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G protein-coupled receptor kinase 4-induced cellular senescence and its senescence-associated gene expression profiling

Pingping Xiao[1,3,1], **Xishi Huang**[1,2,1], **Lanzhen Huang**[2], **Jing Yang**[2], **Ang Li**[1], **Ke Shen**[1], **Philip B. Wedegaertner**[4], and **Xiaoshan Jiang**[1,3]

[1]Cell Signaling Laboratory; Guilin Medical University, Guilin, Guangxi 541004, China

[2]Center for Science Research; Guilin Medical University, Guilin, Guangxi 541004, China

[3]Graduate College, Guilin Medical University, Guilin, Guangxi 541004, China

[4]Department of Biochemistry and Molecular Biology, Sidney Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19083 USA

Abstract

Senescent cells have lost their capacity for proliferation and manifest as irreversibly in cell cycle arrest. Many membrane receptors, including G protein-coupled receptors (GPCRs), initiate a variety of intracellular signaling cascades modulating cell division and potentially play roles in triggering cellular senescence response. GPCR kinases (GRKs) belong to a family of serine/threonine kinases. Although their role in homologous desensitization of activated GPCRs is well established, the involvement of the kinases in cell proliferation is still largely unknown. In this study, we isolated GRK4-GFP expressing HEK293 cells by fluorescence-activated cell sorting (FACS) and found that the ectopic expression of GRK4 halted cell proliferation. Cells expressing GRK4 (GRK4(+)) demonstrated cell cycle G1/G0 phase arrest, accompanied with significant increase of senescence-associated-β-galactosidase (SA-β-Gal) activity. Expression profiling analysis of 78 senescence-related genes by qRT-PCR showed a total of 17 genes significantly changed in GRK4(+) cells (≥ 2 fold, $p < 0.05$). Among these, 9 genes — AKT1, $p16^{INK4}$, $p27^{KIP1}$, $p19^{INK4}$, IGFBP3, MAPK14, PLAU, THBS1, TP73 — were up-regulated, while 8 genes, Cyclin A2, Cyclin D1, CDK2, CDK6, ETS1, NBN, RB1, SIRT1, were down-regulated. The increase in cyclin-dependent kinase inhibitors (p16, p27) and p38 MAPK proteins (MAPK14) was validated by immunoblotting. Neither p53 nor $p21^{Waf1/Cip1}$ protein was detectable, suggesting no p53 activation in the HEK293 cells. These results unveil a novel function of GRK4 on triggering a p53-independent cellular senescence, which involves an intricate signaling network.

*Corresponding Author: Xiaoshan Jiang, M.D., Ph.D., Rm 310, Hong-De Building, Graduate College, Guilin Medical University, 109 North 2nd Huan Cheng Road, Guilin, Guangxi 541004, China; Tel: +86 773 589 1052; Fax: +86 773 589 3516; jiangxs@glmc.edu.cn.

[1]These authors contributed equally to this work.

Keywords

G protein-coupled receptor kinase 4; cellular senescence; gene expression profiling; p53-independent senescence

1. Introduction

In 1965 Hayflick described a state of irreversible cell growth arrest in culture, which is now known as cellular senescence and thought to contribute to tumor suppression, organismal aging and tissue repair [1,2]. Based on the mechanisms involved, cellular senescence is classified into two categories: replicative senescence due to telomere attrition and premature senescence caused by intrinsic and/or extrinsic stress factors including activated oncogenes, DNA damage, or oxidative stress. Both types of senescence exhibit a permanent loss of the capacity for cellular proliferation and similar phenotypes characterized by an enlarged and flattened cell morphology, distinct nuclear structures of heterochromatin, and the induction of senescence-associated β-galactosidase (SA-β-gal) activity [2,3]. Despite having been studied for decades, the signaling mechanisms of cellular senescence are not fully understood.

G protein-coupled receptors (GPCRs) make up the largest group of cell surface receptors and mediate cellular responses to diverse extracellular stimuli. The initiations of intracellular signaling cascades that regulate cell division and cellular proliferation by GPCRs have been demonstrated [4–6]. GPCR signaling is regulated by G protein-coupled receptor kinases (GRKs). GRKs belong to a family of serine/threonine kinases. Together with β-arrestins, GRKs determine the rate and extent of homologous desensitization of GPCR signaling through their role in phosphorylation of ligand bound GPCRs. In mammals, seven GRKs have been identified so far, which are classified into three main groups: the rhodopsin kinase subfamily (GRKs 1 and 7), the β-adrenergic receptor kinases subfamily (GRKs 2 and 3), and the GRK4 subfamily (GRKs 4, 5 and 6). Besides the role in receptor desensitization, GRKs are also found to be capable of phosphorylating various intracellular proteins including those that potentially modulate cell growth such as insulin receptor substrate 1 and p38MAPK (GRK2 [7,8]), p53 (GRK5 [9]), histone deacetylase 6 (GRK2 [10]) and IκBα (GRK6 [11]). Recently, Johnson *et al.* reported that GRK4 subfamily members (GRK4/5/6) contain a functional nuclear localization sequence which can regulate their nuclear translocation and DNA-binding ability [12]. These observations suggest potential roles of GRKs in cell proliferation.

The human GRK4 gene is encoded by 16 exons. Alternative splicing of GRK4 gene generates 4 isoforms that differ in the presence or absence of exon 2 and exon 15: GRK4α (578 amino acids) is the full-length isoform; GRK4β (546 amino acids) misses the sequence encoded by exon 2; GRK4 γ (532 amino acids) misses the sequence encoded by exon 15; and GRK4δ (500 amino acids) misses both sequence encoded by exons 2 and 15 [13]. GRK4 has been the least understood member of the GRKs. Several reports have linked it to hypertension and breast cancer [14,15]. The biological function of GRK4 involves the desensitization of LH, FSH, mGlu, GABA(B), dopamine D1/D3 and angiotensin type 1

receptors [13,16–19]. An effect of GRK4 on cell growth has not been reported. Unlike other non-visual GRKs, GRK4 expression is limited to a few tissues: testis, myometrium, kidney and brain [13].

In current report, we have studied the capability of full-length GRK4 (referred as GRK4 in this manuscript) in induction of cellular senescence in human embryonic kidney HEK293 cells. HEK293 cells with ectopic expression of GFP-GRK4 were isolated by fluorescence-activated cell sorting (FACS) and then analyzed for their proliferative properties and cell cycle distribution as well as senescence-associated phenotype. Our results showed that overexpression of GRK4 halted cell proliferation and arrested cell cycle in the G1/G0 phase. Cellular senescence biomarker SA-β-gal staining was significantly increased cell population expressing GRK4. Furthermore, by comparing the expression profiles of 78 cellular senescence-related genes between the GRK4-expressing positive and negative cells, we found a total of 17 genes significantly changed. These data unveil a novel function of GRK4 on triggering cellular senescence.

2. Materials and methods

2.1. Cell line, construct and materials

HEK293, MCF-7 and MDA-MB 231 cells were originally obtained from the American Type Culture Collection. The full length human GRK4, GRK5 and GRK6 in pRK5 constructs was provided by Richard Premont (Duke University, Durham, NC USA) and was in-frame fused into pEGFP-(N1) plasmid. Rabbit anti-GRK4(K-20), -p53, -p21, -p27 polyclonal antibodies, and mouse anti-p16, anti-Rb and anti-Actin monoclonal antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Mouse anti-GRK4-6 monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse anti-Ki67 monoclonal antibody was purchased from ProMab Biotech (Richmond, CA, USA). Rabbit anti-p38 MAPK (D13E1) monoclonal antibody was purchased from Cell Signaling Technology Inc (Danvers, MA, USA). Goat anti-rabbit and anti-mouse IgG (H+L)-HRP conjugated secondary antibody were purchased from Promega (Madison, WI, USA). An Enhanced Chemiluminescent Detection Kit was obtained from Pierce Biotechnology (IL, USA). 4,6-Diamidino-2-phenyl-indole (DAPI), X-gal, adriamycin (ADM) and 5-fluorouracil (5-FU) were purchased from Sigma-Aldrich. All the cell culture plates were purchased from Corning Inc. (Corning, USA).

2.2. Cell culture and plasmid transfection

HEK293 cell line were cultured in DMEM medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 μg/mL streptomycin, and 100 U/mL penicillin (Hyclone) at 37°C in a humidified atmosphere with 5% CO_2. HEK293 cells were seeded in 10 cm plates and transiently transfected with pEGFP-GRK4 by using lipofectamine 2000 reagent (Invitrogen) at 70%–80% cell confluency according to manufacturer's instructions.

2.3. Cell sorting

Twenty-four hours after transfection, cells were trypsinized and washed with phosphate-buffered saline (PBS) twice. Cells were then resuspended with PBS containing 100 U/mL

penicillin and 100 μg/mL streptomycin and 1% FBS, and applied to the FACS AriaIII flow cytometer (Becton-Dickinson, San Jose, CA, USA). Cells were first gated on their forward- and side-scatter profiles. The gated cells were subsequently differentiated by their side-scatter profile and GFP property. Two distinct populations, the GFP-positive and GFP–negative cells, were sorted. The purity of sorted cells was determined by running the cells to the FACS analysis at the same condition. The sorted cells were collected in DMEM containing 5% FBS with penicillin-streptomycin for further experiments.

2.4. Immunofluorescence microscopy

Sorted cells were seeded on cover slips placed in 6-well plates and incubated for 24 h. Cell immunofluorescence staining was performed as described previously [20]. Briefly, the cover slips were fixed in 3.7% formadeldehyde for 15 minutes followed by incubation in TBS solution supplemented with 1% Triton X-100 (TBS-Triton) and 2.5% milk for blocking for 20 minutes. Cells were incubated with primary antibodies at dilution ratio of 1:200 for 1 hour at room temperature followed by 3 times of washing with TBS-Triton solution with 2.5% milk. Cells were then incubated with secondary anti-rabbit or anti-mouse antibodies that are preconjugated with Alexa 594 (red) (Invitrogen) at dilution ratio of 1:100 for 30 minutes at room temperature. The nuclei were stained by DAPI. Cover slips were mounted to glass slides in Prolong Gold antifade mounting medium (Invitrogen) overnight. Confocal images were acquired using a Zeiss LSM710 confocal microscope (Carl Zeiss, Germany).

2.5. Cell proliferation assay

Cell proliferative ability was determined by Cell Counting Kit(CCK)-8 (Dojindo Co., China). The sorted cells were seeded onto 96-well plates at a concentration of 1×10^3 per well. At each 24 h interval after cell-seeding, 10 μl of CCK-8 reagent was added to each well and cells were incubated for 2 h at 37°C. The absorbance at 490 nm was subsequently read using a microplate spectrophotometer (Tecan Infinite® M200 Pro, Switzerland). Five wells were measured for cell proliferation in each group.

2.6. Cell cycle analysis

The sorted cells were washed twice with PBS and fixed with 70% ethanol overnight at 4°C. Cells were then treated with staining buffer [PBS containing 1 mg/mL propidium iodide and 10 mg/mL RNase A] at 37°C in the dark for 30 min. The DNA contents of cells were determined by analyzing 10,000 ungated cells using the FACS AriaIII flow cytometer and Cell Quest software (Becton-Dickinson, USA).

2.7. SA-β-Gal staining

The sorted cells were plated in a 6-well plate and cultured with 2 ml of DMEM supplemented with 5 % FBS and 1 % penicillin/streptomycin for 5 days. Cells were washed twice with PBS and fixed with 0.5% glutaraldehyde for 5 min. Followed by 3 times of washing with PBS, cells were then stained at 37°C for 6~8 h in 2 ml of X-gal staining solution (1 mg/mL X-gal, 150 mmol/L NaCl, 2 mmol/L MgCl2, 5 mmol/L K3Fe(CN)6, 5 mmol/L K4Fe(CN)6, 40 mmol/L NaPi, pH6.0). The staining solution was replaced with PBS and positive marker was determined by the presence of blue cytoplasmic staining under

a light microscope, which indicates excess senescence-associated lysosomal β-galactosidase activity

2.8. RNA isolation and Real-time RT-PCR

Total RNA was extracted using TRIzol (Invitrogen), and cDNA synthesis was performed using the PrimeScript™ RT reagent Kit (Takara, Dalian, China), according to the manufacturers' instructions. RNA quantity was assessed using the Nanodrop (N2000, Thermo Scientific) with 260/280 ratios ranging from 2 to 2.2. The primer sequences of 81 human cellular senescence-related genes which were selected referring to the gene table of the RT2Profiler™ PCR Array Human Cellular Senescence (Cat No. PAHS-050Z, Sabiosciences, USA) are listed in Supplementary Table 1. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, USA) in an Applied Biosystems 7900 Sequence Detection System using the following PCR parameters: 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 sec, 60°C for 15 sec, and 72°C for 15 sec. The fluorescence threshold value (Ct) was calculated to represent the relative mRNA expression and β-actin was used as an internal control.

2.9. Immunoblotting

Cells were collected by centrifugation and the pellets were lysed with RIPA buffer [25 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1% NP40, 1% sodium deoxycholate] containing a protease inhibitor cocktail (Sigma). Protein concentration was determined using a bicinchoninic acid (BCA) assay (Pierce, USA). Equal amounts of proteins (50μg) were separated with 10% SDS-PAGE electrophoresis, and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Membrane was blocked in TBS-T buffer supplemented with 5% milk for 1 h, and probed with primary antibody overnight at 4°C followed by HRP-conjugated appropriate secondary antibody for 1 h at room temperature. The targeted proteins were detected using an ECL reaction kit (Pierce). Images were acquired and analyzed using a Bio-Rad's ChemiDoc XRS+ system (Bio-Rad, USA).

2.10. Statistics analysis

Student's t-test and scatter plot were performed to analysis the data using SPSS Statistics 20.0 (SPSS Inc., USA). Data are expressed as means±standard deviation (SD) of at least three independent experiments. P-values <0.05 were considered statistically significant.

3. Results

3.1. Isolation of GRK4/5/6-positive and -negative cells by fluorescence activated cell sorting

HEK293 cells were respectively transfected with pEGFP-GRK4, -GRK5 and -GRK6 plasmids. pEGFP(N1) plasmid was used as a control. Twenty-four hours after transfection, cells were collected and applied to fluorescence activated cell sorting (FACS). Cells were first gated on their forward- and side-scatter profiles. The gated cells were subsequently analyzed by their side-scatter profile and GFP property. Two distinct populations were detected for GRK4 transfected cells, which respectively represented GRK4-positive and GRK4-negative cells (Fig 1A), and were sorted out as GRK4(+) and GRK4(−) cells. The

purity of the two sorted populations was determined by re-analysis with FACS and the result showed their purities were 96.5±3.9 % and 95.4±1.2%, respectively (Fig 1B and 1C). Purities of GRK5(+)/GRK5(−), GRK6(+)/GRK6(−) and GFP(+)/GFP(−) cells are shown in Supplementary Figure 1A. Expression levels of GRK4, GRK5 and GRK6 in the flow cytometry sorted cells were further confirmed by both immunofluorescence microscopy and immunoblotting with anti-GRK4 antibody (for GRK4(+)/GRK4(−) and GFP(N1)(+)/GFP(N1)(−)cells) or anti-GRK4-6 an body (for GRK5(+)/GRK5(−) cells and GRK6(+)/GRK6(−) cells)(Fig 1D and 1E; Supplementary Figure 1B and 1C). These results demonstrated that GRK4-, as well as GRK5-, GRK6- and GFP-positive and -negative cells were successfully sorted out.

3.2. Overexpression of GRK4 inhibits cell proliferation and arrests cell cycle progression in G0/G1 phase

To determine whether expression of the GRK4 subfamily proteins influence cell growth, the sorted GRK4/5/6(+) and GRK4/5/6(−) cells were seeded onto 96-well plates and the cellular proliferation was examined with a CCK-8 assay at days 1, 2, 3, 4 and 5. The GFP(+) and GFP(−) cells were used as controls. Comparing to their parallel cell populations that GRK4/5/6 or GFP was negatively expressed, only GRK4-positive cells showed a significant growth inhibition (Supplementary Figure 2). The proliferative inhibition in GRK4-positive cells was further depicted in Fig 2A, comparing to both the GFP(+) and GFP(−) cells and the wild-type HEK293 cells. Since the inhibition of cell proliferation could be a result of the induction of cell growth arrest, we next investigated the regulatory effects of GRK4 overexpression on the cell-cycle distribution. The GRK4(+) and GRK4(−) cells, as well as GFP(+) and GFP(−) cells, were sorted and cultured in DMEM medium. Cells were collected at days 1, 3, and 6, and stained with propidium iodide (PI). Cells were then subjected to flow cytometric analysis. As expected, a significant increase of G1/G0 phase cells were observed in the GRK4(+) cells of 87.83±0.89% at day 1,85.98 ± 1.62 at day 2 and 85.40 ± 1.01 at day 6, compared to that in GRK4(−) cells, GFP(+) and GFP(−) cells, and that in control cells (Fig 2B;Table 1). Taken together, these results showed that overexpression of GRK4 in HEK293 cells is capable of inhibiting cell proliferation and inducing an irreversible cell cycle G1/G0 phase arrest.

3.3. GRK4 functions as an effective inducer of cellular senescence

Cell growth inhibition can be due to a senescence response. Besides showing a characteristic flat and large cellular morphology, increased number of vacuoles in the endoplasmic reticulum and large lysosomal bodies in cytoplasm, senescent cells show negativity of cell proliferative marker Ki67 [21] and present a major shift in biochemical activity of β-galactosidase (SA-β-gal) within lysosomes, which can be detectable at pH 6.0 by cytochemistry and serve as a reliable marker for cellular senescence [3]. To test whether the GRK4 triggers a senescence response, we firstly detect the Ki-67 protein in the GRK4(+) and GRK4(−)cells by Immunofluorescence microscopy. Human nasopharyngeal carcinoma CNE2 cells were used as a control. As shown in Fig 3A, the Ki-67 protein were clearly detected within the cell nuclei of GRK4(−) cells and CNE2 cells, whereas it was barely found in GRK4(+) cells. We next assessed the SA-β-gal activity in the GRK4(+) and GRK4(−) cells by a SA-β-gal staining. The stained cells and total cells were counted under

an inverted phase microscope and the percentage of cells staining positive was calculated. As shown in Figure 3, high stain positive of 99±0.20% in GRK4(+) cells for SA-β-gal was detected (Fig 3B), while this was markedly low to only 1±0.15% in GRK4(−) cells (Fig 3C). These results suggest that overexpression of GRK4 is capable of inducing the cellular senescence response in HEK293 cells.

3.4. Differential expression profile of senescence-related genes in GRK4(+) cells

It has been proposed that cellular senescence is controlled by genes that are activated to initiate or accelerate senescence signaling. To understand the pathways involved in the GRK4-triggered senescence, we investigated the expression profiles of senescence-related genes between the sorted GRK4(+) and GRK4(−) HEK293 cells. mRNA level of 78 genes whose expression is known to be associated with human cellular senescence response were measured by a quantitative real time PCR assay and GAPDH gene was used as inner control for normalizing the expression levels of genes tested. A total of 17 different expression genes (more than 2-fold change) were identified in the GRK4(+) HEK293 cells if the P value was set at <0.05 (Figure 4 and Supplementary Table 2). Among these, 9 genes — AKT1, $p16^{INK4}$, $p27^{KIP1}$, $p19^{INK4}$, IGFBP3, MAPK14, PLAU, THBS1, TP73 — were up-regulated, while 8 genes — Cyclin A2, Cyclin D1, CDK2, CDK6, ETS1, NBN, RB1, SIRT1 — were down-regulated. Genes whose fold expression changes and p-values exceeded the boundaries along with their biological processes involved are listed in the Table 2.

3.5. GRK4-induced cellular senescence in HEK293 cells is p53-independent

The mRNA data provided information that suggests a complicated signaling mechanism of GRK4-induced cellular senescence, in which several important cyclin-dependent kinases inhibitors (CKIs), such as p16 and p27, and mitogen-activated protein kinases (MAPK) are up-regulated. Surprisingly, there was no increase of p53 expression found in the differential profile (see Supplementary Table 2). To further address whether the GRK4-triggered cellular senescence response relies on p53 pathway, we tested the levels of both p53 and p21 proteins in the GRK4-sorted cells by immunoblotting assay and lysate of adriamycin (ADM)-treated breast cancer MCF-7 cells was used as the positive control. Consistent with the expression data of mRNA level tested by qRT-PCR, no positive bands of p53 and p21 were detected in all the HEK293 cell lysates (Fig 5A). In addition, by immunoblotting assay, we checked expression of some cell cycle regulatory factors and found the increases of p16, p27 and p38 MAPK proteins and decrease of Rb proteins in the GRK4(+) cells (Fig 5B and 5C), which corresponded with the findings on the mRNA level test. To further validate that the HEK293 cells are not p53-functional, HEK293 cells were respectively treated with 5 μM adriamycin (ADM) and 500μM5-fluorouracil (5-FU), and breast cancer cell lines MCF-7 ($p53^{WT}$)and MDA-MB 231 ($p53^{MUT}$) were used as controls. As shown in Supplementary Figure 3, the induction of both p53 and $p21^{WAF1}$ proteins by those genotoxic drugs was only observed in MCF-7 cells. There was no p53 activation observed in HEK293 cells. Taken together, these data suggest that the GRK4-induced cellular senescence in HEK293 cells is through a p53-independent pathway, which involves p16, p27 and p38 MAPK.

4. Discussion

Although GRKs participate in a wide range of signaling cascades through desensitization of various GPCRs associated with cell proliferation/desensitization or interactions with non-receptor proteins such as PI3K, Akt, GIT, MEK, p53, p38MAPK or IκBα [22], the role of GRKs in regulating cell growth has not been addressed. GRK4 belongs to GRK4 subfamily. Unlike GRK5 and GRK6, the two other members in GRK4 subfamily, the expression pattern of GRK4 is highly selective and is only detectable in select tissues in the testis, myometrium, kidney and brain in adult mammalian. GRK4 has been the least well-understood member of the GRKs and its potential role in modulating cellular proliferation is not yet known. In this study, we found that overexpression of GRK4, not GRK5 or GRK6 significantly inhibits HEK293 cell growth, along with an at least 6-day irreversible retention of cells in G1/G0 proportion and positive SA-β-galactosidase staining. To our knowledge, this is the first evidence that a GRK protein is capable of inducing cellular senescence. Identification of the different expression of genes involved in this novel GRK4-induced senescence will provide information to understand mechanisms underlying cellular senescence and expand our knowledge of GRKs' biological function. Moreover, it will be important to expand these studies in HEK293 cells to other cell types to gain insight into the physiological or pathophysiological of these initial findings of GRK4-induced cellular senescence.

Cells undergoing senescence represent a permanent inability to progress through the cell cycle. The progression of the cell cycle in mammalian cells is directly controlled by cyclin-dependent kinases (CDKs), which are activated by forming complexes with their respective cyclin partners. It has been known that the complexes of cyclin D1-CDK4/6 and cyclin E1-CDK2 are key regulators that drive G1/S transition [23]. On the other hand, cyclin A2 regulates cell cycle progression through binding CDK2 in S phase or CDK1 during the transition from G2 to M phase and is considered an important regulatory component initiating mitosis [24]. The abnormal expression of these CDKs and cyclins has been linked to cell cycle arrest and cellular senescence. For example, the activity of CDK2 alone or cooperatively with CDK4 or CDK6 was required for cell cycle G1/S transition and down-regulating expression of CDKs or cyclin D1/A2 or both resulted in cellular senescence or cell cycle G1/G0 phase arrest [25–30]. We analyzed differential expression profile of 78 senescence-related genes in the sorted HEK293-GRK4(+) cells that had demonstrated G1 arrest and senescence and found that the expressions of cyclin A2, cyclin D1, CDK2, CDK4 and CDK6 were down-regulated. Additionally, we recognized that the effects of cyclin-CDK complexes are controlled by the cyclin-dependent kinase inhibitors (CKIs). CKIs negatively regulate cell cycle progression through inhibiting the activities of CDKs. In mammalian cells CKIs fall into two classes: INK4 and CIP/KIP families. The INK4 CKI proteins, including $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$, sequester CDKs and inhibit the formation of CDK-cyclin complexes, whereas the Cip/Kip CKIs, including $p21^{Waf1/Cip1}$, $p27^{kip1}$ and $p57^{kip2}$, bind to cyclin-CDK complexes [31]. $p16^{Ink4a}$ is a well known inhibitor of Cdk4 and Cdk6 and $p27^{kip1}$ an inhibitor of Cdk2 and Cdk4. Both are important downstream targets of p38 mitogen-activated protein kinases (MAPK) and the increased expression of $p16^{Ink4a}$ or $p27^{kip1}$ was found to be associated with p38MAPK-activated cellular senescence [32,33]. In

line with these studies, our data showed a significant up-regulation of $p16^{Ink4a}$, $p27^{kip1}$ and p38MAPK at both mRNA and protein levels in HEK293-GRK4(+) cells that underwent cell growth inhibition and demonstrated senescence phenotype. Altogether, the decreased expression of the cyclins-CDKs and the increased expression of CKIs could be a result of the GRK4-induced G1 arrest senescence. Their specific roles in the regulatory pathways in response to overexpression of GRK4 need to be further clarified.

TP53 (p53) and Rb tumor suppressor proteins play critical roles in the induction of senescence [3,33]. Under the intrinsic and/or extrinsic stress, p53 is activated and transactivates expression of its downstream target $p21^{Waf1/Cip1}$, which is essential for cell-cycle exit and senescence [31,35]. $p21^{Waf1/Cip1}$ gene presents binding sites for transactivation domain of p53 and, therefore, the lack of $p21^{Waf1/Cip1}$expression can generally serve as an indicator for inactivation of p53 [36]. Despite the well-documented role of p53 in senescence, recent studies have revealed that cellular senescence can occur independently of p53 activation [3]. In current study, neither p53 nor $p21^{Waf1/Cip1}$ was detectable at both levels of protein and mRNA in the studied cells, suggesting that the novel GRK4-induced senescent response is through a p53-indepentent signaling pathway. Besides p53, pRb also regulates G_1 to S phase transition by reversibly inhibiting E2F-mediated transcription of cell-cycle progressing genes in its active, i.e. hypophosphorylated, form. Narita *et al* demonstrated that the decreased level of Rb protein was associated with Ras-induced senescence [37]. Similar to this result, down-regulation of Rb expression was detected in the HEK293-GRK4(+) cells, which may contribute to the observed senescent phenotype.

In conclusion, we demonstrate here a novel biological effect of GRK4 on triggering a p53-independent cellular senescence, which involves an intricate signaling network. Whether the GRK4-induced senescence is due to desensitization of certain GPCR signaling or through phosphorylation of non-receptor substrates that relate to cell growth inhibition or both needs to be further elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Conflict of interest statement: The authors declare no conflicts of interest.

Abbreviations

GPCR G protein-coupled receptor

GRK	G protein-coupled receptor kinases
SA-β-Gal	senescence-associated-β-galactosidase
FACS	fluorescence-activated cell sorting
CCK-8	cell counting kit-8
CDK	cyclin-dependent kinase
CKI	cyclin-dependent kinase inhibitor

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Highlights

- G protein-coupled receptor kinase (GRK) 4 halts cell cycle progression and induces senescence phenotype in HEK293 cells.
- Seventeen senescence-related genes differentially expressed in GRK4-posivtive cells are presented.
- A novel function of GRK4 on triggering a p53-independent cellular senescence is suggested.

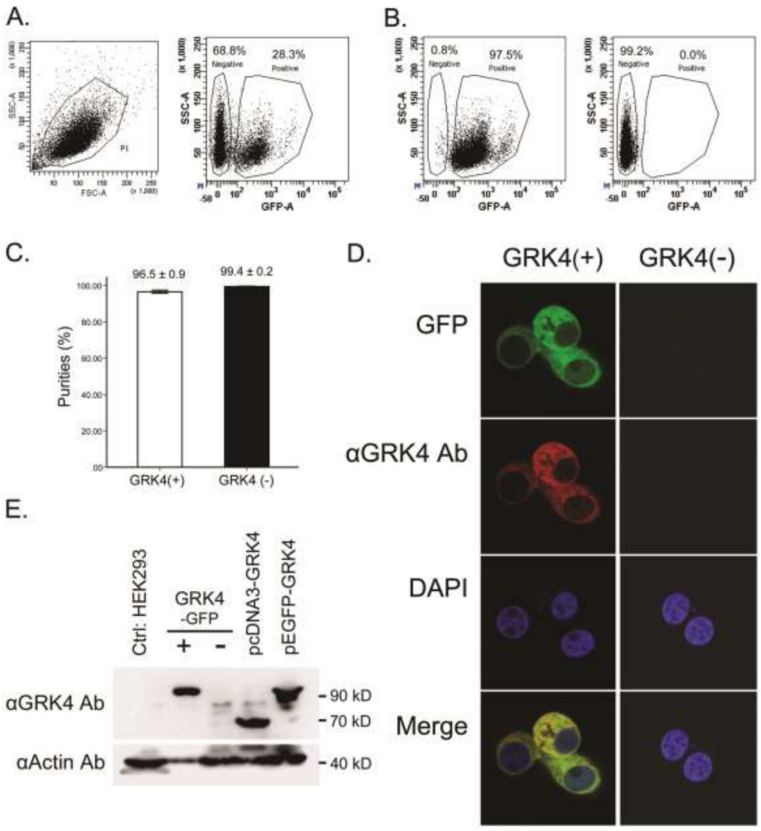

Figure 1. Isolation of GRK4-positive and -negative HEK 293 cells using fluorescence activated cell sorting (FACS)

A, Flow cytometry scatterplots of a representative experiment showing gate preparation for sorting of GRK4-positive and –negative cells. Twenty-four hours after transfection with pEGFP-GRK4 plasmids, HEK293 cells were harvested and subjected to FACS. Numbers shown are percentage of each population in whole cells tested. *B,* Representative re-analysis determining the purities of sorted populations. *C,* Histogram for the FACS re-analysis data of sorted cells. Numbers shown are percentage of the mean ± SD from three separate experiments. *D,* Immunofluorescence microscopy was performed using the GRK4 antibody and the Alexa 594 secondary antibody as described in *Experimental Procedures* (magnification: ×600). *E,* GRK4 protein expression in the sorted cells was determined by immunoblotting with a GRK4 antibody.

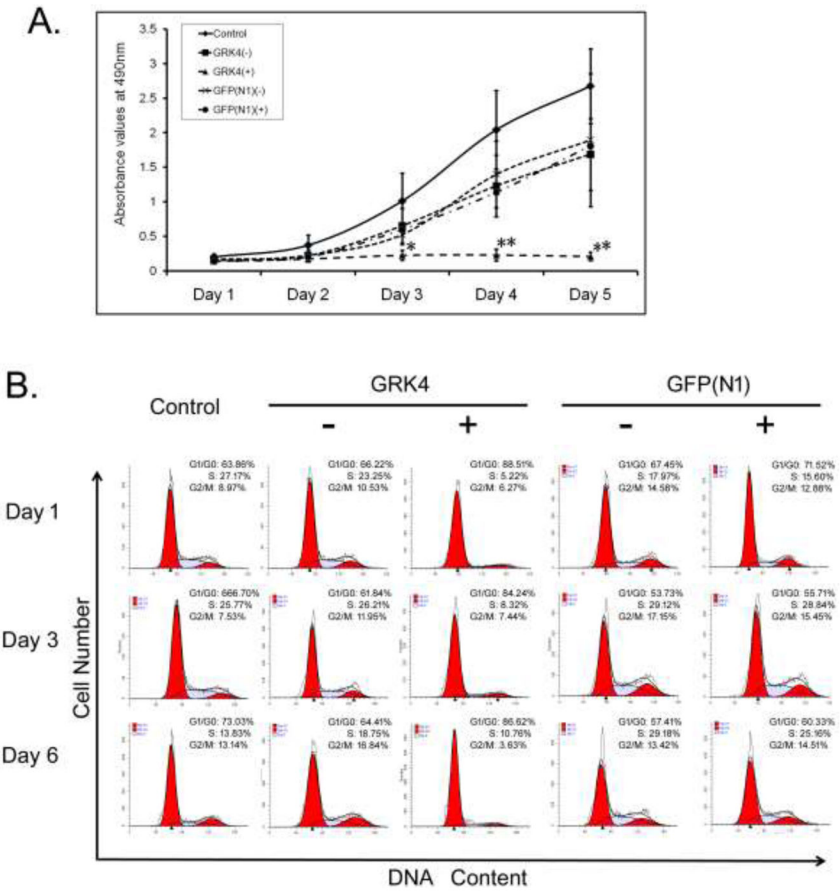

Figure 2.

Effects of GRK4 overexpression on cell proliferation and cell cycle distribution in HEK293 cells. Twenty-four hours after transfection with pEGFP-GRK4 and pEGFP(N1) plasmids, HEK293 cells were harvested. The GRK4-positive (GRK4+) and -negative (GRK4−) cells were sorted by FACS and then. The GFP(+) and GFP(−) cells were sorted as a control. *A,* After the indicated times, the proliferation of GRK4(+) and GRK4(−) cells, GFP(+) and GFP(−) cells was evaluated using the CCK-8 assay. *B,* Cell cycle of the sorted cells was determined by flow cytometry using propidium iodide staining and a representative flow cytometric analysis on cell cycle distribution was shown. Each bar represents mean ± SD of three independent experiments. *: $p<0.05$, **: $p<0.01$ (vs. unsorted).

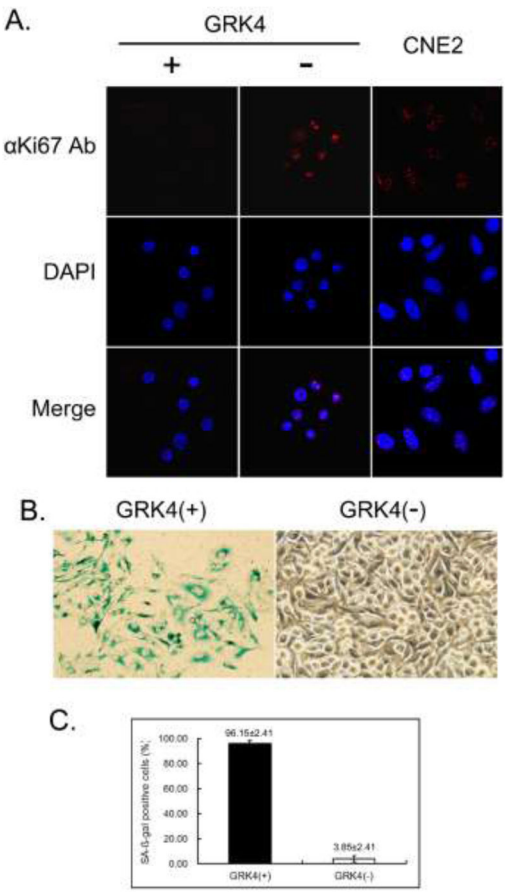

Figure 3. Induction of cellular senescence by GRK4 overexpression in HEK293 cells

The GRK4-positive(GRK4+) and -negative(GRK4−) cells were sorted by FACS after 24 h transfection with pEGFP-GRK4 plasmids. A, The cells were seeded on cover slips placed in 6-well plates and incubated for 24 h. Ki-67 protein was detected by Immunofluorescence microscopy using a Mouse anti-GRK4-6 monoclonal antibody in the GRK4(+) and GRK4(−) cells(magnification: ×600). Human nasopharyngeal carcinoma CNE2 cells were used as a control. *B*, The cells were seeded onto 6-well plates and cultured for 6 days. *A,* Cellular senescence was determined by SA-β-gal staining (magnification: ×400). Senescent cells were stained as blue in cytoplasm. *BC,* Quantitative data of SA-β-gal staining in the sorted cells. Each bar represents mean ± SD of three independent experiments.

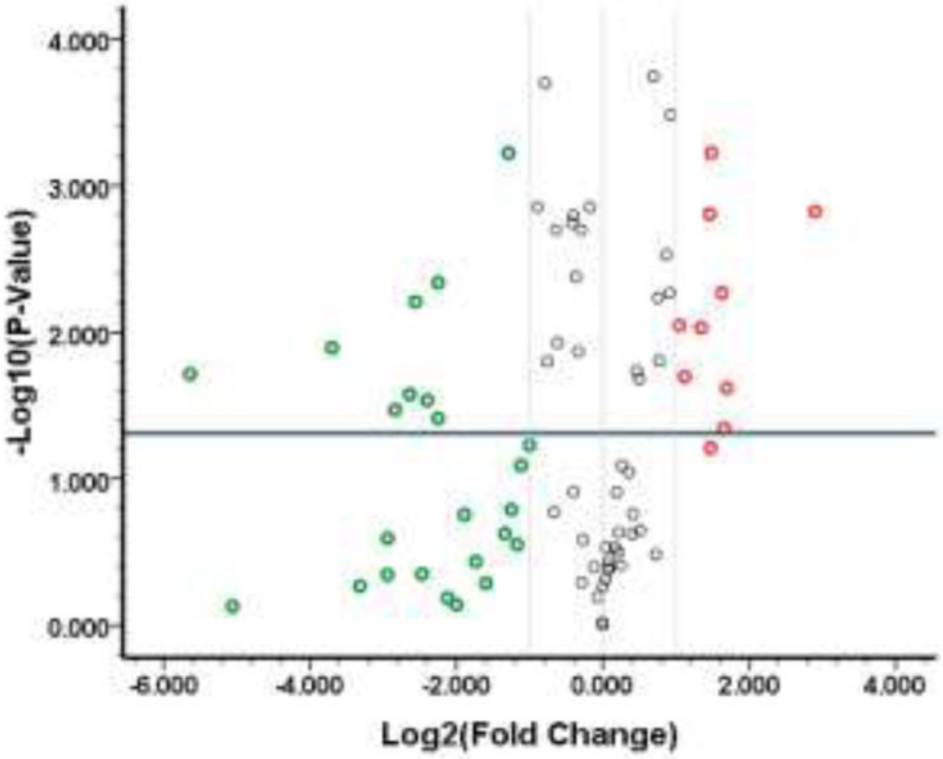

Figure 4. A scatter plot of 81 senescence-related gene expression in GRK4(+) cells versus GRK4(−) cells

Twenty-four hours after transfection with pEGFP-GRK4 plasmids, the GRK4-positive(GRK4+) and -negative(GRK4−) cells were sorted by FACS. The mRNA levels of81 senescence-related genes were tested by qRT-PCR. GAPDH gene was as inner control. Red dots are the genes whose expression increased more than 2 fold and green decreased more than 2 fold. The dots above the blue horizontal line are the genes with $p < 0.05$. The experiments were repeated three times.

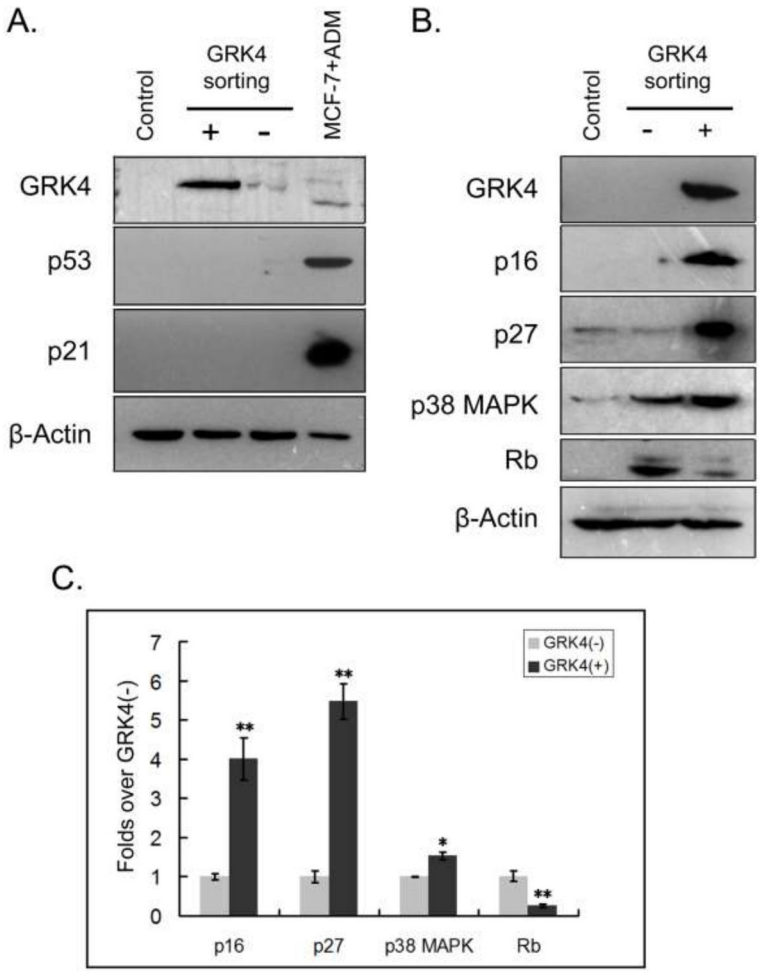

Figure 5. Change of protein levels during GRK4–induced senescence in HEK 293 cells

Twenty-four hours after transfection with pEGFP-GRK4 plasmids, GRK4(+) and GRK4(−) cells were sorted by FACS. The cells were lysed, and cellular proteins were separated by SDS/PAGE and transferred to PVDF membranes. Protein levels of p53, p21, p16, p27, p38MAPK and Rb were determined by immunobloting with correspondent antibodies. Actin is shown as a loading control. *A,* Effects of GRK4 overexpression on the levels of p53 and p21. ADM-treated MCF-7 cells served as antibody control. B, Effects of GRK4 overexpression on the levels of p16, p27, p38MAPK and Rb. *C,* Quantitative data of the protein levels of interest. Each bar represents the mean ± SD of three independent experiments (*P<0.05, ** P<0.01 vs. GRK4(−)). ADM: Adriamycin.

Table 1

Induction of G1/G0 arrestin HEK293 cells by GRK4.

	G1/G0	S	G2/M
Day 1			
Control	61.65±8.06	31.43±5.07	10.21±1.14
GRK4(−)	60.19±5.22	21.77±8.25	18.04±10.60
GRK4(+)	87.83±0.89 *	7.69±2.44	4.48±2.41
GFP-N1(−)	60.75 ± 6.49	25.25 ± 7.33	14.12 ± 1.10
GFP-N1(+)	64.60 ± 7.06	23.21 ± 6.94	12.21 ± 1.66
Day 3			
Control	62.84 ± 4.00	26.29 ± 3.57	10.87 ± 3.19
GRK4(−)	62.33 ± 2.29	25.40 ± 0.71	12.26 ± 2.11
GRK4(+)	85.98 ± 1.62 *	8.16 ± 1.12	5.86 ± 1.46
GFP-N1(−)	64.51 ± 11.12	23.39 ± 8.29	12.10 ± 4.41
GFP-N1(+)	57.82 ± 2.34	27.72 ± 2.23	14.46 ± 1.02
Day 6			
Control	67.47 ± 6.49	17.02 ± 7.09	15.51 ± 2.99
GRK4(−)	65.42 ± 5.66	18.84 ± 5.48	14.47 ± 1.99
GRK4(+)	85.40 ± 1.01 *	9.79 ± 1.28	4.82 ± 1.03
GFP-N1(−)	60.86 ± 3.74	26.40 ± 2.42	12.74 ± 2.17
GFP-N1(+)	64.73 ± 4.36	22.96 ± 3.54	12.31 ± 2.10

HEK293 cell were transfected with pEGFP-GRK4 and pEGFP(N1) plasmids, respectively. Twenty-four hours after transfection, cells were sorted on the basis of their GFP fluorescence and cultured in DMEM medium. At desired time points, cells were collected, fixed, stained with propidium iodide (PI) and then analyzed for cell cycle distribution by flow cytometry. Results are representative of three independent experiments; values are mean ± SD.

* $P<0.01$ vs. vehicle control.

Table 2

Differentially expressed senescence-related genes in GRK4(+) cells.

Genes	Fold-change	GO- biological process	*P-value*
AKT1	2.06±0.25	Regulation of protein phosphorylation and cell proliferation	*P=0.001*
p16,INK4	3.09±0.38	G1/S transition and cell cycle arrest	*P=0.006*
p19,INK4	2.54±0.37	G1/S transition and cell cycle arrest, DNA repair	*P=0.010*
p27,KIP1	2.75±0.17	Cell cycle arrest and response to hypoxia	*P=0.002*
IGFBP3	3.14±1.20	Regulation of cell growth and apoptosis	*P=0.045*
MAPK14	2.79±0.11	DNA damage checkpoint	*P=0.001*
PLAU	3.23±0.88	Angiogenesis and chemotaxis	*P=0.024*
THBS1	7.47±0.61	Response to hypoxia and regulation of cell proliferation	*P=0.002*
TP73	2.17±0.42	Regulation of p53 and MAPK activity	*P=0.020*
Cyclin A2	−3.70±0.25	Cell cycle regulation, G2 checkpoint	*P=0.012*
Cyclin D1	−2.56±0.49	Cell cycle regulation, G1/S transition	*P=0.006*
CDK2	−2.64±0.15	Cell cycle regulation, G1/S and G2/M transition	*P=0.027*
CDK6	−2.25±0.53	Cell cycle regulation, G1/S transition	*P=0.009*
ETS1	−5.64±0.73	Response to hypoxia and positive regulation of cell proliferation	*P=0.004*
NBN	−2.40±0.13	telomere maintenance, DNA damage repair	*P=0.029*
RB1	−2.25±0.38	Cell cycle regulation, G1/S transition	*P=0.005*
SIRT1	−2.84±0.69	p53 binding and histone deacetylase activity	*P=0.011*