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The study of the protective effect of APE

조선대학교 대학원

의학과

김 경 환

The study of the protective effect of APE

APE의 신경세포 보호효과

2008 년 2 월 25 일

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지도교수 박 상 학

이 논문을 의학박사학위신청 논문으로 제출함.

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The study of the protective effect of APE

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ABSTRACT

Human apurinic/apyrimidinic endonuclease (APE) is a multifunctional protein that is capable of repairing abasic sites and single-strand breaks in damaged DNA. In addition, it serves as a redox-modifying factor for a number of transcription factors. Identifying the transcriptional targets of APE is essential for understanding the mechanisms for how it affects various cellular outcomes. Expression array analysis was used to identify glial cell-derived neurotropic factor receptor α1 (GFRα1), which is an encoding receptor for the glial cell-derived neurotropic factor (GDNF) family, whose expression was induced by APE. Activation of NF-κB following APE expression represents a possible mechanism of APE-induced GFRα1 expression. Tumor progression of pancreatic cancers critically depends on activation of GDNF/ GFRα1 signal pathway. Our results indicate that APE-mediated increase in GFRα1 contributes to pancreatic tumor proliferation and invasion. The relationship with GFRα1 suggests that APE is also directly linked to regulation of neuron cell proliferation and survival, which may be important for promoting stress resistance and regulating life span under normal conditions. Thus, APE-mediated increase in GFRα1 may be a potential therapeutic target for

neurodegenerative disorder. These studies indicate that understanding the relationship of APE to activation of GDNF responsiveness may reveal new insights into the important role of APE, such as cancer progression and neuron cell survival

I. INTRODUCTION

The major human apurinic and apyrimidinic (AP) endonuclease APE (also known as Redox factor-1: Ref-1), which is homologous to *E. coli* exonuclease III, plays a key role in both short patch and long-patch base excision repair (1-3). It cleaves the AP sites in DNA and allows them to be repaired by other enzymes involved in base excision repair (4-7). The AP sites can be formed by chemical hydrolysis, the oxygen metabolism, ionizing radiation, UV irradiation, alkylating agents or oxidizing agents (4,8). In addition, AP sites can also arise spontaneously, where it has been estimated that 20,000 purines and 500 pyrimidines are lost in each 24 h cell cycle in human cells (9). The presence of AP sites blocks DNA replication, leading to DNA breakage, mutagenicity and cytotoxicity. APE contributes to more than 95 % of the total cellular AP site-specific activity (10), which is consistent with APE being essential for maintaining the genomic stability.

APE is a multifunctional protein that is not only responsible for the repair of AP sites but also stimulates the DNA binding activity of the AP-1 family of transcription factors via a redox-dependent mechanism (11,12). This effect is mediated via the reduction of a conserved cysteine residue located at the DNA-binding domains of c-*fos* and c-*jun* (13-15). APE is also capable of modulating or activating other classes of transcription factors via a similar reducing action including NF-κB, p53, Egr-1, c-Myb, HLF, and Pax-8 (16-20). The ability of APE to activate the transcription factors involved in the cellular response to various stresses, suggests that APE may play an important role in various cellular processes.

It is not known why APE, which is vital to a critical DNA-repair process, can also affect the functioning of several apparently disparate transcriptional regulators. Whatever the significance of its different roles in cells may be, APE is essential for early development. This is because a deletion of the APE gene is lethal at a very

early stage of embryogenesis (21). Moreover, APE has been implicated in the protection against cell death resulting from various toxic stimuli. The reduction of APE has been reported to sensitizing the cells against oxidative DNA damage (22,23). In contrast, APE overexpression provokes an increase in resistance to some alkylating agents and oxidative stress (24-26).

Although the DNA repair and transcription factor reducing properties of APE are well-known, other fundamental mechanisms by which it may regulate redox-sensitive transcription, and influence cell function need to be elucidated. Identifying the transcription targets of APE is essential for understanding the pathways by which APE affects cellular outcomes. To date, the list of transcription targets of APE is not comprehensive. Expression array analysis was performed using Ad-APE (adenovirus encoding an *APE* gene) infected GM00637 human fibroblast cells was performed in an effort to identify the downstream target genes of APE particularly those that might be involved in APE-mediated cell survival and proliferation. This paper reports an APE target gene, glial cell-derived neurotropic factor receptor α1 (GFRα1), which were identified through this screening, contributes to the APE-mediated increase in glial cell-derived neurotropic factor (GDNF) responsiveness including c-Src activation and cell proliferation. This study is the first to indicate a link between the APE and GDNF/GFRα signaling.

II. MATERIALS AND METHODS

Cell culture and DNA Constructs. The human fibroblast GM00637 cells (Coriell Institute for Medical Research) and mouse neuroblastoma neuro2A cells (American Type Culture Collection) were maintained in Earle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS). The human pancreatic cancer cell line PC3 and DU145, human colon cancer cell line SW480 and DLD1 (American Type Culture Collection) were maintained in RPMI1640 supplemented with 10% fetal bovine serum (FBS). The cells were maintained in 5%CO_2/95% air at 37 °C in a humidified incubator. Human *APE* cDNA, was amplified by RT-PCR using the *APE* oligo primer (5`-TCT AGA ATG CCG AAG CGT GGG AAA AAG G-3`, 5`-GGT ACC TCA CAG TGC TAG GTA TAG GGT G-3`) from human fibroblast GM00637 cells. The nucleotide sequence of each construct was confirmed by cycle sequencing using an ABI PRISM 310 genetic analyzer (PerkinElmer Life Science). The cells were transfected with the Lipofectamine (Gibco BRL) according to the manufacturer's protocol.

The *APE* cDNA was cloned into a pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA, USA) and a *pShuttle* vector (Invitrogen) after confirming the DNA sequence. The newly constructed plasmid *pShuttle-hAPE* was then doubly digested with *PI-SceI/I-CeuI*, and the purified product was ligated using Adeno-X DNA. The DNA was linearized with *PacI* and purified before Liopfectamine (Invitrogen) transfection of HEK293 cells. After transduction, HEK293 cells layers were overlaid with agarose and assessed for viral plaque formation at 10 days. For virus collection, the cells were lysed with three consecutive freeze-thaw cycles, and the virus was collected from the supernatant. The virus titer was approximately $1x10^7$ pfu/ml, which was determined using an end-point dilution assay. A vector carrying the β-galactosidase gene *LacZ* (Ad-

LacZ) was used to monitor efficiency of transduction by the viral vectors and a nonspecific transgene expression controls. The transduction efficiency was tested by *in situ X-Gal staining*, and infection with 50-100 multiplicity of infection (MOI) of Ad-LacZ resulted in 90 – 100 % of cells testing positive in GM00637 human fibroblast cells.

The APE was expressed in the human fibroblast cells using the replication-deficient adenoviral vector harboring the human *APE* (Ad-APE) genes. The transduction efficiency of adenovirus vector in GM00637 human fibroblast cells was evaluated using a different adenovirus vector containing the β-galactosidase (Ad-LacZ) MOI and observing the X-gal staining of β-galactosidase 48 h after infection. It indicated that the cell infection rate increased with the increase of MOI of the viruses: MOI = 1, only a few cells expressed β-galactosidase; MOI = 50, approximate 90 % cells expressed β-galactosidase; MOI = 100, almost all cells expressed β-galactosidase (data not shown). The cells infected with 100 MOI of Ad-LacZ did not show the change in either the cell proliferation or morphology (data not shown). Based on the transduction efficiency of Ad-LacZ in GM00637 cells, cells infected with either Ad-APE or Ad-LacZ at the 50 MOI were used. To assess the adenovirus-mediated expression of APE, Western blot analysis was performed with extracts of cells 48 h after infection with an adenovirus vector, and a high-level of the APE protein was observed in the cells infected with the Ad-APE (examples in Figure 1b, *upper panel*). Comparing genomic DNA sequence (Genebank number: AC005872) and known mRNA sequences of human GFRα1 (Genebank number: NM_005264, NM_145793 or BC 014962), at least two different promoters must be involved in its transcription.

GFRα1 promoter constructs: Genomic DNA from the human fibroblast GM00637 cell line was prepared using Puregene™ DNA purification system (Gentra Systems, Inc. Minneapolis, MN), and the *GFRα1* promoter regions search at the NCBI in the AC005872. The genomic DNA fragments corresponding to GFRα1 upstream sequence were amplified using PCR with the human genomic DNA from GM00637 cells as a template. The amplified fragment was then cloned into the *Kpn*I and *Xho*I sites of pGL3-Basic vector (Promega, Madison, WI) for construction of luciferase reporter vector. The 0.5-kb GFRα1 promoter construct contains the nucleotide sequences from -575 to -66 including about 420-bp upstream sequences and a part of the second exon from Fig. 2a. The 0.96-kb GFRα1 promoter construct contains the sequences from -2291 to -1329 including 0.8-kb upstream sequences and a part of the first exon. The 2.2-kb GFRα1 promoter construct encompasses the nucleotide sequences from -2291 to -66. The site-directed mutagenesis constructs were generated using 0.5-kb GFRα1 promoter construct as a template by Muta-Direct Site-Directed Mutagenesis Kit (Intron Biotechnology, Korea). The mutated nucleotide sequences were as follows and written in capital; mtNF1 5′gttggaaatCGcc3′, mtNF2 5′ggTAgagtctccg3′ and mtNF3 5′cccggagttGGct3′.

Electrophoretic mobility shift assay (EMSA): The ^{32}P-labeled NF1 (5′-GTGTTGGAAATTCCCCAAAG-3′), NF2 (5′-CTGTGGGGGGAGTCTCCGGCGCT-3′), NF3 (5′-GACCCGGAGTTTCCTCTTTC-3′) or biotin-labeled NF-κB consensus sequences (5′- AGTTGAGGGGACTTTCCCAGGC-3′) were used as probes. The double stranded NF1, NF2 or NF3 were end-labeled using T4 polynucleotide kinase (New England BioLabs, Inc. Ipswich, MA) and ^{32}P-γ-ATP (3,000Ci/mmol) (Amersham

Biosciences, Piscataway, NJ). The radioactive probe was purified using Sephadex G-25 Spin columns (Roche, Germany). The double stranded NF-κB consensus sequences were biotin-labeled using Biotin 3′ End DNA Labeling kit (Pierce, Rockford, IL). The nuclear extracts were prepared from parent, pcDNA3- and APE-expressing GM00637 cells. Cells were harvested and resuspended in cold buffer containing 10 mM HEPES-KOH (pH 7.9), 1.5mM $MgCl_2$, 10mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 0.1% NP-40 and 1x Complete protease inhibitor cocktail (Roche, Germany). After incubation in ice for 10 minutes, nuclear fraction was harvested by brief centrifugation. To obtain nuclear extracts, the pellet was resuspended in cold buffer containing 20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF. In each reaction, 10 µg of nuclear extracts,1 µl of the radio-labeled probe (40 pmol) or 20 fmol of biotin-labeled probe were incubated in the reaction buffer (10 mM HEPES-KOH, pH 7.9, 60 mM KCl, 1 mM EDTA, 10% glycerol, 10 mM $MgCl_2$, 0.2 mM DTT and 1 µg of poly dIdC) for 20 minutes at room temperature. Antibodies against c-rel (C, sc-71), Rel B (C-19), p65 (C-20), p50 (H-119) (Santa Cruz Biotechnology, Santa Cruz, CA) and p52 (06-413, Upstate, Temecula, CA) were incubated in the reaction buffer for 20 minutes at room temperature before the binding reaction.

Chromatin immunoprecipitation (ChIP): Chromatin immunoprecipitation was performed using EZ ChIP™ Chromatin Immunoprecipitation kit (Upstate, Temecula, CA) according to the instruction's manual. Formaldehyde solution (1%, final concentration) was added to the cultured cells with 90% confluency in 10 cm culture dish and stored at room temperature for 10 minutes. After quenching unreacted formaldehyde with 10x Glycine solution, the cells were washed with cold PBS and then harvested in the SDS lysis buffer containing Protease Inhibitor

Cocktail II provided in the kit. The cell lysates were sonicated with 5 sets of 5-second pulses using ultrasonic processor (VCX 130) from Sonics & Materials, Inc. (Newtown, CT) equipped with mm tip and set to 70% of maximum power. Immunoprecipitation was performed using 100 µl of sheared crosslinked chromatin, corresponded to 2 x 10^6 cell equivalents, 1 µg of anti-p65 antibody (C-20 ,Santa Cruz Biotechnology, Santa Cruz, CA) and 60 µl of protein G agarose. To reverse the DNA-protein crosslinks, 8 µl of 5 M NaCl was added to 200 µl of eluted protein/DNA complexes and incubated at 65℃ for 4 hours. DNAs were purified with spin columns. PCR was carried out to detect the protected DNA fragments by p65 using the primer sets encompassing 0.5-kb GFRα1 promoter region.

Promoter Luciferase activity assay*s.* The promoter constructs were transiently transfected into the cells using the Lipofectamin (Invitrogen, Carlsbad, CA) according to the manufacture's protocol. The transfection efficiency was determined by β-galactosidase activity of pCMV β-gal (Promega, Madison, WI). The GM00637 cells were cotransfected with promoter constructs and APE expression vector or pcDNA3.1 vector. The cells were harvested 48 hours after the transfection using a lysis buffer (5x PLBR, Promega). The luciferase and β-galactosidase activities were measured using Luciferase assay system and β-galactosidase enzyme assay system, respectively, according to the manufacture's protocol (Promega, Madison, WI). Briefly, luciferase activity was measured by a luminometer (programed to perform a 2-second pre-measurement delay, followed by a 10-second measurement period for each reporter assay). The luciferase activities were normalized based on each β-galactosidase activity and adjusted comparing with the activity using empty pGL3-basic vector to describe as relative luciferase activity. Experiments were repeated at least three times.

Western Blotting-The cells were washed with PBS, and lysed at 0 °C for 30min in a M-PerR Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA). The protein content was determined using a Bio-Rad dye-binding microassay (Bio-Rad, Hercules, CA, USA). 20 μg of protein per lane was electrophoresed on 10% SDS polyacrylamide gels after boiling the protein in a Laemmli sample buffer for 5 min. The proteins were blotted onto Hybond-C membranes (Amersham Biosciences, Piscataway, NJ, USA), and the protein markers (Fermentas, Hanover, MD, USA) were used as the size standards. After electroblotting, the membranes were blocked with 1X Tris-buffered saline containing Tween-20 (TBS-T; 10 mM Tris-HCl, pH 7.4, 150mM NaCl, 0,1% Tween-20) and 5% milk, and incubated with the primary antibody diluted in a 1X TBS-T buffer for 2 h. The primary antibodies were diluted by 1/1000. The membranes were repeatedly washed and incubated with the appropriate secondary antibodies (1/4000) in a 1X TBS-T buffer for 1 h. The blotted protein was detected using an ECL kit (iNtRon Biotech, Korea). The following antibodies were used for immunohistochemistry and immunoblot analyses: rabbit polyclonal antibody GFRα1(H-70) and APE(C-20) (Santa Cruz Biotechnology, Inc) and phospho c-Src(tyr418) (Cell Signaling Technology, Beverly, MA, USA)

Immunofluorescence Microscopy. The paraformaldehyde-fixed cells were incubated with anti-GFRα1 antibody (Santa Cruz Biotechnology). The cells were stained by incubation with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit or anti-goat secondary antibodies (Vector, USA). The immunofluorescence images for the GFRα1 proteins were obtained using FV300 laser microscopy (Olympus, Japan) at an excitation wavelength appropriate for FITC (488nm).

Semiquantative Reverse Transcriptase Polymerase Chain Reaction. RNA extraction was carried-out using RNA-STAT-60 according to the manufacturer's instructions (TEL-TEST, Inc., Friendswood, Texas). Briefly, after homogenizing the cells in the RNA STAT-60, the homogenate was mixed with chloroform (5:1;v:v), shaken vigorously for 15 sec, and centrifuged at 13,000 rpm for 15 min at 4 °C. The RNA present in the upper colorless aqueous phase was precipitated by adding isopropanol, which was washed twice with 70% ethanol, and the residue was air dried for 10 min. The RNA was then resuspended in DEPC. 10 μl RNA aliquots were prepared and stored at -70 °C until needed. 2 μg of the total RNA was reverse transcribed using a M-MLV cDNA synthesis system (Promega), and the reverse transcribed DNA was subjected to a polymerase chain reaction (PCR). The profile of the replication cycles is as follows denaturation at 94 °C for 50 sec, annealing at 58 °C for 50 sec, and polymerization at 72 °C, for 1 min. In each reaction, the same amount of *glyceraldehydes-3-phosphate dehydrogenase* (*GAPDH*) was used as the internal control. The primers used for the PCR are as follows: *APE* forward, 5'-ATG CCG AAG CGT GGG AAA AA-3'; *APE* reverse, 5'-TCA CAG TGC TAG GTA TAG GGT GAT AGG-3' designed to amplify a 954 bp region; GFRα1 forward, 5`-AAG GAG ACC AAC TTC AGC CT-3`; GFRα1 reverse, 5`-TTG CAG ACA TCA TTG GAC AC-3` designed to amplify a 382 bp region; c-Src forward, 5`- ATC GTC ACT GAG TAC ATG GC -3`; c-Src reverse, 5`-CAG AGA AGG ATT CCG AAA CT-3` designed to amplify a 341 bp region; Ret forward, 5`- ATT CGT ACG TGA AGA GGA GC-3`; Ret reverse, 5`-AAA TCA GGG AGT CAG ATG GA -3` designed to amplify a 378 bp region; GAPDH forward, 5'- CCA TGG AGA AGG CTG GGG-3'; and GAPDH reverse 5'- CAA AGT TGT CAT GGA TGA CC-3' designed to amplify a 194 bp region (total number of cycles:26). The PCR products were resolved on 1% agarose gels, stained with ethidium bromide, and then photographed.

Small interfering RNA (siRNA)-based Experiments- The siRNA target sites within the human *APE* and *GFRα1* gene were chosen using the Ambion's siRNA target finder program: APE siRNA (534bp from Atg) GUCUGGUACGACUGGAGUAtt-3' (sense) and 5'-UACUCCAGUCGUACCAGACtt-3' (anti-sense); GFRα1 siRNA(1228bp from Atg) 5'-UACACACCUCUGUAUUUCCtt-3' (sense) and 5'-GGAAAUACAGAGGUGUGUAtt-3' (antisense); LacZ siRNA, 5`-CGUACGCGGAAUACUUCGAtt-3` (sense), 5`-AAUC GAAGUAUUCCGCGUACGtt-3` (antisense) for the *LacZ* gene. These siRNAs were prepared using a transcription-based method with a Silencer siRNA construction kit (Ambion, Austin, TX, USA). The cells were transfected with the siRNA duplexes using Oligofectamine (Invitrogen).

WST-1 Proliferation Assays—GM00637 cells were infected with Ad-LacZ or Ad-APE. 24 h later, the cells were then incubated with or without GDNF (Sigma-Aldrich). To investigate the effect of APE and GRFα1 on the cell proliferation in response of GDNF, Ad-LacZ or Ad-APE infected cells were transfected with control siRNA, APE siRNA or GFRα1 siRNA using the Oligofectamine transfection reagent (Invitrogen). 24 h after transfection, the cells were then incubated with or without GDNF for up to 72 hours. Every 24 h after GDNF treatment, the WST-1 tetrazolium salt (Roche Applied Science) was added to the culture for 2 h to monitor the level of cell proliferation according to the manufacturer's instructions. After the incubation period the production of formazan dye was quantified using a spectrophotometer (450 nm).

Invasion Assay Invasion assays were done using a Matrigel invasion chamber (BD Biosciences Discovery Labware) as described previously (25). Briefly, pancreatic cancer cells were seeded in the upper chamber in serum-free media in the presence of GDNF (10 and 100 ng/mL). In some experiments, cells were preincubated with the MEK-1 inhibitor PD98059 (10 μmol/L), the PI3K inhibitor wortmannin (200 nmol/L), or anti RET antibodies (1 μg/mL) for 30 minutes before adding GDNF. In small interfering RNA (siRNA) studies, cells were transfected with RET siRNA 48 hours before being seeded into the upper chamber. Complete medium containing 20% fetal bovine serum (FBS) served as a chemoattractant in the lower chamber. GDNF was added to the lower compartment at the same concentration as in the upper chamber. After 24 hours, invading cells on the lower membrane surface were stained and counted. All invasion experiments were done at least in triplicate using separate cultures and the same lot of Matrigel chambers.

III. RESULTS

APE down-stream target gene, GFRα1

A significant increase in APE expression has been demonstrated in malignant tissues and a higher APE expression level was also reported to be associated with tumor progression (7). In addition, alteration in APE expression and mutations in the APE gene have been found in patients with a variety of neurodegenerative diseases. Thus, APE has been implicated in the tumor progression and dysfunction of APE may contribute to the development of neurodegenerative disease. However, molecular mechanisms underlying this effect are unclear. In the present study, we sought to determine the downstream target genes regulated by APE, particularly those that might be involved in the APE-mediated cell survival and proliferation, using annealing control primers (ACP)-based RT-PCR analysis.

ACP-based RT-PCR analysis was performed and the expression patterns in a human fibroblast cells GM00637 stably transfected separately with APE expression vector and empty pcDNA3 vector were compared (Fig. 1). Using this approach, we identified partial cDNA that was only expressed in APE-transfected cells but not empty vector transfectec cells (Fig. 2a). The 418-bp amplicon displayed complete homology to glial cell-derived neurotropic factor receptor α1 (GFRα1) genes (Fig. 2b). GFRα1 is a key receptor for the glial cell-derived neurotropic factor (GDNF) family, which promote the survival of various neurons, and is involved in tumor cell proliferation and invasion.

To confirm this ACP-based RT-PCR result, semiquantitative RT-PCR analyses of the Ad-LacZ and Ad-APE transduced GM00637 cells were performed. Semiquantitative RT-PCR analysis using the *GFRα1* primers showed that the expression level of the *GFRα1* genes was increased dramatically by infecting them with Ad-APE, but not with Ad-LacZ (Fig. 3a). In order to determine if this increase

in the *GFRα1* mRNA levels correspond to an increase in the GFRα1 protein level, western blots was carried out using an antibody against the GFRα1. SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) was used to separate the whole-cell extracts of the protein from the APE-transfected cells, as well as to separate the protein from the LacZ-transfected cells. Western blot analysis with the GFRα1 antibody showed that the GFRα1 protein levels were higher in the APE-transfected cells than the LacZ-transfected cells (Fig. 3b). GFRα1 induction was observed as early as 24 h after the Ad-APE infection (Fig. 3c). Immunofluorescence staining using the same antibody also confirmed the expression of endogenous GFRα1 in Ad-APE-infected GM00637 cells but not in Ad-LacZ-infected cells (Fig. 3d).

APE-mediated increase in GDNF responsiveness in GM00637 cells

We next investigated whether the GFRα1 induction mediated by APE could promote a functional interaction with GDNF. The receptor tyrosine kinase, Ret, is a major component in the signaling cascade activated by members of the GDNF family (30,31). However, Ret was not detected in the GM00637 cells (data not shown). Recent *in vitro* studies have shown that exogenously applied GDNF interacts with cells expressing GFRα1, leading to the activation of the Ret-dependent and Ret-independent signal pathways (32-34). Because Src-family kinase has been reported to be the direct downstream target of the GDNF/GFRα signal pathway in the Ret-deficient cell lines (32-34), we examined whether GDNF induced Src activation by investigating the phosphorylation status of Src in the total lysates from GM00637 stably expressing APE and pcDNA3 using antibodies directed against the activated form of Src. It was found that c-Src became phosphorylated on Tyr418 in the GNDF-stimulated, APE-expressing cells but not pcDNA3-transfected cells (Fig. 3e). The increase in c-Src phosphorylation was

maintained for up to 3 h after the GDNF treatment. In order to determine if APE indeed contributes to the enhancement of the GDNF responsiveness, APE-siRNA or GFRα1-siRNA was used in an attempt to inhibit its expression level. Western blot analysis revealed that the transfection of APE-siRNA into APE expressing GM00637 cells led to decrease APE expression as well as GFRα1 expression, compared with control-siRNA-transfected cells (Fig. 3f). In addition, the GDNF-induced c-Src (Tyr418) phosphorylation was markedly suppressed in the APE-expressing cells treated with the APE-siRNA. Moreover, the APE-expressing cells treated with the GFRα1 siRNA showed the greatest inhibition of GFRα1 protein expression as well as the attenuation of the GDNF-induced c-Src phosphorylation.

GFRα1 interacts with the GDNF family, resulting in the activation of the intracellular pathway, which contributes to cell proliferation and differentiation (27-29). Therefore, we examined the effect of GDNF on the proliferation of APE-expressing GM00637 cells. The APE-expressing cells treated with GDNF showed a more rapid increase in the number of cells on days 1, 2 and 3 than the parent cells and pcDNA3-transfected cells treated with GDNF (Fig. 3g). The cell proliferation experiments confirmed that the transfection of GFRα1-siRNA significantly reduces the level of cell proliferation in APE expressing cells in response to GDNF compared with the control-siRNA transfection (Fig. 3g). These results suggest that the APE-mediated increase in GFRα1 expression is contributed to the enhancement of the GDNF responsiveness in GM00637 cells.

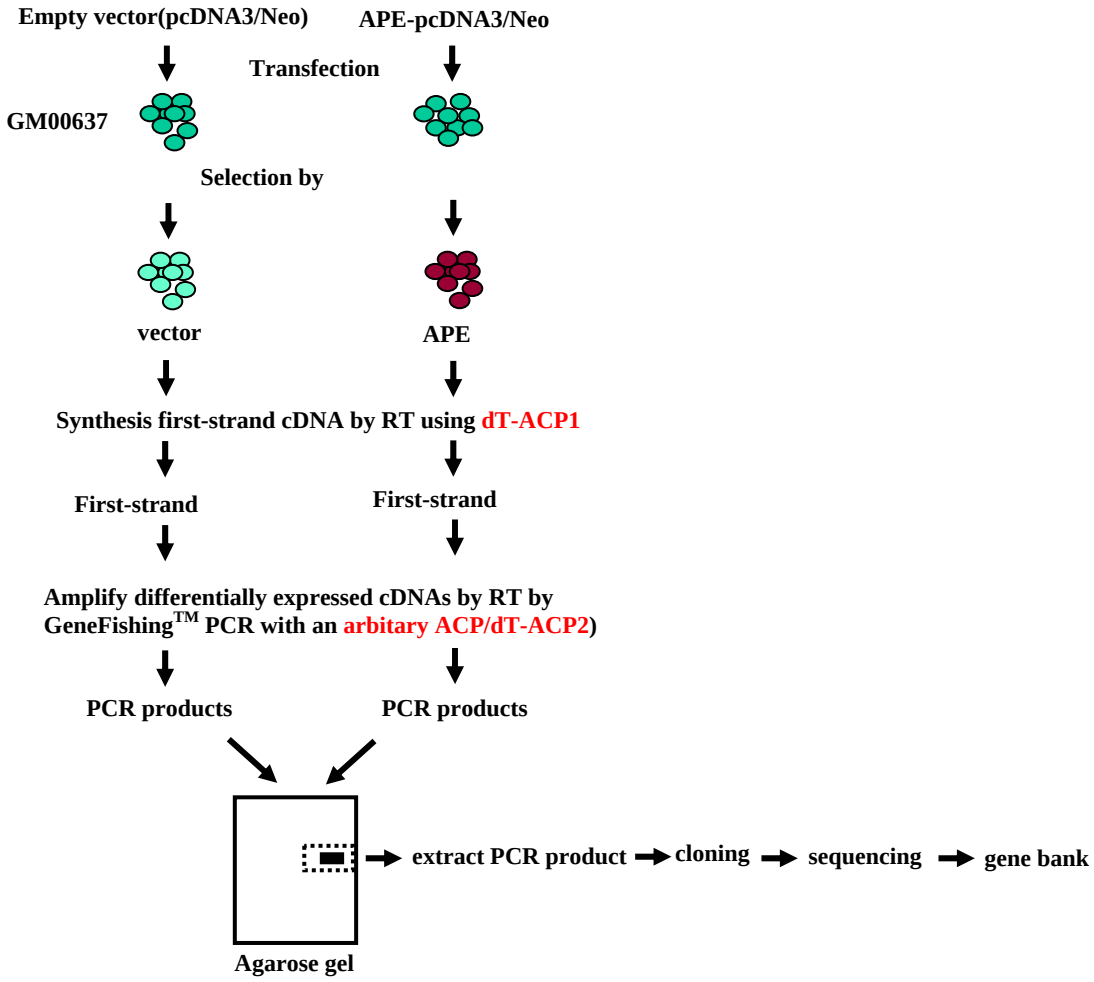
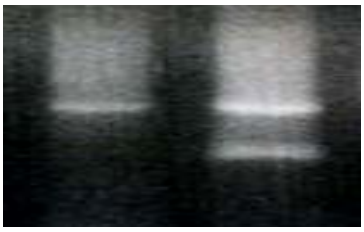

Empty vector(pcDNA3/Neo)
APE-pcDNA3/Neo
Transfection
GM00637
Selection by
vector
APE
Synthesis first-strand cDNA by RT using dT-ACP1
First-strand
First-strand
Amplify differentially expressed cDNAs by RT by
GeneFishing™ PCR with an arbitary ACP/dT-ACP2)
PCR products
PCR products
extract PCR product
cloning
sequencing
gene bank
Agarose gel

Figure 1 Schematic diagram of the ACP-based RT-PCR for identification of differentially expressed genes from empty vector pcDNA3 stably transfected GM00637 cells and stably APE expressing GM00637 cells. The mRNA isolated from these cells was used for the synthesis of first-stand cDNA using the dT-ACP1 primer. Second-strand cDNAs were then amplified during second-stage PCR by using a combination of dT-ACP2 (reverse primer) and one of 20 arbitrary ACP primers (forward primer). The PCR products were separated on an agarose gel and stained ethidium bromide. The differentially cDNA bands were excised from the gel for further cloning and sequencing.

a



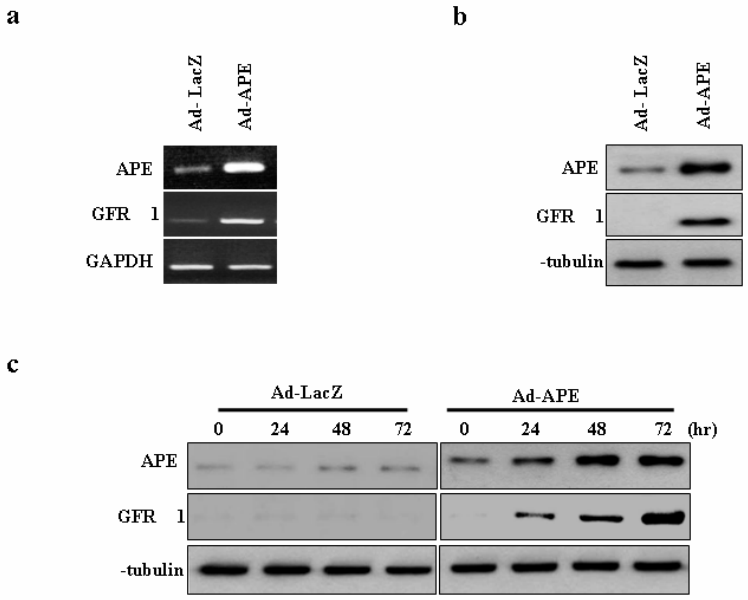
b

cDNA synthesis primer: dT-ACP1: 5'-CTGTGAATGCTGCGACTACGATXXXXX$(T)_{18}$-3'
Reverse primer: dT-ACP2: 5'-CTGTGAATGCTGCGACTACGATXXXXX$(T)_{15}$-3'
Forward primer: arbitrary ACP15: 5'-GTCTACCAGGCATTCGCTTCATXXXXXCCACCGTGTG-3'
Universial primers: 5' universal primer : 5'-GTCTACCAGGCATTCGCTTCAT-3'
3' universal primer : 5'-CTGTGAATGCTGCGACTACGAT-3'

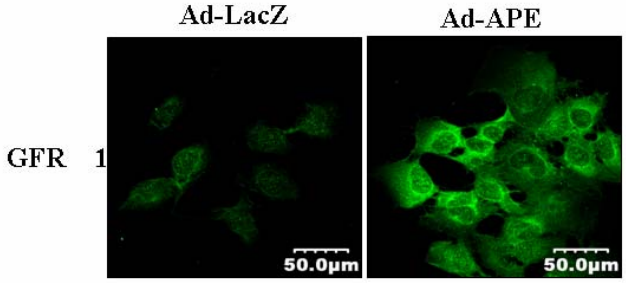
(NM_145793) Homo sapiens GDNF family receptor alpha 1 (GFRA1), transcript variant 2, mRNA (2211-2592) GFRα-1 –contained pGEM®-T easy vector sequence

T7promoter
GGGCGAATTG GGCCCGACGT CGCATGCTCC CGGCCGCCAT GGCGGCCGCG GGAATTCGATT
gtctaccaggcattcgcttcat tttccacgct gatgtttatg tactgtaaac agttctgcac tcttgtacaa
5' universal primer
aagaaaaaac cctgtcaca tccaaatata gtatctgtct tttcgtcaaa atagagagtg gggaatgagt gtgccgattc aatacctcaa tccctgaacg acactctcct aatcctaagc cttacctgag tgagaagccc tttacctaac aaaagtccaa tatagctgaa atgtcgctct aatactcttt acacatatga ggttatatgt agaaaaaaat tttactacta aatgatttca actattggct ttctatattt tgaaagtaat gatattgtct catttttta ctgatggttt aatacaaaat acacagagct tctttcccct ca atcgtagtcgcagcattcacag
3' universal primer
AATCACTAGTG AATTCGCGGC CGCCTGCAGG TCGACCATAT GGGAGAGCTC CCAACGCGTT GGATGCATAG CTTGAGTATT CTATAGTGTC ACCTAAATAG
SP6 promoter

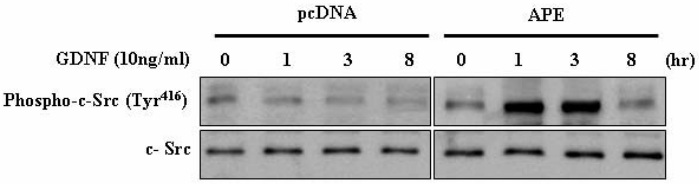
Figure 2 Identification of APE target gene (**a**) Differential display of APE-transfected and empty vector-transfected GM00637 cells using a set of an arbitary ACP15 (forward primer) and dT-ACP2 (reverse primer). The mRNA extracted from APE-transfected and empty vector-transfected GM00637 cells was used for the synthesis of first-strand cDNA using the dT-ACP1 primer. Second-strand cDNA was then amplified during second-stage PCR by using dT-ACP2 and arbitary ACP15 primer. Arrow indicates differential levels of mRNA expression between APE-transfected and empty vector-transfected cells.. The lower panel is a sequence of dT-ACP (cDNA synthesis primer), arbitary ACP15 and dT-ACP2. **(b**) This cDNA band was cloned and the sequence between 251 and 456 (red bold character) has complete homology with glial cell-derived neurotropic factor receptor α1 (GFRα1), whose size is 2.3kb


a
Ad- LacZ
Ad-APE
APE
GFR 1
GAPDH
b
Ad- LacZ
Ad-APE
APE
GFR 1
-tubulin
c
Ad-LacZ
Ad-APE
0
24
48
72
0
24
48
72
(hr)
APE
GFR 1
-tubulin

d


Ad-LacZ
Ad-APE
GFR 1
50.0μm
50.0μm

e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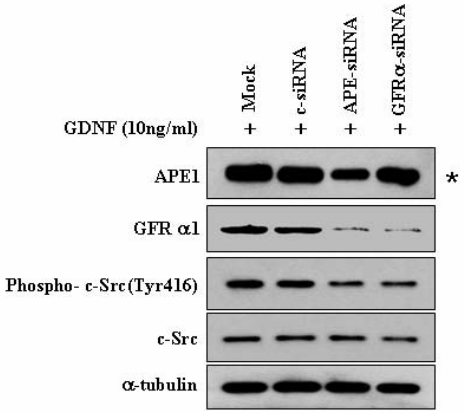
APE
GDNF (10ng/ml)
0 1 3 8 0 1 3 8 (hr)
Phospho-c-Src (Tyr416)
c- Src

f


Mock
c-siRNA
APE-siRNA
GFRα-siRNA
GDNF (10ng/ml)
+
+
+
+
APE1
*
GFR α1
Phospho- c-Src(Tyr416)
c-Src
α-tubulin

g

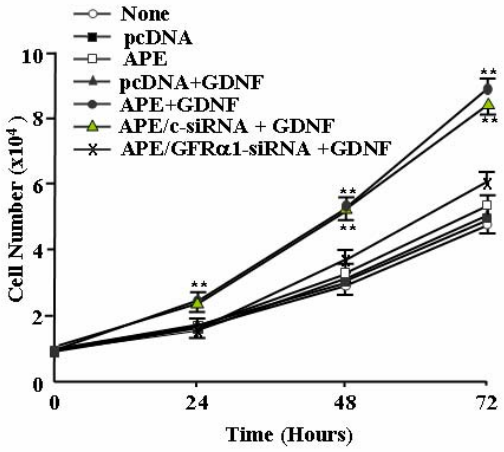

None
pcDNA
APE
pcDNA+GDNF
APE+GDNF
APE/c-siRNA + GDNF
APE/GFRα1-siRNA +GDNF
Cell Number (x10⁴)
Time (Hours)
0
2
4
6
8
10
0
24
48
72
**

Figure 3. APE-mediated increase in GFRα1 leads to GDNF responsiveness in GM00637 cells **(a)** GM00637 cells were transduced with Ad-LacZ or Ad-APE at MOI of 50, and the cells were harvested 48 h after the transduction. The total RNA was extracted and subjected to semiquantitative RT-PCR using the *APE, GFRα1* and *GAPDH*-specific primers on 26 cycles. (**b)** GM00637 cells were transduced with Ad-LacZ or Ad-APE. After 48 h, APE, GFRα1 and α-tubulin levels were analyzed by immunoblotting. (**c)** GM00637 cells were transduced with Ad-LacZ or Ad-APE. At the indicated times, cell extracts were prepared and examined for APE and GFRα1 content by immunoblotting. (**d)** GFRα1 protein expression in APE expression cells was confirmed by Immunofluorescence (**e)** The pcDNA3-and APE-expressing cells were incubated with GDNF (10ng/ml) for the indicated time points. Cell lysates were immunoblotting as indicated. (**f)** The APE-expressing cells were transfected with the mock, control-siRNA, APE-siRNA or GFRα1-siRNA. 48 h after transfection, cells were then incubated with GDNF (10ng/ml) for 1 hr. The total cell lysates were used for immunoblotting analysis as indicated. (**g)** The parent cells (none), pcDNA3- and APE-expressing cells were transfected with control-siRNA or GFRα1-siRNA and then incubated with or without GDNF (10 ng/ml) for up to 72 hours. The number of cells was determined by counting the cells every 24 h after GDNF treatment. Each value is a mean ± SD from three separate experiments. Double asterisks indicate $p < 0.01$.

Promoter analysis of GFRα1 genes

To identify the regulatory elements of GFRα1 gene which can be activated in APE expressing GM00637 cells, we made reporter constructs composed of the 2.2-kb GFRα1 promoter (-2291~-66), 0.96-kb GFRα1 promoter (-2291~-1327) and 0.5-kb GFRα1 promoter (-575~-66) fused to a luciferase reporter gene (Fig. 4a). The full-length promoter construct, 2.2-kb GFRα1 promoter and two truncation constructs 0.96-kb and 0.5-kb GFRα1 promoter were transfected into parent, pcDNA3- and APE-transfected GM00637 cells. Luciferase results indicated that truncated constructs exhibited similar transcriptional activity as the full-length promoter construct in parent and pcDNA3-transfected cells (Fig. 4b). However, the 2.2-kb GFRα1 promoter construct consistently displayed an approximately 3.1-fold increase in luciferase activity after transfection into APE expressing cells as did truncated promoter construct 0.5-kb GFRα1 promoter. In contrast 0.96-kb GFRα1 promoter construct, showed no significant increase in promoter activity after transfection, indicating that the 575 bp upstream sequences from the translational start site of GFRα1 gene is sufficient for inducible expression by APE.

By examining the 0.5-kb 5′ flanking region of the human GFRα1 gene, we have found three putative NF-κB binding sites within -349 to -335, -300 to -287 and -155 to -143, denoted NF1, NF2 and NF3, respectively (Fig. 5a). To determine whether these putative NF-κB binding motifs indeed participate in the APE-mediated induction of GFRα1, we performed several assays in human fibroblast GM00637 cells. In reporter assay using three mutant constructs of 0.5-kb GFRα1 promoter, NF1-mutated construct (0.5-kb GFRα1 mtNF1) showed a striking decrease in APE-induced promoter activities but not NF2- or NF3-mutated construct (0.5-kb GFRα1 mtNF2 or 0.5-kb GFRα1 mtNF3, respectively) (Fig. 5b),

suggesting that the presence of putative NF-κB binding sites NF1 is important for the APE-mediated activation of the GFRα1 promoter in GM00637 cells.

We used an electrophoretic mobility-shift assays (EMSA) to investigate the possibility that APE expression contributes to the enhancement of binding of NF-κB to DNA, the NF-κB binding activity in GM00637 cells following APE transfection was analyzed using the oligonucleotides corresponding to the consensus NF-κB binding motif. The DNA binding ability of NF-κB in APE expressed cells was significantly increased and this band was efficiently removed with an excess of the unlabeled NF-κB oligonucleotides (Fig. 5c). To identify the members of the NF-κB family of transcription factors involved in the NF-κB binding activity observed in APE expressed cells, we selected antibodies specific for different members of the NF-κB family for their ability to interfere with DNA binding activity. The p65 antibody totally inhibited the formation of the NF-κB-DNA binding complex in APE expressed cells and resulted in a supershift, whereas the other members of NF-κB family (p50, p52, c-Rel and RelB) did not affect NF-κB-DNA binding activity at all. To determine whether the NF-κB protein binds to the GFRα1 promoter and to determine whether APE contributes to the NF-κB binding activity of the GFRα1 promoter, the NF-κB binding activity in the APE transfected GM00637 cells was analyzed by EMSA employing the oligonucleotides corresponding to three putative NF-κB binding sites (NF1, NF2 and NF3) of GFRα1 promoter. As shown in Fig. 5d&e, APE transfected cells exhibited significantly elevated protein-NF1 binding activity. These protein-NF1 complexes could be complete out by a molar excess of the unlabeled consensus NF-κB oligonucleotides and supershifted DNA-protein complexes were observed after adding the anti-p65 antibodies. In contrast, APE expression did not affect the proteion-NF2 &3 binding activity.

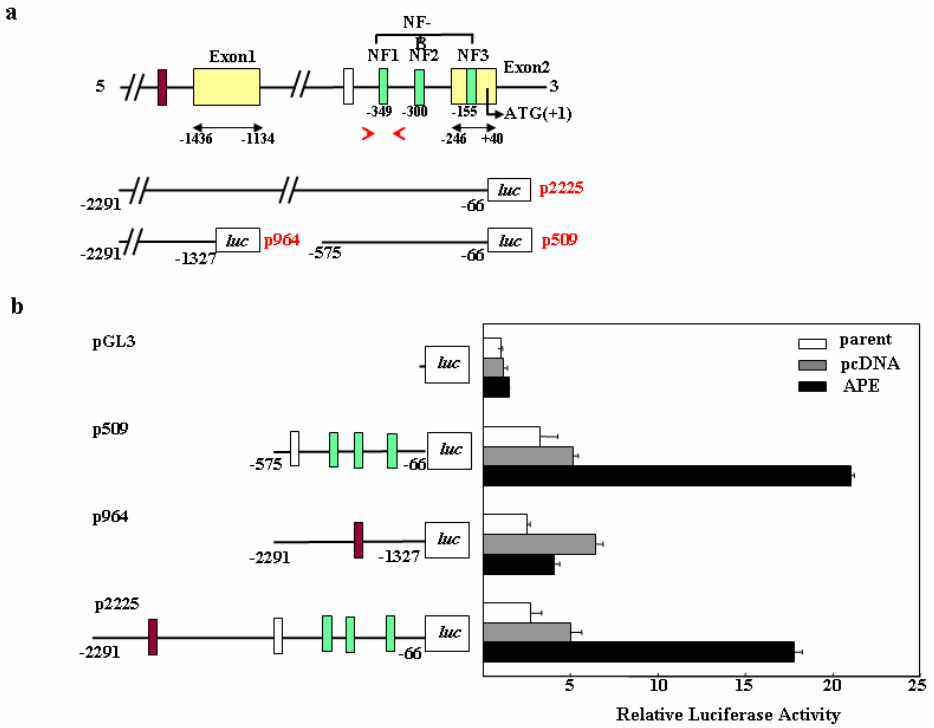
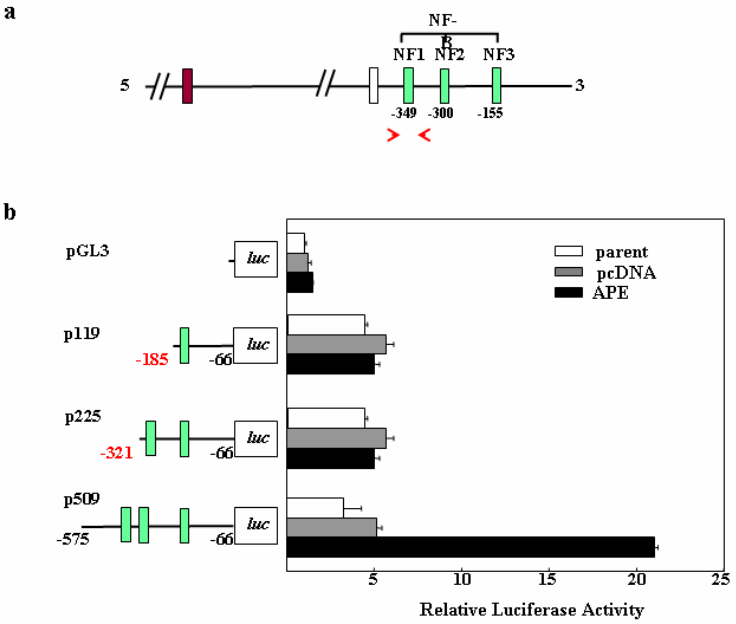
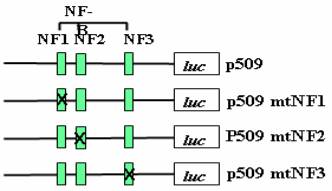
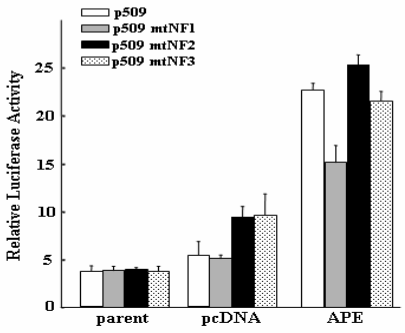

a
NF-κB
Exon1
NF1
NF2
NF3
Exon2
5
3
-349
-300
-155
ATG(+1)
-1436
-1134
-246
+40
-2291
luc
p2225
-66
-2291
luc
p964
-1327
-575
-66
luc
p509
b
pGL3
luc
p509
-575
-66
luc
p964
-2291
-1327
luc
p2225
-2291
-66
luc
parent
pcDNA
APE
5
10
15
20
25
Relative Luciferase Activity

Figure 4 GFRα1 promoter analysis (**a**) The upper panel shows schematic representation of the human GFRα1 promoter region. The putative three NF-κB binding sites (NF1, NF2 and NF3), the GAGA box, the GC box, Exon1 and 2 are shown in greens, red, whites and yellows, respectively. Three putative NF-κB binding sites within -349 to -335, -300 to -287 and -155 to -143, denoted NF1, NF2 and NF3, respectively. The middle and lower panel shows the GFRα1 promoter region of the promoter-reporter construct, p2225 (-2291 ~ -66), p964 (-2291 ~ -1327) and p509 (-575 ~ -66). (**b**) The parent cells, pcDNA3- and APE-expressing cells were transfected with the indicated plasmids. pGL3, promoterless luciferase vector; p2225, promoter-reporter construct containing position -66 to -2291 of the GFRα1 promoter; p964, promoter-reporter construct containing position -1327 to -2291 of the p2225; p509, promoter-reporter construct containing position -66 to -575 of p2291. The cells were harvested 24 hours after transfection, and a luciferase assay was performed. In order to determine the transfection efficiency, the β-galactosidase expression vector was co-transfected with each of the plasmids tested, and the β-galactosidase activity level was detected as the internal control of transfection. Diagram representations of the promoters are shown. The values are reported as a mean ± s.d. from six separate experiments. ** denotes $p<0.01$.

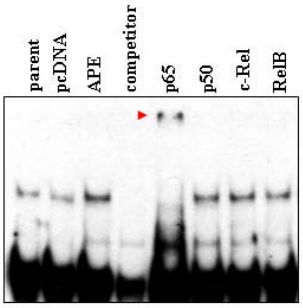

a
NF-κB
NF1
NF2
NF3
5
3
-349
-300
-155
b
pGL3
p119
-185
-66
p225
-321
-66
p509
-575
-66
luc
parent
pcDNA
APE
5
10
15
20
25
Relative Luciferase Activity

c


NF-
B
NF1 NF2 NF3
luc
p509
luc
p509 mtNF1
luc
P509 mtNF2
luc
p509 mtNF3

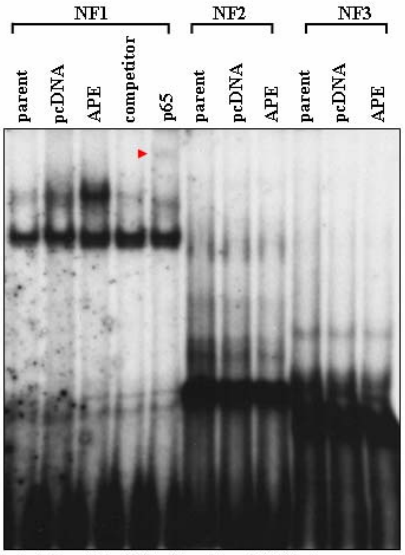

p509
p509 mtNF1
p509 mtNF2
p509 mtNF3
Relative Luciferase Activity
0
5
10
15
20
25
parent
pcDNA
APE

d



NF- B consensus oligonucleotides

e



Putative NF- B binding sites of GFR 1 promoter

Figure 5. APE enhances GFRα1 promoter activity by enhancing p65 NF-κB activation. (**a)** The upper panel shows schematic representation of the human GFRα1 promoter region. The putative three NF-κB binding sites (NF1, NF2 and NF3), the GAGA box and the GC box are shown in greens, red and whites, respectively. (**b**) The parent cells, pcDNA3- and APE-expressing cells were transfected with the indicated plasmids. pGL3, promoterless luciferase vector; p509, promoter-reporter construct containing position -66 to -575 of the GFRα1 promoter; p225, promoter-reporter construct containing position -66 to -321 of p509; p185, promoter-reporter construct containing position -66 to -185 of p509. Diagram representations of the promoters are shown. The values are reported as a mean ± s.d. from six separate experiments. ** denotes $p<0.01$. (**c)** The parent cells, pcDNA3- and APE-expressing cells were transfected with p509, p509 mtNF1, p509 mtNF2 or p509 mtNF3, respectively. Diagram representation of the mutated NF1, NF2 and NF3 are shown. The cells were harvested 24 hours after transfection, and a luciferase assay was performed. **(d)** In an electrophoretic mobility-shift assay (EMSA), 22 bp of biotin-labeled oligonuleotide probes containing the consensus NF-κB binding sequence were incubated with the nuclear extracts isolated from parent cells, pcDNA3- and APE-transfected cells. The unlabeled oligonucleotide was used as a competitor. For supershift assays, anti-p65, anti-p50, anti-p52, anti-c-Rel and anti-RelB antibodies were added to the reaction mixtures and incubated for 30 min prior to separating the DNA-protein complexes. The DNA-protein complexes were run on 6% nondenatured polyacrylamide gels and detected by Chemiluminescent Nucleic Acid Detection Module (Pierce). (**e)** The radiolabeled or unlabeled oligonucleotides spanning the individual putative NF-κB binding sites on the GFRα1 promoter region (NF1, NF2 and NF3) were used as probes or a competitor. For the supershift, anti-p65 antibody was used. Triangles indicate antibody supershifted complexes (**f)** The chromatins from parent, pcDNA3- and

APE-expressing GM00637 cells were fixed with formaldehyde and fragmented by sonication. Fragmented chromatins were immunoprecipitated using anti-p65 antibody and goat anti-rabbit IgG and then analyzed by polymerase chain reaction with primer sets covering GFRα1 promoter region. The positions of the ChIP primers are delineated in an upper panel by arrowheads. The primers for the 5′ and 3′ far distal regions were used as control.

APE-mediated GFRα1 expression contributes in the tumor progression

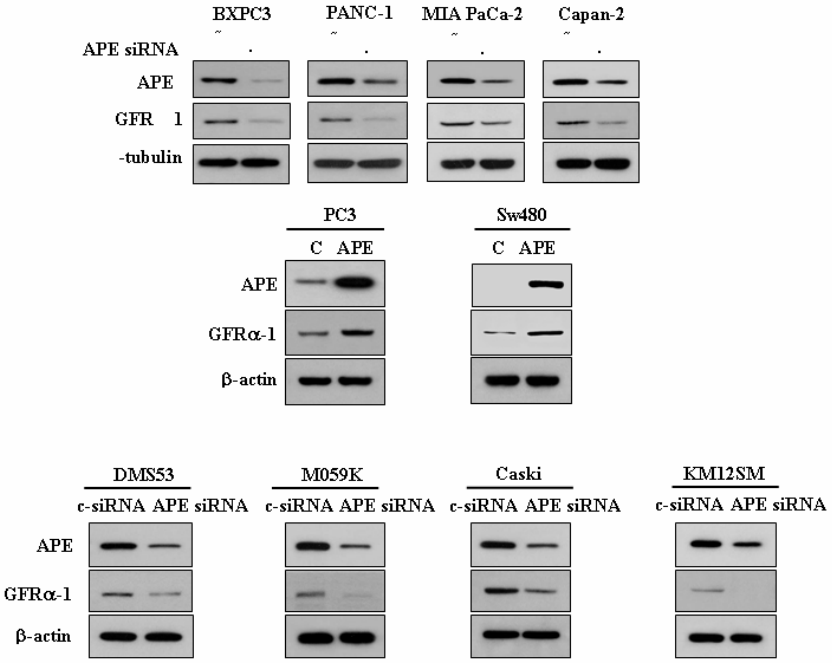
Many malignant cancer cell lines express GFRα, which is involved in tumor cell progression. Thus, we next asked whether this expression could be attributed to APE. Using immunoblotting, APE together with GFRα1 were detected at similar levels on human BXPC3, Capan-2, PANC-1 and MIA PaCa-2 pancreas and DLD1 colon and DU145 prostate cancer cell lines, whereas SW480 colon and PC3 prostate cancer cell lines expressed marginal APE level and no detectable GFRα1 (Fig. 6a). To test the effect of APE on the GFRα1 expression, APE-specific siRNA was transfected into four pancreas cancer cell lines, DLD1 colon and DU145 prostate cancer cell lines. Western blot analysis revealed that the APE-specific siRNA oligonucleotides decreased expression of APE by more than 80% as well as significantly suppressed the levels of GFRα1 expression when compared with the control siRNA transfected cells. Immunofluorescence staining using APE and GFRα1 antibody also showed that APE- siRNA transfection led to suppression of GFRα1 expression in four pancreas cancer cell lines (Fig. 6b). In addition, the GFRα1 expression levels in the two individual APE-expression vector transfected SW480 and PC3 cells were significantly higher than those of the empty vector-transfected cells (Fig. 6a). Thus expression of APE is responsible for GFRα1 expression in human cancer cells.

We chose BXPC3 and MIA PaCa-2 cells to test the effects of APE-mediated GFRα1 expression on tumor cell growth and invasion, as those two cells lines highly expressed APE and GFRα1, and GDNF increases proliferation and invasion of those two cell lines. We first examined the effect of GDNF on the proliferation of BXPC3 and MIA PaCa-2 cells. These cells treated with GDNF showed a rapid increase cell proliferation. However, cells transfected with either APE siRNA or GFRα1 siRNA showed significant suppression of GDNF-induced cell proliferation

(Fig. 6c). To show that the effects of the APE siRNA on BXPC3 and MIA PaCa-2 cell proliferation were due to GFRα1 suppression, these cells were transduced by a lentivirus endcoding the GFRα1. Before transfection with APE siRNA, BXPC3 and MIA PaCa-3 cells were transduced by GFRα1 lentiviral vector. Cells transduced by the GFRα1 lentivirus did not undergo decreased proliferation in response of GDNF seen with the APE siRNA (Fig. 6d).

We next determined whether APE-mediated increase in GFRα1 modulates BXPC3 and MIA PaCa-3 pancreatic cancer cell invasion using the Matrigel double-chamber assay. It was clear that GDNF stimulated the in vitro invasive activity in control siRNA-transfected BXPC3 and MIA PaCa-2 cells, but not in the GFRα1 siRNA-transfected cells (Fig. 6e). Moreover, GDNF-induced in vitro invasive activity was inhibited by APE siRNA in BXPC3 and MIA PaCa-2 cells. To confirm whether the GDNF effect was specifically APE-mediated GFRα1 expression in these cells, BXPC3 and MIA PaCa-2 cells were transduced by a lentivirus endcoding the GFRα1 and then transfected by APE siRNA. Transduction of APE siRNA transfectants to GFRα1 lentivirus resulted in an increase in GDNF-induced invasion (Fig. 6f). These results suggest that APE-mediated GFRα1 expression is involved in the GDNF-induced cancer cell proliferation and invasion.

a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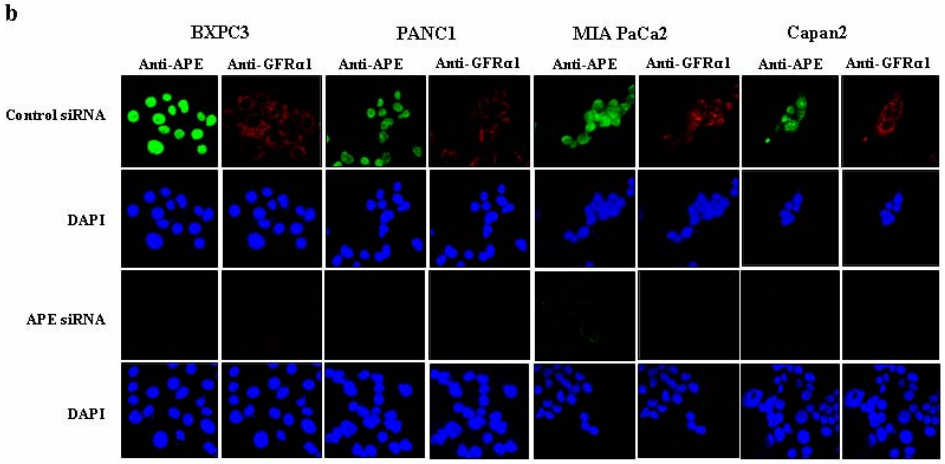
MIA PaCa-2
Capan-2
APE siRNA
APE
GFR 1
-tubulin
PC3
Sw480
C APE
C APE
APE
GFRα-1
β-actin
DMS53
M059K
Caski
KM12SM
c-siRNA APE siRNA
c-siRNA APE siRNA
c-siRNA APE siRNA
c-siRNA APE siRNA
APE
GFRα-1
β-actin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PANC1
MIA PaCa2
Capan2
Anti-APE
Anti-GFRα1
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Anti-GFRα1
Anti-APE
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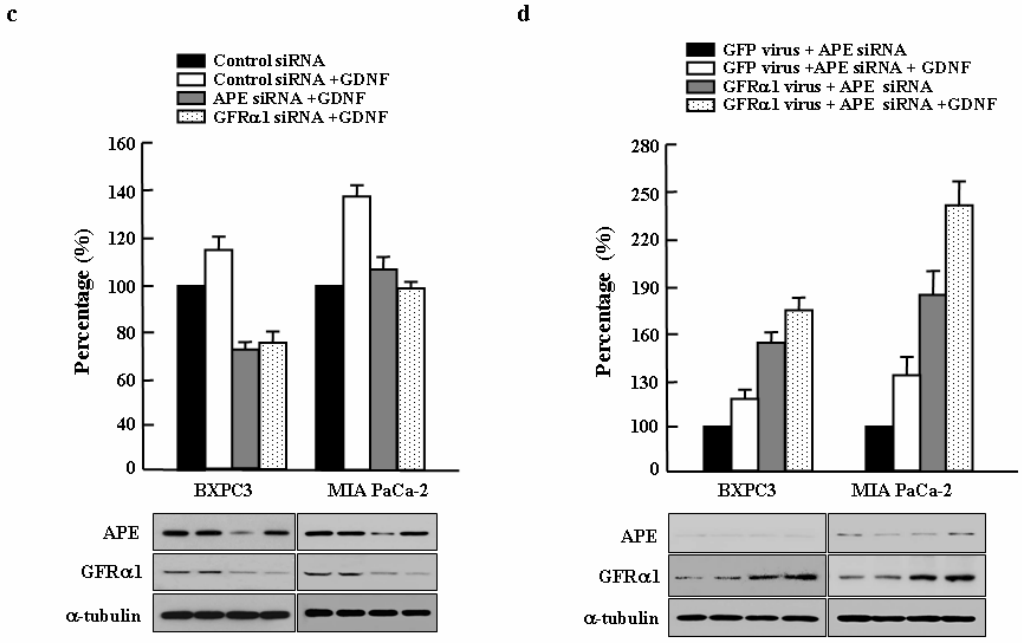
Anti-APE
Anti-GFRα1
Control siRNA
DAPI
APE siRNA
DAPI


c
Control siRNA
Control siRNA +GDNF
APE siRNA +GDNF
GFRα1 siRNA +GDNF
Percentage (%)
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140
120
100
80
60
40
0
BXPC3
MIA PaCa-2
APE
GFRα1
α-tubulin
d
GFP virus + APE siRNA
GFP virus +APE siRNA + GDNF
GFRα1 virus + APE siRNA
GFRα1 virus + APE siRNA +GDNF
Percentage (%)
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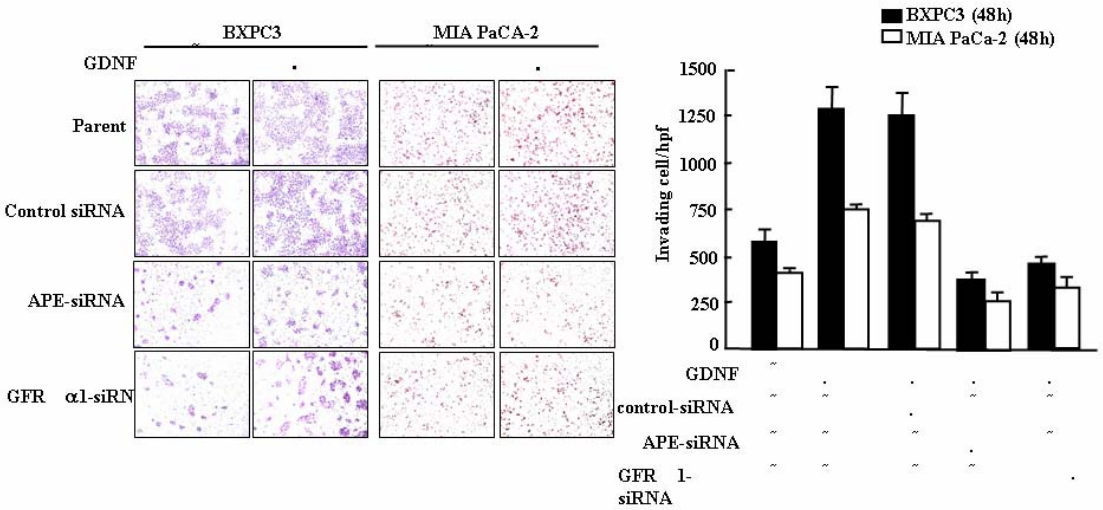
BXPC3
MIA PaCa-2
APE
GFRα1
α-tubulin

e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MIA PaCA-2
GDNF
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Control siRNA
APE-siRNA
GFR α1-siRN
BXPC3 (48h)
MIA PaCa-2 (48h)
Invading cell/hpf
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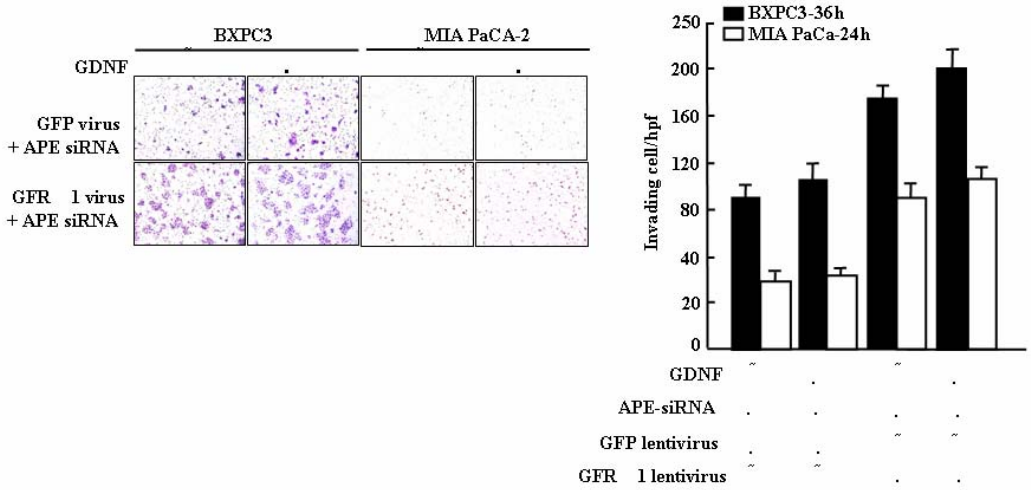
1250
1000
750
500
250
0
GDNF
control-siRNA
APE-siRNA
GFR 1-
siRNA

f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MIA PaCA-2
GDNF
GFP virus
+ APE siRNA
GFR 1 virus
+ APE siRNA
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MIA PaCa-24h
Invading cell/hpf
250
200
160
120
80
40
20
0
GDNF
APE-siRNA
GFP lentivirus
GFR 1 lentivirus

Figure 6. APE regulates GDNF-mediated proliferation and invasion through GFRα1 expression in human pancreas cancer cells. **(a)** Effect of APE siRNA on the GFRα1 expression. Four pancreas cancer cells were transfected with control siRNA or APE-siRNA. Cell lysates were immunoblotting as indicated. (**b)** APE and GFRα1 protein expression in APE siRNA-transfected cells was confirmed by immunofluorescence. (**c**) APE siRNA and GFRα1 siRNA inhibited GDNF-induced increase in BXPC3 and MIA PaCa-2 proliferation. Lower, expression levels of APE and GFRα1 as visualized by immunoblotting. (**d**) APE siRNA-transfected BXPC3 and MIA PaCa-2 cells following tranduction by a GFRα1-expressing lentivirus rescued GDNF-induced proliferation. Cells were transduced with either GFRα1 lentivirus or control GFP lentivirus at MOI of 50, then transfected with APE siRNA. After transfection, cells were treated for 24 hours with or without GDNF. Lower, expression levels of APE and GFRα1 as visualized by western analysis. **(e)** Photomicrographs show the effect of APE siRNA and GFRα1 siRNA on GDNF-induced increase in BXPC3 and MIA PaCa-2 invasion. The histograms show the average number of invading cells. (**f**) Photomicrographs show the influence of GFRα1 lentivirus on GDNF-induced invasion of APE siRNA-transfected BXPC3 and MIA PaCa-2 cells. The histograms show the ability of GFRα1 lentivirus to rescue the GDNF-induced invasion of BXPC3 and MIA PaCa-2 cells expressing APE siRNA. We repeated the experiments in **c, d, e and f** three times, each in duplicate. Data are mean ± s.d.

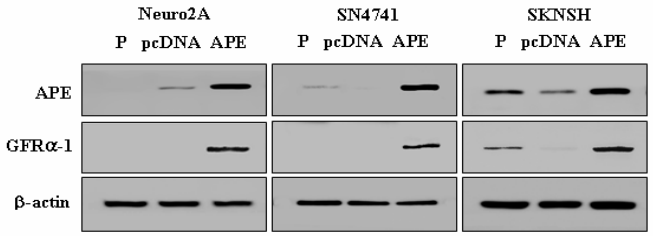
APE triggers GFRα1 responsiveness in neuronal cells

To determine whether APE can induces GFRα1 expression in neuronal cells, APE expression vector was stably transfected into Neuro2a, SN4741 and SKNSH cells. Western blot analysis revealed that the GFRα1 expression levels in these APE stable cells were significantly higher than those of the parent and empty vector (pcDNA3)-transfected cells (Fig. 7a). GFRα1 induction was observed 48 h after APE expression in Neuro2A cells (Fig. 7b). Immunofluorescence staining using the same antibody also confirmed the expression of endogenous GFRα1 in APE-transfected Neuro2A cells but not in pcDNA-transfected cells (Fig. 7c).

To investigate the physiological role of APE-mediated GFRα1 expression in neurons, we chose Neuro2a cells, because Neuro2a cells express endogenous Ret but not GFRα1. It has been reported that GFRα1 binds to Ret in response to GDNF in neurons, leading to phosphorylation of Ret tyrosine kinase, which subsequently associated with and activates cytoplasmic Src family tyrosine kinase. To investigate whether this is the case in APE-expressing Neuro2A cells, immunoprecipitation using a Ret antibody was performed, followed by immunoblotting with phosphotyrosine and phospho-Src (p60). There was increase in Ret phosphorylation and detectable association between Ret and phospho-Src in GDNF-stimulated, APE-expressing cells but not parent and pcDNA3 transfected cells (Fig. 7d). We further investigate the activation of several well studied Ret receptor tyrosine kinase signaling pathways that are involved in cell survival, proliferation and differentiation. A time-course study indicated that stimulation of APE-expressing Neuro2A cells with GDNF caused rapid and transient phosphorylation of p44/p42 ERK and phosphorylation of Akt (Fig. 7e). GDNF also led to PLCγ phosphorylation from 5 min to 8 hr of stimulation in APE-expressing Neuro2A cells. No obvious increase in phosphorylation was observed in pcDNA3-

transfected cells after GDNF stimulation. To ensure that the APE-mediated induction of GFRα1 expression is contributed to the activation of the Ret receptor tyrosine kinase signaling pathways by GDNF, GFRα1-siRNA was transfected into APE-expressing Neuro2A cells and then measured the GDNF-induced ERK, Akt and PLCγ phosphorylation. As shown in Figure 7f, GFRα1-siRNA transfection markedly diminished concomitantly GDNF-induced ERK, Akt and PLCγ1 phosphorylation.

a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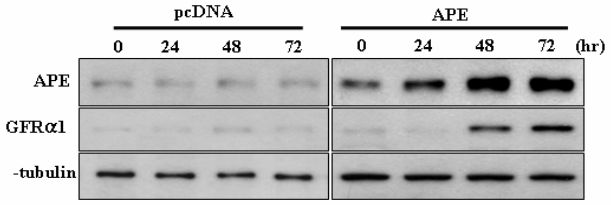
SN4741
SKNSH
P pcDNA APE
P pcDNA APE
P pcDNA APE
APE
GFRα-1
β-actin

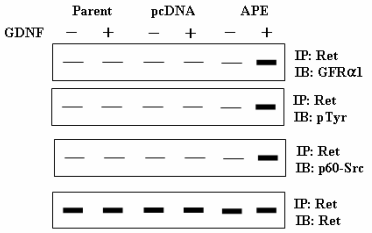
b


pcDNA
APE
0 24 48 72
0 24 48 72 (hr)
APE
GFRα1
-tubulin

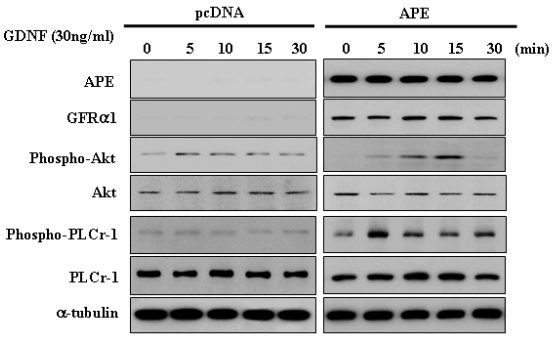
c


pcDNA
APE
GFRα1

d


Parent
pcDNA
APE
GDNF
− + − + − +
IP: Ret
IB: GFRα1
IP: Ret
IB: pTyr
IP: Ret
IB: p60-Src
IP: Ret
IB: Ret

e


pcDNA
APE
GDNF (30ng/ml)
0 5 10 15 30 0 5 10 15 30 (min)
APE
GFRα1
Phospho-Akt
Akt
Phospho-PLCr-1
PLCr-1
α-tubulin

f

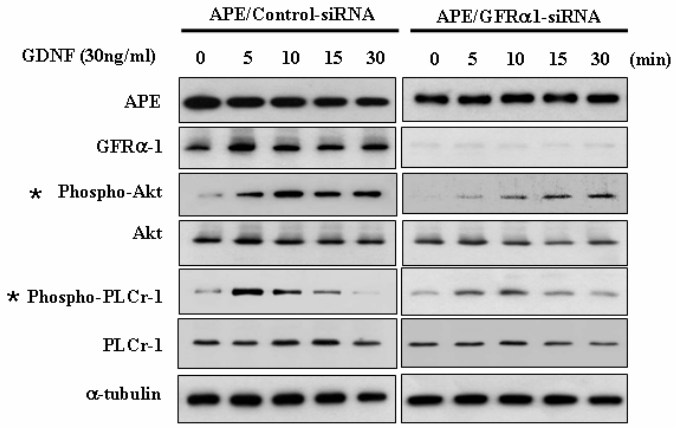

APE/Control-siRNA
APE/GFRα1-siRNA
GDNF (30ng/ml)
0 5 10 15 30 0 5 10 15 30 (min)
APE
GFRα-1
* Phospho-Akt
Akt
* Phospho-PLCr-1
PLCr-1
α-tubulin

Figure 7. Activation of the GFRα1/Ret signaling pathway in Neuro2a/APE cells in response to GDNF **(a)** Neuro 2A, SN4741, SKNSH and PC12 cells were transfected with empty vector (pcDNA) or APE expression vector (APE). Cell lysates were immunoblotting as indicated. **(b)** Neuro2a cells were transfected with empty vector (pcDNA) or APE expression vector (APE). At the indicated times, cell extracts were prepared and examined for APE and GFRα1 content by immunoblotting. (**c)** Neuro2a/APE and vector/Neuro2a cells were immunostained using anti-GFRα1 polyclonal antibodies tagged with Alexa fluor (red color). **(d)** Neuro2a/APE and vector/Neuro2a cells were treated with or without GDNF (10ng/ml) for 1 hr. The cells were lysed and subjected to immunoprecipitation(IP) with antibody to Ret and then analyzed for phosphotyrosin (upper panel) and phosphor-Src (middle panel) by immunoblotting. The bottom panel shows a reprobing of the same filter with Ret antibody. **(e)** Neuro2a/APE and vector/Neuro2a cells were treated with GDNF (10ng/ml). At the indicated times cell extracts were prepared and immunoblotting as indicated.(**f)** Neuro2a/APE and vector/Neuro2a cells were transfected with the control-siRNA, APE-siRNA or GFRα1-siRNA and incubated with GDNF (10ng/ml) for the indicated times. Total cell lysates were immunoblotting as indicated.

APE-induced GFRα1 expression leads to neuronal cell survival

Next, we observed neurite outgrowth using confocal microscopy with fluorescence dye, and found that the expression of APE did not lead to an obvious change in the extension of neurite, but did seem to increase the amount of branching (Fig. 8a). Some of the APE-expressing Neuro2A cells had three or more branches, whereas this was rarely seen in the controls. To investigate whether APE influences GDNF-induced neurite outgrowth, GDNF was applied at a concentration of 50 ng/ml to the control and APE-transfectant cultures for 48 hours. In the APE-expressing Neuro2A cells which were treated with GDNF, the length of the neurite averaged 37±11 μm (n=200) after 48h, whereas the control neurons which were treated with GDNF averaged 11±6 μm (n=190) showing an increase of 70% (Fig. 8a,b&c). Approximately 65% of the GDNF-treated APE-transfected Neuro2A cells had three or more branches compared with 11% for the GDNF-treated control, and 8% had five or more branches compared with 1% for control Neuro2A cells. Thus the increase in length and amount of branching induced by GDNF was greater in APE-transfectant than the control. We next examined the effect of siRNA-mediated knockdown of GFRα1 expression on neurite outgrowth from APE-transfected Neuro2A cells following GDNF stimulation for 48 hours. Compared with control siRNA-transfected cells, GFRα1 siRNA-transfected cells showed short neuritis in response to GDNF (Figure 8 a,b&c). These results suggested that APE-mediated induction of GFRα1 expression led to GDNF-induced neurite outgrowth.

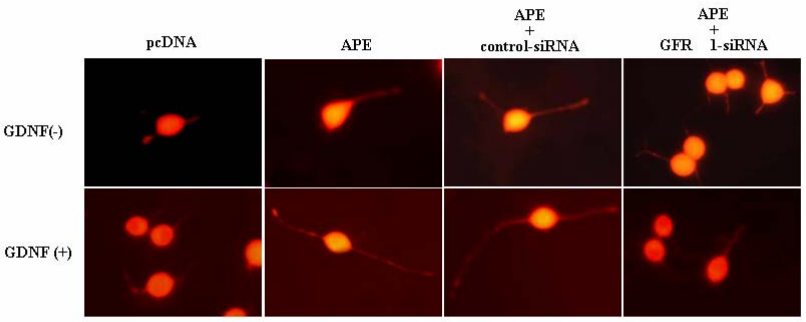
We next studied the effect of GDNF on the localization of the GFRα1 in the plasma membrane of APE-expressing Neuro2A cells. Control and APE-expressing Neuro2A cells were treated for 3 h with GDNF or left untreated; subsequently, the medium was removed and the cells were incubated with an anti-GFRα1 antibody

on ice before fixation to stain for the receptor at the cell surface of the living cells. Control siRNA-transfected Neuro2a cells display no GFRα1 imunostaining, whereas Neuro2a/APE cells reveal staining of clustered receptors (Fig. 8d). This is in accordance with the finding that in these APE stable transfectants the GFRα1 protein predominantly located in lipid rafts at the plasma membrane. The localization of GFRα1 in lipid rafts can be visualized by double labeling of the cells with the anti- GFRα1 antibody and FITC-coupled cholera toxin B which specifically binds tot eh lipid raft marker GM1, a cell surface ganglioside. In Neuro2a/ APE cells, GFRα1 is higly colocalized with GM1 (Fig. 8d)

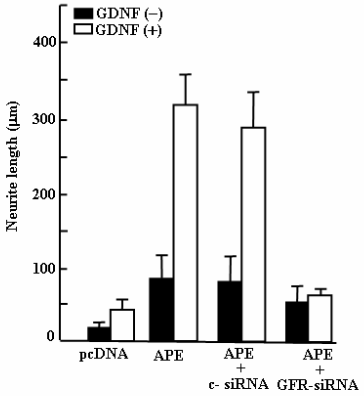
Finally we examined whether APE-mediated GFRα1 expression is contributed to the neuronal survival. The ability of the APE to stimulate cell proliferation in neuro2A cells was first studied. Vector/Neruo2a control and Neuro2a/APE cells were cultured for 36 hr with or without GDNF, and cell numbers were measured. During these culture conditions, there is little or no cell death, and cell numbers should therefore reflect cell proliferation. Neuro2a /APE cells treated with GDNF showed a more rapid increase in the number of cells than the parent and pcDNA3-transfected cells treated with GDNF. In addition, the transfection of GFRα1-siRNA significantly reduces the level of cell proliferation in APE expressing Neuro2a cells in response to GDNF compared with the control-siRNA transfection (Fig. 8e). We next examined the effect of APE overexpression on the susceptibility of cells to several kinds of stressors. Treatment with10 μM of β-amyloid precursor protein for 24 h resulted in severe cellular damage in vector/Neuro2a control cells with GDNF (Fig. 8f); most cells were rounded up and detached from the plate. In contrast, the same treatment caused only a little damage to cells overexpressing APE in the presence of GDNF, indicating that the expression of APE rendered Neuro2a cells more tolerant to β-amyloid toxicity. To determine the specific role of GFRα1 for the survival effects of APE, siRNA against GFRα1 was used. In the cell survival

assay using GFRα1 siRNA-transfected Nuero2A/APE cells, siRNA against GFRα1 abolished the GDNF-mediated survival at β-amyloid. Similar results were obtained in experiments where cell death was induced by starvation (Fig. 8g).

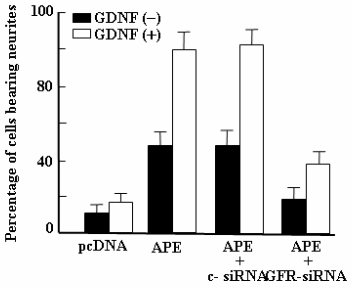
a


pcDNA
APE
APE
+
control-siRNA
APE
+
GFR 1-siRNA
GDNF(-)
GDNF (+)

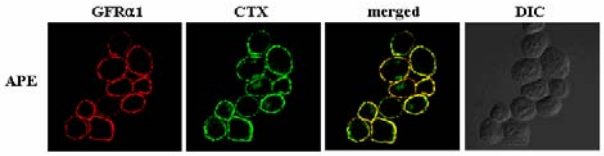
b


GDNF (–)
GDNF (+)
Neurite length (μm)
400
300
200
100
0
pcDNA
APE
APE
+
c- siRNA
APE
+
GFR-siRNA

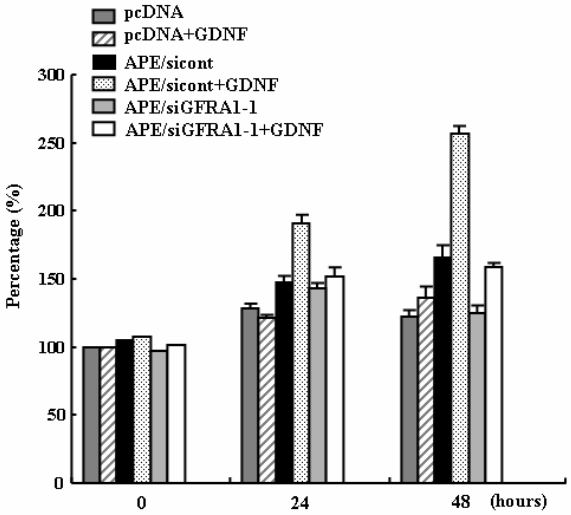
c


GDNF (–)
GDNF (+)
Percentage of cells bearing neurites
100
80
40
0
pcDNA
APE
APE
+
c- siRNA
APE
+
GFR-siRNA

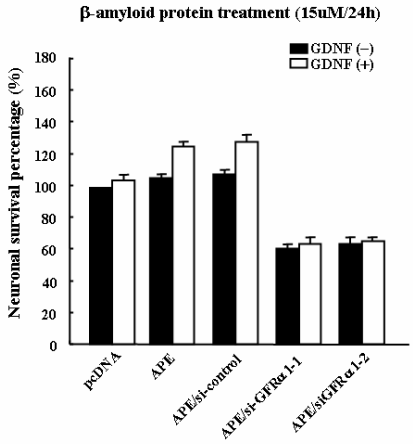
d


GFRα1
CTX
merged
DIC
APE

e


pcDNA
pcDNA+GDNF
APE/sicont
APE/sicont+GDNF
APE/siGFRA1-1
APE/siGFRA1-1+GDNF
Percentage (%)
300
250
200
150
100
50
0
0
24
48
(hours)

f


β-amyloid protein treatment (15uM/24h)
GDNF (–)
GDNF (+)
Neuronal survival percentage (%)
180
160
140
120
100
80
60
40
20
0
pcDNA
APE
APE/si-control
APE/si-GFRα1-1
APE/sIGFRα1-2

g

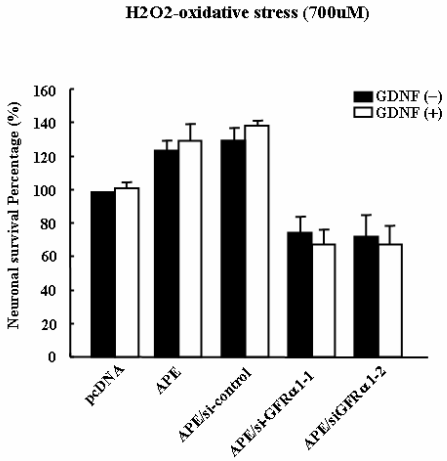

H2O2-oxidative stress (700uM)
GDNF (–)
GDNF (+)
Neuronal survival Percentage (%)
160
140
120
100
80
60
40
20
0
pcDNA
APE
APE/si-control
APE/si-GFRα1-1
APE/sIGFRα1-2

Figure 8. APE expression increases GDNF-induced survival and differentiation of Neuro2a cells. **a)** Neuro2a/APE and vector/Neuro2a cells transfected with control-siRNA or GFRα1-siRNA were serum-starved for h and then incubated with or without GDNF for 3 days and visualized by microscopy. The pcDNA- and APE-expressing Neuro 2A cells were also analyzed. (**b-c**) Mean of the longest neurite (b) and of the neurite baring cells (c) was determined for each culture from measurements of 100 neurons for three different experiments. Each data point represents the mean ± s.e.m. (** $p < 0.01$). (**d)** Correlation between APE overexpression and GFRα1 upregulation in pancreas cancer. Archival pancreas-cancer tissue specimens from 6 patients were analyzed for APE and GFRα1 expression by immunohistochemistry. The three rows represent three tumors. Magnification, x40. (**e**) Neuro2a/APE and vector/Neuro2a cells were transfected with control-siRNA or GFRα1-siRNA and then incubated with or without GDNF (10 ng/ml) for up to 72 hours. The number of cells was determined by counting the cells every 24 h after GDNF treatment. Each value is a mean ± SD from three separate experiments. Double asterisks indicate $p < 0.01$. (**f-g)** Neuro2a/APE and vector/Neuro2a cells were cultured under the serum-free condition or cultured under normal serum media with with β-amyloid precursor peptides (f) or hydrogen peroxide (g) in the prescence or absence of GDNF (10 ng/ml) for 48 hr, cell survival was assessed as described in Methods. Each value is a mean ± SD from three separate experiments. The asterisk indicates significantly different from the GFRα1-siRNA + GDNF at $p < 0.01$.

IV. DISCUSSION

Ad-APE (adenovirus encoding a human APE)-infected GM00637 human fibroblast cells were used in this study to examine the effect of APE on gene expression. The results showed that APE mediates the increase in the *GFRα1* mRNA, GFRα1 promoter activity and GFRα1 protein levels, which is a key receptor for the glial cell-derived neurotropic factor (GDNF) family. It was further shown that c-Src, a downstream target of GFRα1, is functionally activated by GDNF in APE expressing cells, as determined by its phosphorylation. Moreover, it was found that GDNF could stimulate cell proliferation in the APE expressing cells, as measured by counting the number of cells. APE specific RNA experiments demonstrated that the downregulation of APE by siRNA caused a marked reduction in the GFRα1 expression level, as well as the diminished ability of GDNF to phosphorylate c-Src (Tyr418) and to stimulate cell proliferation. Furthermore, the suppression of GFRα1 by siRNA in APE expressing cells leads to a decrease in the GDNF-induced phosphorylation of c-Src (Tyr418) and cell proliferation. These findings suggest that GFRα1 is a direct target of APE.

The GDNF was originally characterized as a potent neurotropic factor specific for the survival and differentiation of the midbrain dopaminergic neurons (35). Subsequently, the biological effects of GDNF on the uterine branching in kidney morphogenesis, spermatogenesis, and survival as well as the differentiation of several other neuronal populations have considerably extended the range of activities of this polypeptide (36-38). Currently, four GFRα proteins, GFRα1, 2, 3, and 4 have been identified. GFRα1 mainly binds GNDF, and GFRα2, 3, and 4 bind neurturin (NTN), artemin (ART), and persephin (PSP), respectively, which are the GDNF family of growth factors (39-43). The GDNF protein signals through a

multi-component receptor complex, which consists of a glycosyl-phosphatidylinositol (GPI) binding subunit, which is known as the GDNF family receptor α (GFRα), and the transmembrane receptor tyrosine kinase (Ret) (27-29). This study demonstrated the functional involvement of APE in the GDNF/GFRα signal pathway. The induction of GFRα1 correlated with the initiation of signaling downstream of the GDNF in the APE expressing cells. Src was phosphorylated by GDNF in the APE-expressing cells. This agrees with a recent report showing that GDNF triggers Src-family kinase activation through GFRα1 independently of Ret (32-34). This suggests that APE can trigger the GDNF/GFRα signal pathway indicating that APE plays a role in cell survival and proliferation, as well as in normal development by modulating the GDNF/GFRα signal pathway.

GDNF-mediated activation of the GFRα/Ret system induces the subsequent signal transduction pathway and transactivation of its target genes, which leads to cell survival and proliferation (27-29). Most of the existing data on the biological effects of GDNF/GFRα were observed in the neuronal cells. Although the biological effects in non-neuronal cells are still unclear, several studies have indicated that GDNF/GFRα/Ret system might be involved in tumor cell proliferation, invasion and migration. For example, older mice overexpressing GDNF develop testicular carcinoma after one year of age as a result of an invasion of undifferentiating spermatogonia to the interstitium, suggesting that the GDNF/Ret/GFRα signal pathway might be implicated in human germ cell carcinogenesis (44). In addition, the pancreatic cancer cell line contained both GFRα1 and Ret and GDNF increased the invasive capacity of human pancreatic cancer cell lines (45). Despite finding no GFRα1 expression in the normal bile duct, it was expressed clearly in a bile duct carcinoma, indicating that carcinogenesis leads to the aberrant expression of GFRα1 (46). Interestingly, a significant increase

in APE expression has been demonstrated in malignant tissues, such as epithelial ovarian cancers, cervical cancer tissues and cell lines, prostate cell tumors, gliomas, rhabdomyosarcoma and germ cell tumors (47-52). A higher APE expression level was also reported to be associated with tumor progression (7). Therefore, the APE-mediated increase in the GDNF responsiveness, via GFRα1, might be an underlying mechanism of the migratory and invasive behavior of cancer cells.

During development, high level of APE expression is present in all somatic tissues (53). The presence of widespread and high level of APE expression during development is expected to play an important role in embryogenesis. *APE* null mice exhibits die during the embryonic stage, which results from a developmental defect (21). The phenotype of embryonic death observed in the *APE*-/- mice may be a consequence of defective DNA repair as well as inappropriate gene regulation whose expression is dependent on APE. This study demonstrated that a defect in APE expression by siRNA suppressed GFRα1 expression and the GDNF responsiveness. Mice lacking GDNF (54,55) and GFRα (56,57) all die soon after birth and share a similar phenotype of kidney agenesis and absence of enteric neurons below the stomach, suggesting GNDF/GFRα signaling pathway plays an important role in morphogenesis during embryonic development. Although little is known about why *APE* null mice are embryonic lethal, one may speculate that arising from APE functional defect in APE-null embryos, a failure of GDNF/GFRα signal pathway needed to stimulate morphogenesis may contribute to embryonic death.

The GDNF/GFRα signaling pathway promotes the survival of various neurons including peripheral autonomic and sensory neurons as well as central motor and dopamine neurons (27). Moreover, in various animal models of Parkinson's disease, GDNF can prevent the neurotoxin-induced death of dopamine neurons and can promote functional recovery (58,59). The ability of GDNF to rescue dopaminergic

neurons supported idea that GDNF might ameliorate degeneration of dopaminergic neurons in patients with Parkinson's disease. Therefore, GDNF has been expected as a therapeutic candidate for the treatment of Parkinson's disease (60). The GDNF is also a good candidate molecule for studies and possible treatment of motor neuron diseases, such as amyotrophic lateral sclerosis or acute neuronal trauma (61). Indeed, GFRα are upregulated in axotomized motor neurons and in regions distal to axotomized sciatic nerves (62). This further supports the search for a role for GDNF/GFRα signaling pathway in the studies of motor neuronal disease.

Recently, several lines evidence have suggested that dysfunction of APE may contribute to the development of neurodegenerative disease. For example, alteration in APE expression and mutations in the APE gene have been found in patients with a variety of neurodegenerative diseases (63-65). In addition, the young rats exhibited a transient increase in APE protein expression in the brain, including hippocampus, in response to the oxidative stress, whereas the aged rats had no response (66), suggesting that adaptation to oxidative stress is compromised in aged rats. Moreover, reducing APE sensitized neuronal cells to a variety of DNA damaging agents, such as H_2O_2 and MMR (67). Therefore, it is possible that APE-mediated increase in the GFRα expression might involve in neuronal function and survival. Accordingly, investigations aimed at determining the biological significance of APE-mediated increase in the GFRα expression in neuronal cells is currently under investigation.

APE is important in mediating DNA binding of the AP-1 protein complex (11-15). This occurs via a posttranslational mechanism in which conserved cysteine residues in the DNA binding domains of Fos and Jun proteins are reduced, allowing DNA binding to occur. Additionally, phosphorylation of APE by CKII and PKC stimulates redox-activation of the transcription factor Ap-1, leading to the regulation of gene expression (68,69). APE is also involved in the reduction of the

Cys-62 residues of p50, which is essential for DNA binding activity of NF-κB, suggesting APE may act as a redox-sensitive regulator of NF-κB (70). Moreover, upregulation of APE significantly potentiates hypoxia-induced expression of a reporter construct containing the HIF-1-binding site (17), and APE is thought to be critical in the linking of two coactivator proteins, CBP/p300 and SRC-1, to HIF-1α (71,72). Furthermore, APE is found to be a component of protein complexes that binds to negative calcium response element (nCaRE)(73), Ku70(Ku86)(74) and heterogeneous nuclear ribonucleoprotein L (hnRNP-L)(75) in the promoter of the parathyroid hormone (PTH) gene, renin gene and APE gene itself, where it may down-regulate expression of those genes (76,77). Recent work by Bhakat *et al* (78) suggests that APE is acetylated by CBP/p300 both *in vivo* and *in vitro*, and acetylation stimulates binding to nCaRE in the PTH promoter, leading to downregulation of the PTH gene. Therefore, it appears that APE stimulates the transcriptional activation by at least two independent mechanisms: by stimulating DNA binding activity directly, and indirectly by enhancing the transactivation activity. The results showed that transfecting the GM00637 cells with mutant APE (APE^{C65}) leads to a dose-dependent increase in the GFRα1 promoter activity. Previous *in vitro* studies using recombinant human APE proteins suggest that cysteine 65 is the redox-active site of APE (15). It suggests that cysteine may confer redox activity to APE by maintaining the active site in an appropriate conformation and allow other residues to act as reductants. However, recent *in vivo* and *in vitro* studies have shown that APE cysteine 65 is not essential for the direct reduction of cysteines within the DNA binding domains of Fos and Jun or for the maintenance of a protein conformation critical for the redox regulatory of APE (79). Our studies ruled out the possibility that cysteine 65 are responsible for APE-mediated increase in the transcriptional activation of GFRα1, but could not rule out the possibility that another residues of APE capable of redox activation function in

this effect. It is also possible that APE may regulate the GFRα1 transcription through the activation of redox-independent transcription factors. Reporter gene analysis identified the region of the GFRα1 promoter, -236 and -13, that appeared to be important for regulating the GFRα1 promoter by APE. The activity of the fragments spanning -236 and -13 of the GFRα1 promoter was analyzed using the Transfac software (www.genomatix.de), which found that this segment contains four putative NF-κB binding sites and one putative early growth response 1 (Egr-1) binding site (data not shown). NF-κB is known to be an important redox-sensitive transcription factor and is activated by APE (12, 19). Egr-1 is another class of transcription factor that is activated by APE (16). Thus, it is likely that APE may be involved in the regulation of GFRα1 expression through the activation of NF-κB and/or Egr-1 transcription factors. Further studies will be needed to determine the detailed mechanism by which APE promotes GFRα1 expression in addition to determining how many genes are regulated by this mechanism.

In conclusion, this study showed that the GDNF receptors, GFRα1, are induced by APE. It was also demonstrated that APE activates the GDNF responsiveness through GFRα1, resulting in c-Src phosphorylation and cell proliferation in the GM00637 human fibroblast cells. These results highlight the potential role of APE in normal development and cell proliferation mediated through GDNF/GFRα signaling.

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<국문초록>

APE의 신경세포 보호효과

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사람 apurinic/apyrimidinic endonuclease (APE)는 AP 부분을 복구시키는 효소이자 redox 조절에 관여하여 여러 종류의 전자인자 활성을 조절하는 다기능 단백질이다. 본 연구에서는 APE 에 의한 타킷 단백질인 glial cell-derived neurotropic factor receptor α1 (GFRα1)을 발굴하였는데 이 단백질은 glial cell-derived neurotropic factor (GDNF)의 수용체로 잘 알려져 있다. APE 에 의하여 증가된 GFRα1 은 사람 상피세포인 GM00637 세포에서 GDNF/ GFRα1 신호전달을 통하여 세포내 c-Src (tyr 418)의 인산화를 촉진 시켜 세포 분열을 증가 시켰다. 또한 사람 췌장 암세포를 포함한 다양한 암세포에서 APE 에 의하여 GFRα1 의 발현이 증가되었다. 췌장암세포에서 APE 에 의하여 증가된 GFRα1 이 췌장 세포의 성장과 암세포가 주위조직으로 퍼지는 invasion 을 촉진 시켰다. 신경세포에서는 APE 에 의하여 증가된 GFRα1 이 신경세포 생존에 관여하는 신호전달 시스템을 활성화 시켰다. AP 에 의하여 증가된 GFRα1 은 신경세포 생존에 관여하는 신호전달 황성을 통하여 퇴행성 뇌질환을 유발하는 베타 아밀로이드 전구체 및 산화성 손상에 대한 세포 생존 능력을 증가 시켰다.

저작물 이용 허락서

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본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB 구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집・형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

동의 여부: 동의 (O) 반대 ( )

2008 년 2 월 일

저작자: 김 경 환 (서명 또는 인)

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