagged with EGFP in SKOV3 cells using a viral vector. The appearance of EGFP fluorescence in transduced cells was considered as
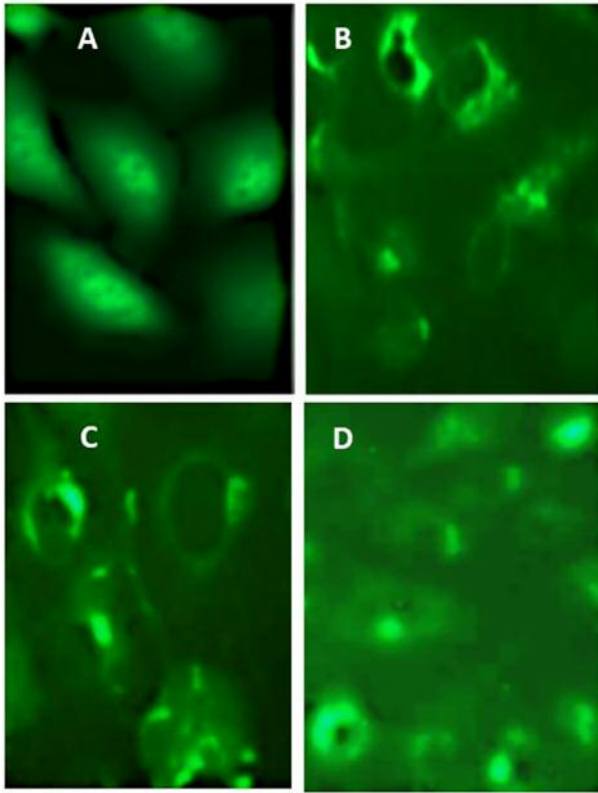
Figure 1. *Fluorescence microscopy images of glutamate ionotropic receptor kainite type subunit 2 (GRIK2)-transduced cells. SKOV3 cells transduced with constructs of enhanced green fluorescence protein (EGFP) (panel A), EGFP-GRIK2E (panel B), EGFP-GRIK2C (panel C), and EGFP-GRIK2B (panel D). The images were obtained with ×100 objective.*

Figure 2. *Viability of SKOV cells transduced with glutamate ionotropic receptor kainite type subunit 2 (GRIK2) isoforms. A: The proliferation of parental SKOV3 cells and GRIK2 isoform-transduced cells was monitored for 7 days using (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTT) assay. B: The expression of GRIK2 isoforms was confirmed by probing the western blot with anti-green fluorescence protein (GFP) antibody. The amount of protein loaded onto the gel is shown by probing the blot with glyceraldehyde 3-phosphate (GAPDH) antibody. P: Parental SKOV cell line; V: SKOV cells transduced with an empty vector; B-E: SKOV cells transduced with GRIK2 isoforms B-E. The numbers following the isoforms designate the specific clones used for the experiment.*

proof of the translation of these isoforms. The expression of *GRIK2* in transduced cells was evident in organelles, as well as the cell surface. These results are indicative of proper processing of *GRIK2*-EGFP in endoplasmic reticulum and Golgi before their transport to cell surface. The expression of EGFP alone, however, was observed throughout the cell including the nucleus. Figure 1 demonstrates the expression of EGFP fused to *GRIK2* isoforms.

Expression of GRIK2 isoforms in SKOV3 cells. SKOV3 cells were transduced with vector with EGFP alone and EGFP-tagged *GRIK2* isoforms, and the transduced cells were grown in the presence of a selectable marker. The surviving colonies were stained with crystal violet for visualization and counting. The numbers of surviving colonies in the selection medium were indicative of the efficiency of gene transfer. The vector transduction experiment indicated the presence of 160 surviving colonies on the plate. The transduction of A, B, C, D and E isoforms led to survival of 3, 5, 7, 18 and
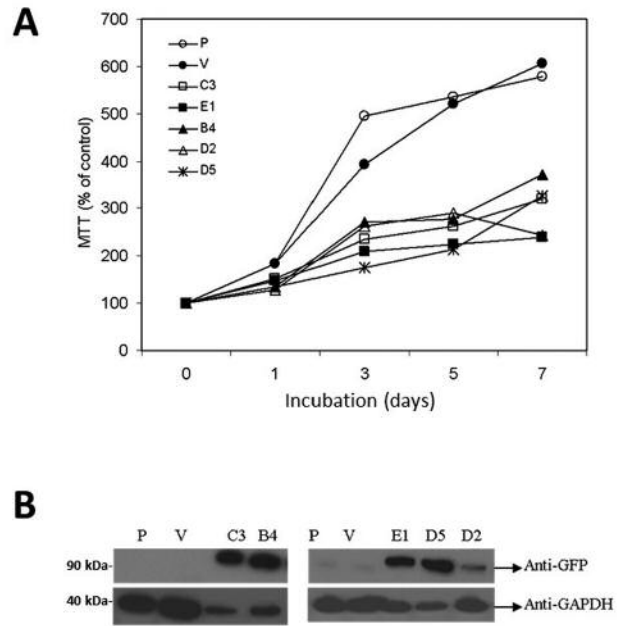
5 colonies, respectively. These results demonstrate the different efficiency of gene transfer for the different *GRIK2* isoforms. However, the transfer efficiency was significantly greater than previously observed with other non-retroviral transfection protocols (4).

The selected clones were analyzed for proliferation by counting the cells growing in culture at an interval of 2-3 days for up to 39 days. While *GRIK2A*-transduced clones did not multiply, the proliferation period for colonies containing isoforms B, C, D and E ranged between 21 and 37 days. These data indicate that the transduced clones have a finite potential for multiplication as compared to parental SKOV3 cells and cells transduced with vector.

Assessment of viability of transduced clones by MTT assay. MTT assay was performed on parental, vector-transduced control and SKOV3 cells transduced with different *GRIK2* isoforms. As shown in Figure 2A, the parental and vector-transduced SKOV3 cells grew with an expected doubling
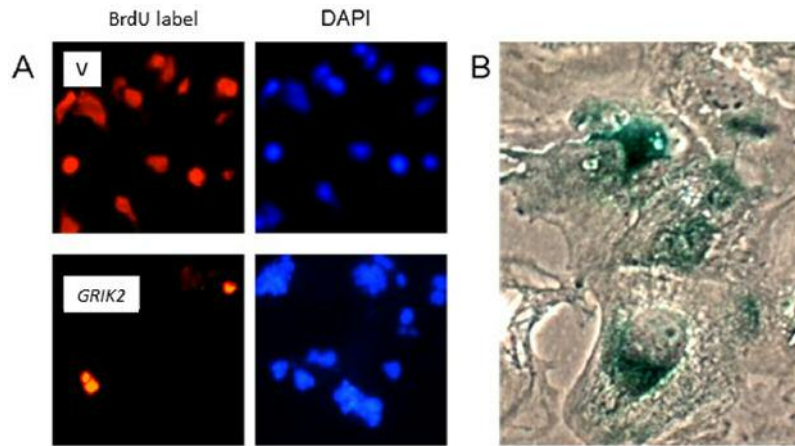
Figure 3. *Replication and replicative senescence in control and glutamate ionotropic receptor kainite type subunit 2 (GRIK2)-transduced SKOV3 cells. A: The cells were transduced with either an empty vector (V) or GRIK2B isoform and then incubated with 5-bromo-deoxy-uridine (BrdU) to quantify replicating cells. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). B: Senescence-associated β-galactosidase activity was assessed by incubating the GRIK2B-transduced cells with X-Gal reagent. The images were taken with ×100 objective.*

time during the course of the experiment that lasted for 7 days. The doubling time for SKOV3 cells transduced with *GRIK2* isoforms was considerably increased. The expression of *GRIK2* isoforms in these clones was confirmed by probing the total cell lysate with anti-GFP. The abundance of the expressed protein is quite clear from the western blot presented in Figure 2B.

GRIK2 isoforms induce replicative senescence in SKOV3 cells. We evaluated DNA replication in transduced cells by determining the extent of BrdU incorporation. As shown in Figure 3A for SKOV3 cells transduced by empty vector, all DAPI-stained DNA in nuclei had incorporated BrdU. However, in *GRIK2*-transduced clones of SKOV3 cells only a small fraction of DAPI-stained nuclei had incorporated BrdU. These results indicate that GRIK2-transduced cells had ceased to replicate.

The absence of DNA replication in transduced cells suggested the induction of replicative senescence. The induction of senescence in *GRIK2*-transduced SKOV3 cells was tested by determining the expression of β-galactosidase activity. The transduced cells were plated at a density of 2×10^4 cells in a petri dish for 14 h, and subsequently stained with X-Gal reagent. The blue stain was visualized by phase contrast microscopy (Figure 3B). The presence of β-galactosidase activity in cells that did not incorporate BrdU clearly demonstrates the induction of senescence in these cells.

Abundance of cell-cycle regulators in GRIK2-transduced SKOV3 cells. SKOV3 cells are known to lack p53 and other proteins responsible for G_1/S arrest. Therefore, transduction
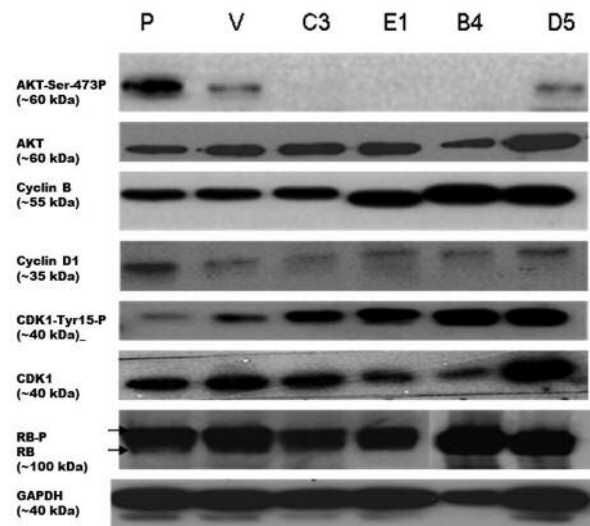

Figure 4. *The abundance of cell-cycle regulator proteins in native and GRIK2 -transduced SKOV3 cells. The cell lysate from parental SKOV3 cells (P) and cells transduced with either empty vector (V) or GRIK2 isoforms B, C, D and E were probed with antibodies against the indicated proteins. The amount of protein loaded on each lane was quantified by probing the gel with anti- glyceraldehyde 3-phosphate (GAPDH). The numbers following the isoforms designate the specific clones used for the experiment. CDK1: Cyclin-dependent kinase 1; RB: retinoblastoma; P: phosphorylated form.*

of *GRIK2* into SKOV3 cells can allow investigation of *GRIK2* involvement in cell-cycle/growth arrest. The transduced cells were lysed, proteins electrophoresed and immunoblotted with antibodies against cell cycle regulators

cyclin D1, cyclin D3, cyclin A, cyclin B, RB, CDK1, CDK1-Tyr15-P, AKT-Ser473-P and AKT. As shown in Figure 4, the level of active AKT (AKT-Ser473-P) decreased in *GRIK2*-transduced cells as compared to parental SKOV3 cells and vector-transduced cells. Furthermore, the level of inactive CDK1 (CDK1-Tyr15-P) was elevated in transduced cells. However, no significant difference was observed in the abundance of the other proteins listed above.

Discussion

Chromosomal transfer has been valuable in identifying chromosomes and regions of chromosomes that encode tumor-suppressor genes and senescence genes (11-13). Transfer of chromosome 6q14-21 or a yeast artificial chromosome clone of 1.0 Mb from the 6q16.3 region led to the senescence of ovarian carcinoma cells (2, 4). It is noteworthy that chromosome 6q27 encoded *RNASET2* has been designated a senescence-inducing gene (5). However, we previously showed that *RNASET2* did not induce senescence in SV40 immortalized cells (7). In light of these observations, chromosome 6q16.3-encoded *GRIK2* appeared to be an attractive candidate gene for consideration of senescence activity. As described below *GRIK2*, indeed, has the ability to induce senescence in ovarian carcinoma cell line SKOV3.

To investigate senescence activity of putative candidates, it is important that gene transfer be highly efficient. If the gene transfer efficiency is low, then molecular investigations of small numbers of transfected cells become difficult. We circumvented these technical difficulties by using PQCXIP, a retroviral vector. Our results clearly demonstrate the efficient transfer of *GRIK2* isoforms cloned in PQCXIP vector. The standardization of such efficient transfer of *GRIK2* constructs has set the stage for future molecular profiling of transduced cells.

The temporal and spatial expression of GRIK2 remains unaltered upon its fusion with EGFP, as shown by its trafficking to the cell surface. The retention of the signal sequence assists the fusion protein translation in the endoplasmic reticulum and subsequent trafficking *via* Golgi and endosomes to the cell surface. However, introduction of EGFP vector alone appeared to localize nonspecifically in the transduced cells. Such uniform expression of EGFP is not surprising. These results demonstrate accurate localization of the fusion protein, and confirm the utility of EGFP–*GRIK2* constructs for evaluating phenotypic characteristics of the transduced cells.

The presence of senescence-associated β-galactosidase activity and enlarged shape are demonstrative of an acquired senescence phenotype in *GRIK2*-transduced cells. The lack of BrdU incorporation in transduced cells confirms the cessation of DNA replication. It is noteworthy that senescent cells did not incorporate BrdU, and replicating cells did not show activation of β-galactosidase. The activation of β-galactosidase has already been established in a variety of senescent cells and appears to be a reliable marker for senescence (3, 18).

Our results demonstrate selective changes in the levels of cell-cycle regulatory proteins. In particular, altered levels of phosphorylated AKT protein and phosphorylated CDK1 suggest mechanisms operative in transduced cell. The phosphorylation of AKT is necessary for its activation and subsequent downstream signaling to promote growth and proliferation. The inactivation of CDK1 by phosphorylation of tyrosine residue 15 is indicative of cell-cycle arrest (19). The distinct phosphorylation of serine residue 473 of AKT and tyrosine residue 15 of CDK1 in transduced clones corresponds to a senescent phenotype. The senescent cells appear to suspend their growth and proliferation by inactivation of AKT signaling pathway. The withdrawal from the cell cycle, on the other hand, can be attributed to the presence of the inhibitory phosphate on CDK1 protein. The removal of the inhibitory phosphate is necessary for progression of cells through the G_1 phase.

Although the mechanisms of senescence induced by GRIK2 isoforms remain unanswered, our results allow us to speculate on the involvement of non-neuronal GRIK2 isoforms as important mediators of cell growth and proliferation signaling pathways. The alterations in the phosphorylation status of AKT and CDK1 support the involvement of GRIK2 isoforms in senescence. The confirmation of these observations by abrogating activation/inactivation of AKT and CDK1 would open new avenues for molecular and mechanistic insight into GRIK2-mediated signaling.

The involvement of AKT and CDK1 has been extensively characterized in normal and tumor cells. Specifically, alteration of AKT activity is known to be modulated by hyperactivation of phosphoinositide 3-kinases in tumors, and subsequent relay to the mechanistic target of rapamycin (mTOR) *via* AKT (14). Decrease in AKT phosphorylation and its association with senescence has been observed in skin-derived precursor cells. In fact, inhibitors of AKT significantly reduced the self-renewal potential of skin-derived precursors (15). AKT activation has also been implicated in maintaining pluripotency of embryonic stem cells (16). The switch between cell-cycle arrest and proliferation is regulated by the presence of growth factors (17). The inhibition of CDK1 by phosphorylation indicates that senescent cells are withdrawn from the cell cycle permanently. The mechanisms that transduce signals from GRIK2 are not evident from our results, but they do suggest non-channel roles of GRIK2 isoforms in non-excitable cells.

We demonstrated that retroviral constructs of *GRIK2* isoforms transduce SKOV3 cells with a high efficiency. The transduced cells show reduced proliferation and undergo

cessation of growth after culturing for 37 days in tissue culture medium. These phenotypic changes accompany the activation of senescence-associated β-galactosidase and alterations in the levels of phosphorylated AKT and CDK1 proteins. In conclusion, our results clearly indicate the emergence of senescent phenotype in *GRIK2*-transduced SKOV3 cells.

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