Epigenetic regulation by G protein alpha12 in human cancer cells and vascular endothelial cells

HYEONJEONG LEE

Department of Medical Science

The Graduate School, Yonsei University

Epigenetic regulation by G protein alpha12 in human cancer cells and vascular endothelial cells

Directed by Professor Eun Jig Lee

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HYEONJEONG LEE

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This certifies that the Doctoral Dissertation of Hyeon Jeong Lee is approved.

이 은 직

Thesis Supervisor: Eun Jig Lee

안 철 우

Thesis Committee Member#1: Chul Woo Ahn

윤 호 근

Thesis Committee Member#2: Ho Geun Yoon

김 형 표

Thesis Committee Member#3: Hyoung-Pyo Kim

서 미 란

Thesis Committee Member#4: MiRan Seo

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Yonsei University

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ABSTRACT

Epigenetic regulation by G protein alpha12 in human cancer cells and vascular endothelial cells

Hyeon Jeong Lee

Department of Medical Science
The Graduate School, Yonsei University

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Heterotrimeric guanine nucleotide binding proteins (G proteins) transmit a variety of extracellular signals from cell surface G protein-coupled receptors (GPCRs) to intracellular effector molecules.

Gα12/13-mediated signals formed networks with other signaling proteins at various levels, from cell surface receptors to transcription factors, to regulate cellular responses. The G12 subfamily is composed of Gα12 and Gα13. Accumulating evidence indicates that Gα12/13-mediated signaling pathways are involved in a variety of physiological processes, including embryonic development, cell growth, cell polarity and migration, angiogenesis, platelet activation, immune response, apoptosis, and neuronal responses.

Epigenetic mechanisms denote gene expression variability without coding sequence alteration. Epigenetic machinery includes DNA methylation, histone modifications and regulation of gene expression posttranscriptionally by small, non-coding RNA molecules. Epigenetic modifications play essential roles in the regulation of gene expression and are important in mammalian development and disease processes.

In this study aim to examine whether Gα12 signaling pathway regulates epigenetic modification and to evaluate this change has any physiological meaning.

In Part Ⅰ, we investigated Gα12 action on apoptosis which is mediated by epigenetic modification of XAF1 promoter in NSCLCs. Basal XAF1 mRNA level was very weakly detected in NSCLCs. However, XAF1 expression was restored in A549 cells when blocked Gα12 signal. According to data from MSP and MeDIP, XAF1 promoter methylation was reduced by siGα12. In our data shown that siGα12 was enhanced demethylation of XAF1 promoter and was induced apoptosis.

In Part Ⅱ, we aimed to investigate the effect of Gα12 on cellular proliferation and its underlying mechanism in HepG2 human hepatoma cells. p16 expression was significantly enhanced in HepG2 cells after siGα12 transfection. From MSP, MeDIP and DNMT1 expression data, we can show that Gα12 regulates p16 expression by epigenetic modification mechanism, by inducing DNMT1 expression and activation. Therefore, Gα12 siRNA inhibits the proliferation of HepG2 cells by upregulating p16 expression, suggesting that the abnormal proliferation of HepG2 cells might be resulted from Gα12 signaling to suppress p16 expression of HepG2 hepatoma cells.

In part Ⅲ, we aimed to investigate the role of Gα12 in serum withdrawal -induced apoptosis of HUVECs, and its underlying mechanisms. Gα12 siRNA markedly increased the serum deprivation-induced apoptosis of HUVECs. Because miR-155 has been reported to regulate apoptosis of HUVECs, we examined apoptotic effect of Gα12 on the miR-155 expression. Results indicated that Gα12 regulates the apoptosis of vascular endothelial cells by regulating miR-155 expression. These results suggest that Gα12 protects vascular endothelial cells against vascular injuries causing endothelial dysfunction by regulating miR-155 expression.

From these results, we conclude that Gα12 regulates epigenetic modification in cancer cell and vascular endothelial cells.

Key words : Gα12, epigenetic modification, cancer, vascular disease, XAF1, p16, miRNA

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The Graduate School, Yonsei University

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Part Ⅰ. Up-regulation of XAF1 by Gα12 knock down induces cell death in A549 lung cancer cell line

1. INTRODUCTION

The term epigenetics refers to heritable changes in gene expression that does not involve changes to the underlying DNA sequence; a change in phenotype without a change in genotype. At least three systems including DNA methylation, histone modification and non-coding RNA (ncRNA)-associated gene silencing are currently considered to initiate and sustain epigenetic change. [1]

DNA hypomethylation can activate oncogenes and initiate chromosome instability, whereas DNA hypermethylation initiates silencing of tumor suppressor genes. An accumulation of genetic and epigenetic errors can transform a normal cell into an invasive or metastatic tumor cell. Additionally, DNA methylation patterns may cause abnormal expression of cancer-associated genes. [2] Global histone modification patterns are also found to correlate with cancers such as prostate, breast, and pancreatic cancer. Subsequently, epigenetic changes can be used as biomarkers for the molecular diagnosis of early cancer. [3,4] Deregulation in any of these histone modifications may shift the balance of

gene expression leading to alterations in critical cellular processes such as transcription, proliferation, apoptosis, and DNA repair, ultimately resulting in cellular transformation and malignant outgrowth. [2,5]

Heterotrimeric guanine nucleotide binding proteins (G proteins) transmit a variety of extracellular signals from cell surface G protein-coupled receptors (GPCRs) to intracellular effector molecules. G proteins consist of two functional signaling units, a guanine nucleotide binding α subunit and βγ subunit dimer. Upon receptor activation, the α subunit undergoes a conformational change that leads to the exchange of GTP for GDP and the dissociation of the α subunit from the βγ dimer, allowing the subunits to engage their downstream effectors. Because of the array of extracellular signals that activate them and their increasingly large number of intracellular targets, G proteins have been implicated in many physiologic and pathophysiologic processes. [6]

Gα subunits are classified into four subfamilies, Gs, Gi, Gq and Gα12. Gαs and Gαi are mainly involved in the stimulation and inhibition of adenylyl cyclases, respectively, to regulate the intracellular concentration of cAMP. PLCβ (phospholipase C-β) isozymes are well established effectors for all members of the Gq sub family and generate IP3 (inositol 1, 4, 5 triphosphate) and DG (diacylglycerol) from PIP2 (phosphatidylino sitol 4, 5 bisphosphate) at the plasma membrane. IP3 increases intracellular $Ca2^{+}$ levels and DG is involved in the activation of PKC (protein kinase C). [7,8] In addition to the regulation of soluble intracellular second messengers, it has been clearly demonstrated that Gα12/13 and Gαq are directly involved in the activation of RhoGTPases. The RhoGTPases are members of the RAS superfamily of monomeric GTP binding proteins. It has been demonstrated that the activation of RhoGTPase results in a variety of cellular responses including gene transcription, embryogenesis and rearrangement of the actin cytoskeleton. [7,9]

The two members of the G12 family in mammals, Gα12 and Gα13, have

received considerable attention in the context of cell proliferation and morphology. Gα12/13-mediated signals formed networks with other signaling proteins at various levels, from cell surface receptors to transcription factors, to regulate cellular responses. Gα12/13 have slow rates of nucleotide exchange and GTP hydrolysis, and specifically target RhoGEFs containing an amino-terminal RGS homology domain (RH-RhoGEFs), which uniquely function both as a GAP and an effector for Gα12/13. [10,11]

Gα12-mediated signaling has been shown to stimulate the activity of a diverse set of downstream proteins that have not been demonstrated to interact directly with Gα12 or Gα13. These include phospholipase D, phospholipase C, phospholipase A2, JNK and p38MAPK, and NF-κB. [12,13] Gα12-mediated signaling has also been shown to trigger phosphorylation of vasodilator -stimulated phosphoprotein, as well as phosphorylation of the focal adhesion proteins paxillin, focal adhesion kinase, and p130 Crk-associated substrate. [13] In addition to the responses described above, Gα12 proteins have been reported to promote signaling through GSK-3β, stimulate ERK5, and control Na+-H+ exchange. [14] More recently, several groups have begun to examine the biologic significance of Gα12-stimulated cell growth and neoplastic transformation in human cancers. The earliest of these studies demonstrated that Gα12 expression is stronger in cell lines derived from human breast, prostate, and colon cancers compared to cell lines derived from nontransformed human tissue. This finding suggested that the Gα12 proteins are upregulated during neoplastic transformation of these common forms of cancer. [13,14]

Gα12 is involved in Rho family GTPase signaling and have been linked to several cellular regulatory processes, including cytoskeletal rearrangement and oncogenic transformation. [15] Expression of the Gα12 proteins is significantly elevated in prostate cancer. Expression of the activated forms of Gα12 in PC3 and DU145 cell lines induced cell invasion through the activation of the RhoA

family of G proteins. JNK activation is required for Gα12-induced invasion of breast cancer cells and that JNK is downstream of Rho and ROCK on this pathway. [12,16,17]

XIAP associated factor 1 (XAF1) antagonizes the anticaspase activity of XIAP and may be important in mediating apoptosis resistance in cancer cells. The pro-apoptotic effects of XAF1 may be mediated by direct sequestration of XIAP from the cytosol to the nucleus, thus antagonizing the inhibition of caspases [18]. XAF1 could sequester XIAP protein to the nucleus, it was proposed that loss of XAF1 in tumor may decrease the functional pool of cytoplasmic XIAP, which in turn deregulates the apoptotic process and thus contributes to malignant tumor progression, growth suppressive role. [19]

Loss of XAF1 expression through promoter methylation has been implicated in the process of tumorigenesis in a variety of cancers. XAF1 reduction is associated with promoter hypermethylation in human stomach, bladder, kidney, and prostate carcinomas.

G proteins regulate apoptosis in addition to other cellular functions, but the roles of specific G protein in apoptosis signaling are not well characterized. Herein, we examined whether Gα12 regulates apoptosis in lung cancer cells through changed XAF1 promoter epigenetic modification.

2. MATERIALS AND METHODS

A. Cell culture

A549, FRO, HepG2, HCT116, MCF7 cells were grown in RPMI1640 medium containing 10% FBS, 100 units/ml penicillin and 100 mg/ml. TPC-1, H1299 cell lines were grown in DMEM medium supplemented with 10% FBS, 100 units/ml penicillin and 100 mg/ml. Cells were cultured in a 5% CO2 incubator at 37℃.

B. Small interfering RNA transfection

Gα12 was knocked down using ON-Target plus-SMARTpool pooled siRNAs (Thermo Scientific Dharmacon, Lafayette, CO, USA) that contains four different siRNAs (D-008435-00-0010). A nonspecific control pool was used for the negative control (Invitrogen Life Technology, CA, USA). Cells were transfected with siRNA using Lipofectamine 2000 reagents according to the manufacturer's guidelines. Briefly, 100 pmol of siRNA and Lipofectamine 2000 were added separately to OPTI MEM medium. After 5 min, the two solutions were mixed and incubated for 20 min at room temperature. The mixture was added to monolayer of cells seeded in 10cm tissue culture plates. The media were replaced with complete cell culture medium after 6 h and whole-cell lysates were prepared 48 h later for detection of protein expression.

C. Western blot analysis

The whole cell lysates were prepared with lysis buffer (50 mM Tris-HCl, 150 mM Sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.5, and 2mM EDTA, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, protease inhibitor cocktail.) The protein concentration was determined by the bicinchoninic acid assay (BCA

protein assay kit, Pierce) with bovine serum albumin as the standard. Equal aliquots of total cell lysates (50 ug) were solubilized in sample buffer and electrophoresed on denaturing SDS-polyacrylamide gel (10% and 15% separating gel). The proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in TBS containing 0.05% Tween 20 and incubated with primary antibodies overnight at 4℃ and then with horseradish peroxidase-conjugated secondary antibodies for 2hr at RT. Antigen-antibody complexes were detected with WEST-SAVE Up luminol-based ECL reagent (ABfrontier, Seoul, Korea).

D. RNA isolation and RT-PCR

Total RNA from each cell was isolated using Isol-RNA Lysis Reagent (5-Prime, Gaithersburg, MD, USA) according to the manufacturer's protocol. The RNA samples were treated with DNase I, quantified, and reverse-transcribed into cDNA using the ReverTra Ace-α First Strand cDNA Synthesis kit (Toyobo, Osaka, JAPAN).

RT–PCR was performed using primers specific for XAF1 (sense primer: 5'-CTTCAGCTCCACAGAGAAGAACTGC-3', antisense primer: 5'-CACG ATCATGTTGGACAACTGCTCC-3') and GAPDH (sense: 5'- CAAGGTCA TCCATGACAA CT-3', antisense: 5'- TTCACCACCTTCTTGATGTC -3'). The cycling conditions were as follows: initial denaturation at 95℃ for 5min, followed 35cycles at 95℃ for 45s, 60℃ for 45s and 72℃ for 45s. Band intensities of the amplified DNAs were compared after visualization on an UV transilluminator.

E. Immunofluorescence.

A549 cells were grown on poly-D-lysine coated confocal dishs. Cells were transfected with Gα12 siRNA for 48h, washed in PBS, and fixed 10 min with 4% methanol. Cells were permeabilized 5 min with 0.1% Triton X-100, and blocked in 5% donkey serum. Cells were incubated with rabbit anti-XAF1 primary antibody at 1:50 in blocking buffer, followed by donkey anti-rabbit-FITC secondary antibody at 1:100. Antibody incubations were performed for 1 h at RT. All cells were also stained with DAPI.

F. Immunoprecipitation

Protein extracts were prepared from cells 48 h after Gα12 siRNA transfection in lysis buffer containing 10% Triton X-100, 10% glycerol, 400 mM NaCl, 10 mM Tris pH 8.0, 1 mM phenyl methyl sulphonyl fluoride and aprotinin. After being sonicated with an ultrasound sonicator, whole lysates were centrifuged at 16,000 g for 10 minutes to remove cell debris. Lysates were pre-cleared with 10 ml of rabbit pre-immune serum coupled to protein A/G agarose for 2 h, followed by low-speed centrifugation. Immunoprecipitations were performed using 5 mg of affinity-purified rabbit polyclonal anti- XAF1 antibody for 2 h. Protein A/G-agarose was added for 90 min, and antibody complexes were collected and washed three times in lysis buffer. Immunoprecipitation samples were resolved by SDS-PAGE and analyzed by immunoblotting. The protein samples were subjected to SDS-PAGE and transferred on to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in TBS containing 0.05% Tween 20 and incubated with primary anti-XIAP antibody and then with horseradish peroxidase-conjugated secondary antibody. Antigen-antibody complexes were visualized by the ECL system (ABfrontier, Seoul, Korea).

G. TUNEL assay

A549 cells were seeded on confocal dish and transfected with Gα12 siRNA . After Gα12 siRNA transfection for 48h, apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using a kit methodology (Roche, Nutley, USA). Briefly, cells were washed once with PBS and fixed in 4% paraformaldehyde for 1 h at 25℃. Cells were washed with PBS and incubated in permeabilization solution (0.1% triton X-100 in 0.1% sodium citrate) for 2 min on ice. They were then washed twice with PBS and treated with TUNEL reaction mixture for 1 h at 37℃ in the dark in a humidified atmosphere. Finally, the cells were washed three times with PBS ananalyzed under fluorescence microscope (Carl Zeiss, Oberkochen, Germany), using an excitation wavelength in the range of 450 - 500 nm and a detection wavelength in the range of 515 - 565 nm (green).

H. Annexin V staining

Apoptosis was determined by staining cells with Annexin V-fluorescein isothiocyanate (FITC, PharMingen, San Diego, CA, USA; Ex/Em =488 nm/519 nm) and propidium iodide (PI; Sigma-Aldrich;Ex/Em = 488 nm/617 nm). In brief, $1x10^6$ cells in 100 mm culture dish were incubated with transfected siGα12 for 24 h. Cells were washed twice with cold PBS and then resuspended in 500 μl of binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) at a concentration of $1x10^6$ cells/ml. 5 μl of Annexin V-FITC and PI (1 μg/ml) were then added to these cells, which were analyzed with a FACScan/CellFit system flow cytometer (Becton-Dickinson, San Jose, CA, USA).

I. Methylation-specific PCR (MSP)

Treatment of DNA with bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. We used reagents supplied in Imprint DNA modification kit (Sigma, St. Louis, MO, USA) for bisulfite treatment, according to the one-step modification procedure recommended by the manufacturer. Briefly, 10 μl of DNA solution was combined with 110 μl of Imprint Bisulfite Modification Reagent, denatured at 99°C for 6 min and incubated at 65°C for 90 min. For purification of bisulfite-treated DNA, we used the Spin column in Imprint DNA modification kit. The modified DNA was then stored at −20°C until use.

For MSP, the modified DNA (100 ng) was amplified with Taq polymerase. The methylated or unmethylated XAF1 primer sets were: unmethylated XAF1 sense, 5'- TTTGGAAAAGGGATGGAGATTTAGATG-3', antisense, 5'- ACAA ACTTTCAATTAAATTTCA-3'; methylated XAF1 sense, 5'- TTTATTTTATTG GTAGACGTTACG -3', antisense, 5'-ATAACTCCTAAACTTCCAAAC G-3'.

J. Methylated DNA Immunoprecipitation (MeDIP)

1 mg of DNA extracted from cell lines. 5mC immunoprecipitation was carried out using the EpiQuik Methylated DNA Immunoprecipitation (MeDIP) Kit (Epigentek Inc, Brooklyn, NY, USA) according to the manufacturer's specifications. First, wells were washed once with Wash Buffer (WB; CP1) and then incubated at RT for 1 h in the presence of 100 ml of Antibody Buffer (AB; CP2) supplemented with 1 ml of 5mC antibody (or 1 ml of Normal Mouse IgG, as a negative control). DNA samples, diluted with ChIP Dilution Buffer (CP4), were added into the assay wells and incubated at RT for 90 min on an orbital shaker. The wells were washed six times with 1 WB, followed by the addition of TE Buffer. Afterwards, the DNA Release Buffer containing proteinase K

were added to each well and samples were incubated at 65℃for 15 min. Then, samples were incubated in Reverse Buffer at 65℃ for 30 min, Binding Buffer were subsequently added to the wells, and the released samples, transferred to the F-Spin column, were centrifuged at 14 000 g for 20 s. purified DNA was eluted in 15 ml of Elution Buffer. PCR was performed using immuno-precipitated DNA. PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining.

K. DNMT Activity Assay

DNMT activity was determined in the nuclear extracts using the EpiQuik DNA Methyltransferase Activity Assay Kit (Epigentek Inc., Brooklyn, NY, USA) following the manufacturer's protocol. Briefly, DNMT enzymes transfer a methyl group to cytosine from Adomet to methylate the DNA substrate, and the methylated DNA can be recognized with an anti-5-methylcytosine antibody. The ratio or amount of methylated DNA, which is proportional to enzyme activity, can then be measured using an ELISA-like reaction by reading the absorbance at 450 nm by the Tecan Infinite M2000 spectrophotometer. The DNMT activity was expressed as a percentage of the corresponding siRNA control.

L. Statistical analysis

Results are expressed mean ± S.D. Statistical analysis are as performed by student's t-test. Relationships were considered statistically significant when p- value was less than 0.05.

3. RESULTS

A. Expression of XAF1 was regulated by Gα12

We screened the expression of the XAF1 in various cancer cell lines. To measure the endogenous expression level of XAF1, RT-PCR analyses was performed on various cancer cell lines. The results indicate that XAF1 express in HepG2, MCF7 cells compared with HUVEC normal control cells, and not detectable in A549, FRO, TPC-1, and HCT116 cells (Figure 1.A). To determine whether Gα12 regulated XAF1 expression level, cancer cells were transient transfected with Gα12 siRNA. Knockdown of Gα12 increased the basal expression level of XAF1 in A549, HepG2, and MCF7 cells, but not changed XAF1 expression in other cell lines. Particularly, Western blot and RT-PCR for Gα12 in A549 cells were predominantly re-expressed XAF1 protein and mRNA level by Gα12 down regulation. (Figure 1.B, C). These results demonstrate that Gα12 adjusted XAF1 expression in A549 cells.

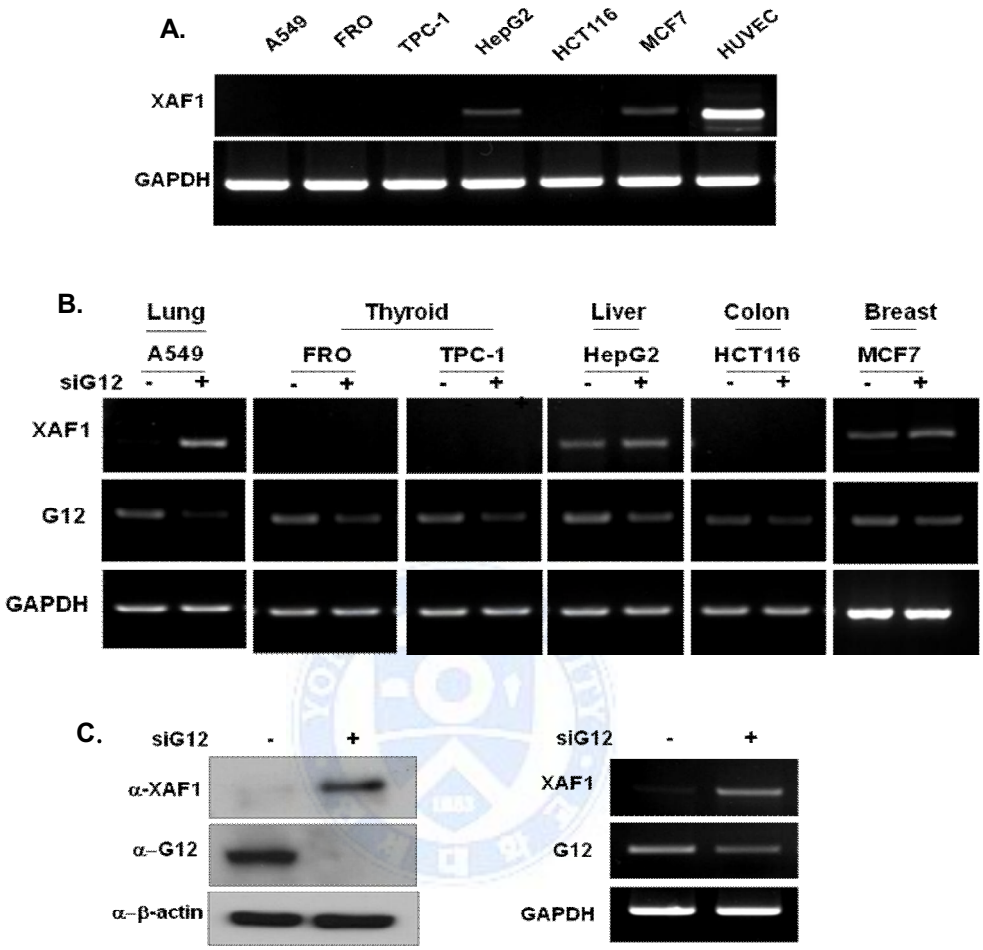


Figure 1. Expression level of XAF1 in various cancer cells and XAF1 expression was controlled by Gα12. (A) Endogenous XAF1 mRNA level was measured by RT-PCR in various cancer cell lines. (B) Effect of Gα12 on XAF1 expression level. Cancer cells were transient transfected with Gα12 siRNA for 48h. XAF1 mRNA level detected by RT-PCR (C) Western blot and RT-PCR for Gα12 in A549 cells reveal restoration of XAF1 protein and mRNA levels by Gα12 siRNA.

B. Restoration of XAF1 by Gα12 down-regulation was appeared in NSCLCs

To describe the influence of Gα12 in XAF1 expression, we screened the expression of the XAF1 in other lung cancer cell lines. XAF1 basal mRNA level was very weakly detected in all NSCLC cells compare with normal cell line (Figure 2.A). However, XAF1 expression was markedly restored by blocking of Gα12 signal in A549 cells and H1299 cells (Figure 2.B).

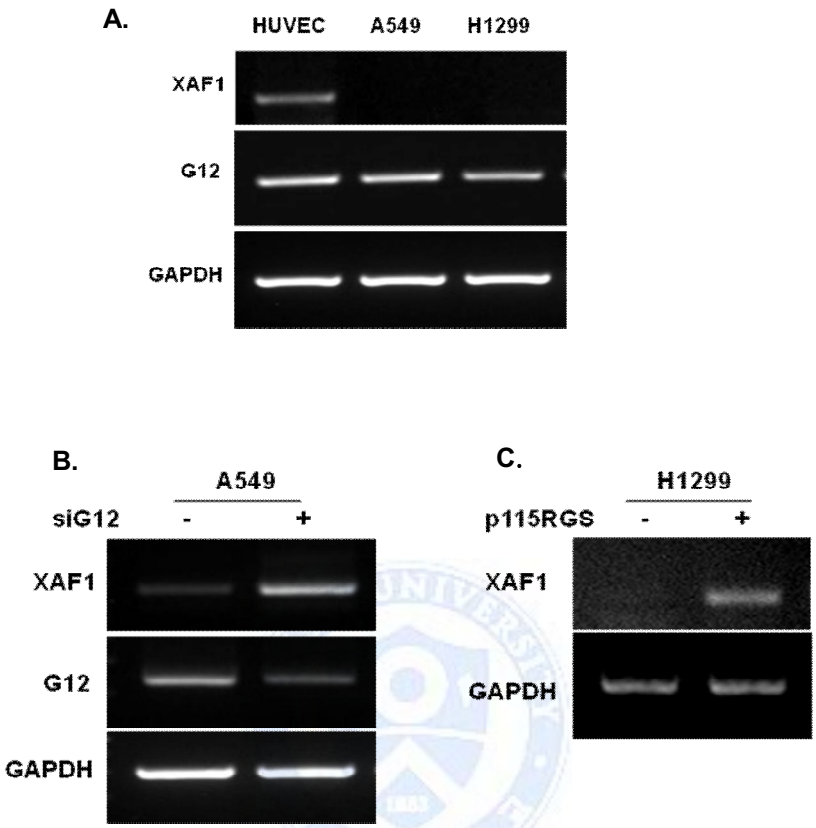


Figure 2. Gα12 regulates expression level of XAF1 in human NSCLC cell lines. (A) Total RNA isolated from 2 NSCLC cell lines and HUVEC normal control cells. Gα12 and XAF1 mRNA basal level detected by RT-PCR (B, C) A549 cells were transfected with Gα12 siRNA for 48h. H1299 cells were transfected with p115RGS that inhibit downstream signals of Gα12. XAF1 expression level was measured by RT-PCR.

C. DNA methylation of XAF1 controlled by Gα12 in A549 cells

To elucidate whether aberrant DNA methylation is associated with gene silencing of XAF1, A549 cell were treated the demethylating agent 5-Aza-2′deoxycytidine (5-Aza-dC). 5-Aza-dC treated in A549 cells was XAF1 re-expression that was shown same aspect increased XAF1 by Gα12 siRNA transfection (Figure 3.A). To determine whether Gα12 regulate methylation of XAF1 promoter region, we used MSP and Methylated DNA Immunoprecipitation (MeDIP) after siGα12 transfection. According to data from MSP, XAF1 was methylated in A549 cells. However, XAF1 promoter methylation was reduced by siGα12. The same effect was obtained when cells were treated 5-Aza-dC (Figure 3.B). Moreover, the data from MeDIP show that siGα12 lowers the frequency of 5-methylcytosine on the promoters of XAF1 (Figure 3.C)

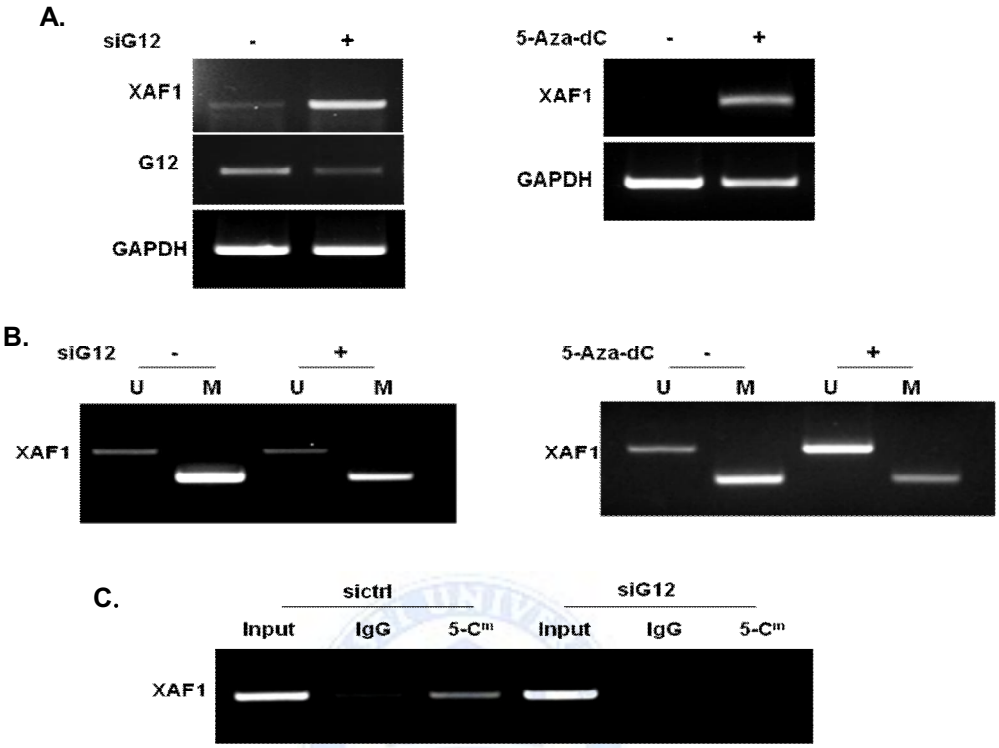


Figure 3. Knock down of Gα12 up-regulates expression of XAF1 via promoter demethylation. (A) Effects of Gα12 and 5-Aza-dC on promoter methylation and expression of XAF1 in A549 cells. A549 cells were transfected with siGα12 RNA for 48h or treated 5-Aza-dC (10 μM) for 5days. XAF1 expression was evaluated by RT-PCR. (B) MSP analysis was performed to determine the promoter methylation of XAF1. 100 ng of bisulfite modified genomic DNA was subjected to PCR amplification of the XAF1 promoter sequences using unmethylation specific and methylation specific primer sets, respectively. M; methylated, U unmethylated. (C) Methylated DNA immunoprecipitation assay was performed to promoter methylation status. Immunoprecipitation of genomic DNA from A549 cells with antibody against 5-methylated-cytosine followed by RT- PCR analysis of MeDIP. Input as DNA control and IgG as negative control.

D. DNMT1 mediate DNA methylation change by Gα12 knock down

As we confirmed that methylation in XAF1 was influenced by Gα12, we tried to find the mediating factors. First of all we checked the DNMT1 expression which is most common methyltransferase. In both siGα12 and 5-Aza-dC treatment, DNMT1 expression was decreased (Figure 4.A). In addition to complete loss of DNMT1 protein in the whole cell lysates and nuclear extracts, DNMT activity was severely reduced upon both siGα12 and 5-Aza-dC treatment (Figure 4.B).

From these results, we can show that Gα12 regulates XAF1 expression by epigenetic modification mechanism by inducing DNMT1 expression and activation.

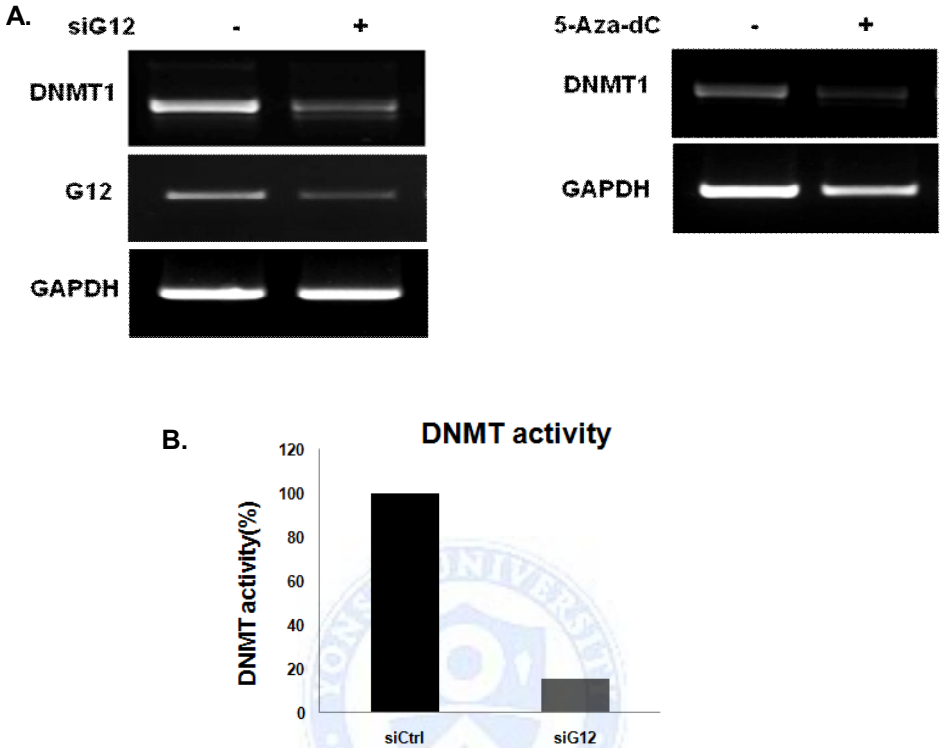


Figure 4. Gα12 regulates both expression and activity of DNMT1. (A) A549 cells were transfected with siGα12 RNA and exposed to 5-Aza-dC for 48h and 5days, respectively. Expression of DNMT1 and Gα12 were analyzed by RT-PCR. Results were representative of three separated experiments. (B) A549 cells were transfected with Gα12 siRNA. DNMT activity was determined in the nuclear extracts, and the methylated DNA can be recognized with an anti-5-methylcytosine antibody. The ratio or amount of methylated DNA, which is proportional to enzyme activity, can then be measured using an ELISA-like reaction by reading the absorbance at 450 nm.

E. Effect of the up-regulation of XAF1 expression by Gα12 knockdown on XIAP expression and localization

XAF1 was a nuclear protein to exert its proapoptotic effect by directly interacting with XIAP and inducing XIAP sequestration in nuclear inclusion. To investigate interaction between XAF1 and XIAP in siGα12 transfected A549 cells, we used fluorescence confocal microscopy to observe the localization of XAF1 and XIAP. XAF1 was not detected and endogenous XIAP was observed predominantly in the cytoplasm in control siRNA transfected cells. However, restoration of XAF1 expression by siGα12 triggered a edistribution of XIAP from the cytosol to the nucleus (Figure 5.A). The possibility of recovering XIAP by immunoprecipitation with an anti- XAF1 antibody was investigated. Coimmunoprecipitation analysis revealed the XIAP protein was precipitated in cellular nucleus and increased in Gα12 knockdown cells (Figure 5.B). As shown in these data, siGα12 increased XAF1 interaction with XIAP.

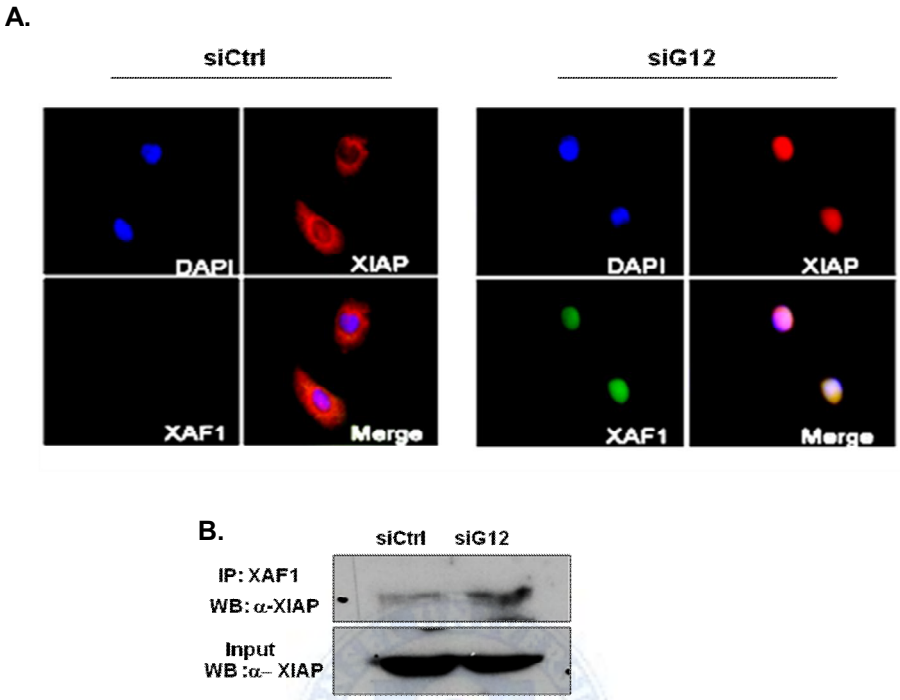


Figure 5. Restoration of XAF1 by siGα12 sequestrates XIAP in nuclear. (A) A549 cells were transfected with siGα12 for 48h. Confocal microscopy observed for the cellular localization of XAF1 with XIAP in siGα12 transfected A549 cells. Immunodetection was performed using specific antibodies linked to fluorescence performed using specific antibodies linked to fluorescence probes: Anti XIAP with cy3 (red) and anti XAF1 with FITC (green). Nuclei were stained with DAPI (blue). (B) Nuclear proteins were extracted from siGα12 transfected A549 cells to evaluate the coimmunoprecipitation of XIAP with anti-XAF1 antibodies. input as positive control.

F. **Effect of Gα12 knockdown on apoptosis**

Next, to determined physiological roles of XAF1 in lung cancer, we performed functional studies. TUNEL assays revealed apoptotic DNA fragments after siGα12 transfection (Figure 6.A). I tried to investigate whether Gα12 enhance cisplatin action, because A549 is a cisplatin resistant cells. As shown in westernblot data, Gα12 knock down induced the cleaved caspase3 in A549 cells. Moreover, siGα12 transfected cells were increased apoptosis in the presence of cisplatin compared with only cisplatin (Figure 6.B). In flowcytometry analysis, there was a 3-fold increase in the percentage of apoptotic cells with siGα12 transfected cells + cisplatin when compare with siCtrl transfected cells+ cisplatin (Figure 6.C). Restoration of functional XAF1 by siGα12 could be effective in overcoming cisplatin resistance by sensitization of tumor cells to cisplatin induced apoptosis.

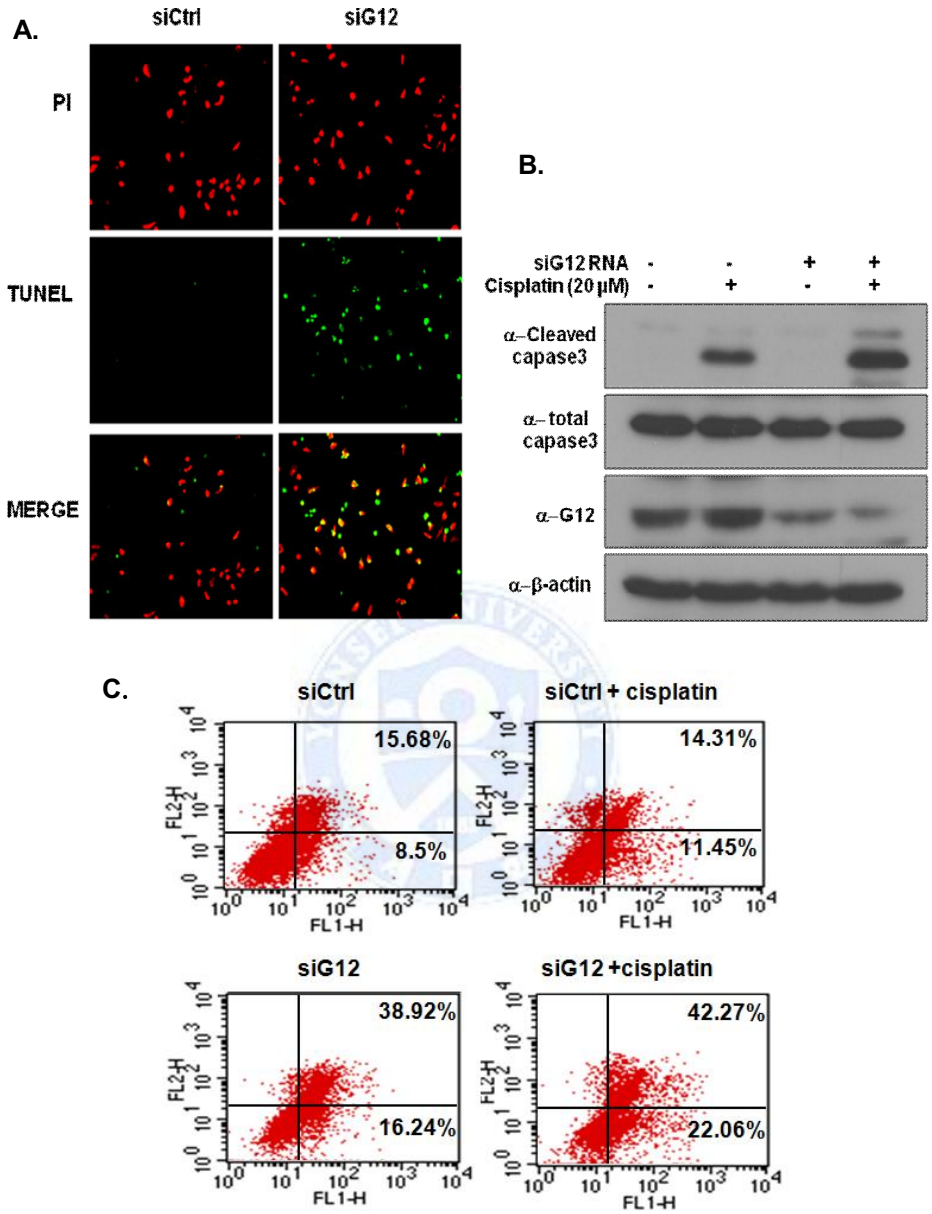


Figure 6. Effect of Gα12 knockdown on apoptosis. (A) Microscopic detection of apoptotic cells by TUNEL after siGα12 RNA transfection for 48h. (B) Casapse3 activation by Gα12 downregulation. Cells were transfected with siGα12 and its effect on cisplatin induced caspase3 activation was examined by

immunoblotting assay using antibody specific for cleaved caspase3. The β-actin control using the same blot indicates equal protein loading. (C) The effect of siGα12 on either early or late stage of A549 apoptosis as detected by flowcytometry. A549 cells were transfected with Gα12 siRNA for 24h and then incubated without (control) or with 20 μM cisplatin. After 24 h, the cells were harvested, stained with Annexin V-FITC and PI, and analysed by flowcytometry. Diagrams of FITC-Annexin V/PI flowcytometry in a representative experiment are presented below the graphs. The lower right quadrants represent the cells in the early stage of apoptosis. The upper right quadrants contain the cells in the late stage of apoptosis or necrosis.

4. DISCUSSION

In this study, we characterized the role of Gα12 in the regulation of XAF1 function and demonstrate that downregulation of Gα12 with Gα12 siRNA mediates XAF1 induced apoptosis in A549 lung cancer cells.

Heterotrimeric G proteins regulate variety of cellular processes including proliferation, differentiation, junctional assembly, and apoptosis. The α-subunits of heterotrimeric G proteins can be divided into four families based on sequence homology: Gs, Gi, Gq, and G12.The last of the four families to be identified, the G12 family has been of particular interest to cancer researchers, since its members were found to promote the growth and oncogenic transformation of murine fibroblasts. [17] These findings led to the hypothesis that GPCRs may signal through the Gα12 proteins to promote tumorigenesis and tumor cell growth. Many studies have suggested a role for the members of the G12 family of heterotrimeric G proteins in oncogenesis and tumor cell growth. The Gα12 proteins are up-regulated in breast cancer and that Gα12 signaling promotes breast cancer metastasis by stimulating breast cancer cell invasion. Signaling by Gα12 proteins through the RhoA family of GTPases is a potent stimulator of prostate cancer cell invasion. [16]

In preliminary experiments, we used the Gα12 siRNA transfection system to the study on new roles of Gα12. We confirmed that expression of XIAP gene was controlled by siGα12 transfection in NSCLCs. XAF1 is one of the XIAP interacting partners and induce apoptosis. Therefore, this finding led to the hypothesis that Gα12 may adjust apoptosis of cancer cells via modulation of XAF1.

XAF1 was first identified as an interacting protein of XIAP. XAF1 antagonizes the anticaspase activity of XIAP. XAF1 is a nuclear protein that directly interacts with endogenous XIAP and results in XIAP sequestration in nuclear inclusions. The sequestering of XIAP in nuclear inclusions is

compatible with the observed increase in the sensitivity of cancer cell lines to apoptotic triggers when XAF1 is overexpressed.[18] Consistent with this, we observed that restoration of XAF1 expression by Gα12 knockdown reversed XIAP-mediated protection against apoptosis. Subcellular distribution studies revealed that XAF1 resides in the nucleus, and can effect a marked relocalization of endogenous XIAP protein from the cytoplasm to the nucleus. We also could detect the nuclear translocation of XIAP protein in tumor cells undergoing apoptosis by XAF1 restoration.

This study also demonstrated the mechanisms by which Gα12 regulates the apoptosis of lung cancer cells. XAF1 is a putative tumor suppressor that its mRNA is present ubiquitously in all normal tissues but is expressed at low or undetectable levels in various cancer cell lines. In many cancers, the CpG islands of selected genes are aberrantly methylated, resulting in repression of transcription of these genes. [19] Recent studies have suggested that loss of XAF1 expression may occur in different human cancers because of aberrant DNA methylation. [20,21] Zou et al found that loss of XAF1 expression is associated with tumor progression in human gastric and colon cancers. [21] Lee et al also discovered that downregulation of XAF1 expression is correlated with human urogenital malignancies.[22]

In this study, we found that XAF1 expression is upregulated in low expression A549 cells following Gα12 siRNA transfection and aberrant hypermethylation of the promoter is tightly associated with downregulation of XAF1 expression in A549 cells. And we show that XAF1 promoter methylation was reduced by siGα12. Thus this suggests that Gα12 regulates XAF1 expression by epigenetic modification mechanism. DNA methylation, catalyzed by DNA methyltransferase, involves the addition of a methyl group to the carbon 5' position of the cytosine ring in the CpG dinucleotide to form methylcytosine. We found that the amount of DNMT1 expression and DNMT activity have correlation with the expression and methylation status of the

XAF1 gene. The eEF2 methylation is preceded by ras-raf-mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK1/2)-p21Cip/WAF1 activation. eEF2, a key factor involved in protein translational elongation is symmetrically arginine-methylated in a reversible manner, being regulated by bFGF through MAPK signaling pathway.[23] Soberanes, S. et al. observed that exposure to PM results in a mitochondrial-oxidant and JNK-mediated increase in the transcription and abundance of DNMT1 and increased methylation of the p16 promoter in the lung epithelium. Aberrant upregulation of DNMT1 could result in early and coordinated hypermethylation of key tumor suppressor genes in PM exposed patients, increasing the risk for the development of lung cancer. [24] c-Jun NH(2)-terminal kinase (JNK) is a key downstream effector of G12 on this pathway. Expression of constitutively-active Gα12 or activation of G12 signaling by thrombin leads to increased JNK and c-Jun phosphorylation. These observations predict mechanism that G12 adjusts methylation of XAF1 promoter through MAPK pathway (e.g. ERK1/2 or JNK) mediated DNMT modulation.

Byun et al reported the importance of transcriptional regulation by 7 CpG dinucleotides located in the 5' proximal region from -23 to -234 nucleotides in both gastric cancer cell lines and tumor tissues.[25] A cluster of methylated CpG sites instead of CpG islands located in the promoter area resulted in gene silencing of XAF1, and CpGs at -2nd, -1st, and +3rd positions are functionally more important in its transcriptional regulation.[21] While we showed a correlation between aberrant methylation of XAF1 and Gα12 regulation and lung cancer progression, the exact promoter region could not be confirmed. Therefore, confirmation of XAF1 promoter methylation site in lung cancer cell may be need further study.

Some lung cancer cells are resistant against to cisplatin-induced tumor regression, leading to the conjecture that defective response to growth suppression effect of cisplatin may give the tumors a selective advantage. In this

context, it could be suspected that lung cancers with XAF1 inactivation might be more resistant to cisplatin drug therapy than cancers with normal XAF1 expression, and restoration of functional XAF1 could be effective in overcoming cisplatin resistance by sensitization of tumor cells to cisplatin induced apoptosis.

XAF1 induces cell cycle arrest during G2/M phase and mitotic catastrophe, and the restoration of XAF1 expression inhibits tumor growth in many types of human cancers. [26] However, we could not observe that restoration of XAF1 expression inhibit tumor cell growth.

Several regulatory pathways of XAF1 expression have been reported. In addition to hypermethylation of XAF1 promoter, Wang, Jide et al reported that heat shock factor 1 negatively while interferon regulatory factor-1 and STAT1 positively modulated XAF1 transcription.[27,28] Inhibition of ERK1/2 stimulated XAF1 expression through indirect transcription regulation. Furthermore, XAF1 was an effector in ERK inhibition-induced cell apoptosis.[29] The over-expression of XAF1 led to activation of wild-type p53 via post-translational modification in cells with or without DNA damage, which resulting in p53 nuclear accumulation and its increased transcriptional activity and enhancing p53-dependent apoptosis.[30] However, we could not observation that Gα12–mediated mechanism for regulation of XAF1 expression and methylation. Therefore, other mechanisms of transcriptional regulation of the XAF1 gene may be present and need further research.

In conclusion, XAF1 undergoes epigenetic silencing in a considerable proportion of lung cancer cell lines by aberrant CpG sites hypermethylation of the gene promoter. In our data shown that siGα12 was enhanced demethylation of XAF1 promoter and was induced apoptosis. Therefore, our data presented here demonstrate that Gα12 was caused cancer cell progression and maintenance by controlling the methylation of XAF1.

Part Ⅱ. Up-regulation of p16 by siGα12 inhibits cellular proliferation of HepG2 cells

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common human malignancies worldwide, and is closely associated with infection of HBV and HCV and contamination of aflatoxin B1. Although the molecular mechanisms of hepatocarcinogenesis remain poorly understood, an increasing number of genetic abnormalities have been recognized.[31]

Cyclin-dependent kinase inhibitor 2A, (CDKN2A, $p16^{Ink4A}$) also known as multiple tumor suppressor 1 (MTS-1), is a tumor suppressor protein, that in humans is encoded by the CDKN2A gene. P16 plays an important role in regulating the cell cycle, and mutations in p16 increase the risk of developing a variety of cancers.[32]

Inactivation of p16 by aberrant methylation of CpG islands is a frequent event in carcinomas and precancerous lesions of various organs, including the stomach. Gastric cancers with higher numbers of methylated genes have more distinct DNA methylation profiles than the originally defined CIMP-positive GCs. DNA methylation of tumor-related genes accumulates in conjunction with tumor progression. HepG2 cells treated with 5-Aza-cdR, showed demethylation of the p16 gene. The p16 mRNA and protein were all increased dramatically, cell cycle was arrested in G1, apoptotic rate increased and implanted tumor grew more slowly.[33,34]

Gα12 is reported to be involved in tumor cell invasion and progression. However, the precise mechanisms by which Gα12 regulates proliferation of cancer cells are poorly understood. Thus, we aimed to investigate the effect of Gα12 on cellular proliferation and its underlying mechanism in HepG2 human hepatoma cells.

2. MATERIALS AND METHODS

A. Cell culture

A549, FRO, HepG2, HCT116, MCF7 cells were grown in RPMI1640 medium containing 10% FBS, 100 units/ml penicillin and 100 mg/ml. TPC-1, PLC/PRF5 cell lines were grown in DMEM medium supplemented with 10% FBS, 100 units/ml penicillin and 100 mg/ml. Cells were cultured in a 5% CO2 incubator at 37℃.

B. Small interfering RNA transfection

Gα12 was knocked down using ON-Target plus-SMARTpool pooled siRNAs. A nonspecific control pool was used for the negative control. Cells were transfected with siRNA using Lipofectamine 2000 reagents according to the manufacturer's guidelines. Briefly, 100 pmol of siRNA and Lipofectamine 2000 were added separately to OPTI MEM medium. After 5 min, the two solutions were mixed and incubated for 20 min at room temperature. The mixture was added to monolayer of cells seeded in 10 cm tissue culture plates. The media were added with complete cell culture medium after 6 h and whole-cell lysates were prepared 48 h later for detection of protein expression.

C. Western blot analysis

The whole cell lysates were prepared with lysis buffer (50 mM Tris-HCl, 150 mM Sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.5, and 2 mM EDTA, 1 mM sodium orthovanadate, 0.1 mM phenylmethyl sulfonyl fluoride, 0.5% NP-40, protease inhibitor cocktail.) The protein concentration was determined by the bicinchoninic acid with bovine serum albumin as the standard. Equal aliquots of total cell lysates (50 ug) were solubilized in sample buffer and electrophoresed on denaturing SDS-

polyacrylamide gel (10% and 15% separating gel). The proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in TBS containing 0.05% Tween 20 and incubated with primary antibodies overnight at 4℃ and then with horseradish peroxidase-conjugated secondary antibodies for 2h at RT. Antigen-antibody complexes were detected with WEST-SAVE Up luminol-based ECL reagent.

D. RNA isolation and RT-PCR

Total RNA from each cell was isolated using Isol-RNA Lysis Reagent according to the manufacturer's protocol. The RNA samples were treated with DNase I, quantified, and reverse-transcribed into cDNA using the ReverTra Ace-α First Strand cDNA Synthesis kit.

RT–PCR was performed using primers specific for p16 (sense primer: 5'-GGAAATTGGAAACTGGAAGC -3', antisense primer: 5'- CTGCCCATCAT CATGACCTG-3') and GAPDH (sense: 5'- CAAGGTCATCCATGACAACT -3', antisense: 5'- TTCACCACCTTCTTGATGTC -3'). The cycling conditions were as follows: initial denaturation at 95℃ for 5min, followed 35cycles at 95℃ for 45s, 60℃ for 45s and 72℃ for 45s. Band intensities of the amplified DNAs were compared after visualization on an UV transilluminator.

E. Immunofluorescence.

HepG2 cells were grown on poly-D-lysine coated confocal dishs. Cells were transfected with Gα12 siRNA for 48h, washed in PBS, and fixed 10 min with 4% methanol. Cells were permeabilized 5 min with 0.1% Triton X-100, and blocked in 5% donkey serum. Cells were incubated with rabbit anti-p16 primary antibody at 1:50 in blocking buffer, followed by donkey anti-rabbit-FITC secondary antibody at 1:100. Antibody incubations were performed for 1 h at RT. All cells were also stained with PI.

F. Methylation-specific PCR (MSP)

Treatment of DNA with bisulfite converts cytosine residues to uracil, but leaves 5-methylcytosine residues unaffected. We used reagents supplied in Imprint DNA modification kit (Sigma, St. Louis, MO, USA) for bisulfite treatment, according to the one-step modification procedure recommended by the manufacturer. Briefly, 10 μl of DNA solution (100 ng) was combined with 110 μl of Imprint Bisulfite Modification Reagent, denatured at 99°C for 6 min and incubated at 65°C for 90 min. For purification of bisulfite-treated DNA, we used the Spin column in Imprint DNA modification kit. The modified DNA was then stored at −20°C until use. For MSP, the modified DNA was amplified with Taq polymerase. The methylated or unmethylated p16 primer sets were: unmethylated p16 sense, 5'- TTTGAGGGATAGGGTTGGAG -3', antisense, 5'- CTCCCCTTTTTCCAAAAAATCA -3'; methylated p16 sense, 5'-TTGAGGGA TAGGGTCGGAG-3', antisense, 5'- CCCTTTTTCCGAAAAA TCGAAA -3'.

G. Methylated DNA Immunoprecipitation (MeDIP)

1 mg of DNA extracted from cell lines. 5mC immunoprecipitation was carried out using the EpiQuik Methylated DNA Immunoprecipitation Kit according to the manufacturer's specifications. First, wells were washed once with Wash Buffer (WB; CP1) and then incubated at RT for 60 min in the presence of 100 ml of Antibody Buffer (AB; CP2) supplemented with 1 ml of 5mC antibody (or 1 ml of Normal Mouse IgG, as a negative control). DNA samples, diluted with ChIP Dilution Buffer (CP4), were added into the assay wells and incubated at RT for 90 min on an orbital shaker. The wells were washed six times with 1 WB, followed by the addition of TE Buffer. Afterwards, the DNA Release Buffer containing proteinase K were added to each well and samples were incubated at 65℃ for 15 min. Then, samples were incubated in Reverse Buffer at 65℃ for 30 min, Binding Buffer were subsequently added to

the wells, and the released samples, transferred to the F-Spin column, were centrifuged at 14 000 g for 20 s. Purified DNA was eluted in 15 ml of Elution Buffer. PCR was performed using immuno- precipitated DNA. PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining.

H. DNMT Activity Assay

DNMT activity was determined in the nuclear extracts using the EpiQuik DNA Methyltransferase Activity Assay Kit (Epigentek Inc., Brooklyn, NY, USA) following the manufacturer's protocol. Briefly, DNMT enzymes transfer a methyl group to cytosine from Adomet to methylate the DNA substrate, and the methylated DNA can be recognized with an anti-5-methylcytosine antibody. The ratio or amount of methylated DNA, which is proportional to enzyme activity, can then be measured using an ELISA-like reaction by reading the absorbance at 450 nm by the Tecan Infinite M2000 spectrophotometer. The DNMT activity was expressed as a percentage of the corresponding siRNA control.

I. Cell cycle analysis

The DNA contents of cell were measured by the PI (Sigma, St Louis, MO, USA) staining method. Cells (1 X 10^6) were harvested by trypsinization and fixed with 70% ethanol for at least 2 h at -20℃. Fixed cells were rinsed twice with PBS, and re-suspended in PBS containing 50 μg/ml RNase A (Sigma, St Louis, MO, USA), then incubated in 37℃ for 30 min. PI was added to the cells suspension and incubated in the dark for 20 min. Stained cells were analyzed by a FACScan flow cytometry.

J. Cell Growth Assay

Cell growth was determined by cell count. A total of 1×10^4 cells were plated in a 6cm plate in complete medium. The following day, cells were transfected with Gα12 siRNA . Cell numbers were counted using a hemocytometer for 3days at 24h intervals.

K. Soft Agar Assay

1.5×10^4 HepG2 cells, expressing sictrl or siGα12, were mixed with 0.35% soft agar and plated on top of a 0.5% bottom agar in a 6-well plate. Cells were incubated at 37°C for 2 weeks to allow colony formation. Each cell line was plated in triplicate. Three random low-power view fields were chosen, and the total number of colonies was counted.

L. Statistical analysis

Results are expressed average ± S.D. Statistical analysis are as performed by student's t-test. Relationships were considered statistically significant when p-value was less than 0.05.

3. RESULTS

A. Effect of Gα12 knockdown on p16 expression in various cancer cell lines

We screened the expression of the p16 gene in various cancer cell lines. p16 basal mRNA level was low detected in HepG2, A549, MCF7 cell lines compare with other cell lines. To determine whether Gα12 regulated p16 expression, cancer cells were transiently transfected with Gα12 siRNA. However, down-regulation of Gα12 was restored p16 expression in HepG2 cells.

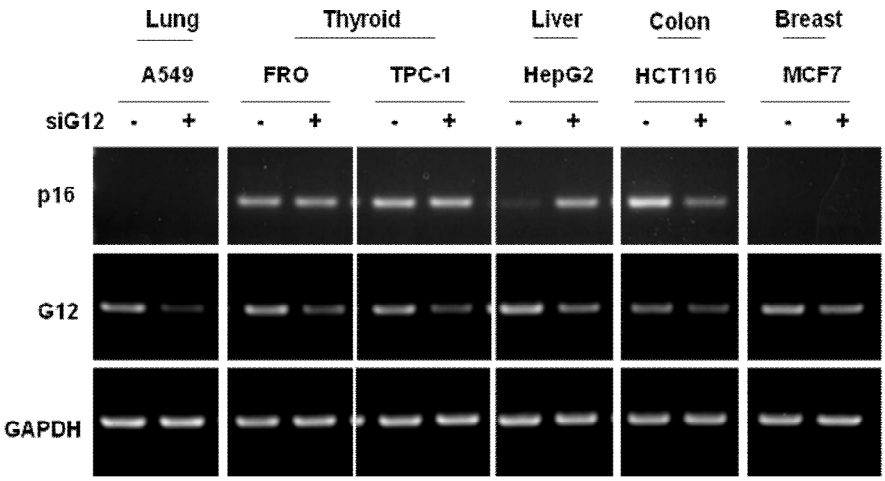


Figure7. Effect of Gα12 knockdown on p16 expression in various cancer cell lines. Cancer cells were transient transfected with Gα12 siRNA for 48h. p16 mRNA level detected by RT-PCR.

B. **Re-expression of p16 by siGα12 revealed in HepG2 cells**

We screened the expression of the p16 in 2 hepatoma cancer cell lines. p16 basal mRNA level was very weakly detected in HepG2 cells and PLC/PRF5 cell lines (Figure 8.A). p16 expression was significantly enhanced in HepG2 cells after siGα12 transfection (Figure 8.B). We confirmed increased p16 expression level when blocked Gα12 signal from immunofluorescence study, western blot, and RT-PCR in HepG2 cells (Figure 8.C). Therefore all experiments were proceeding with HepG2 cells.

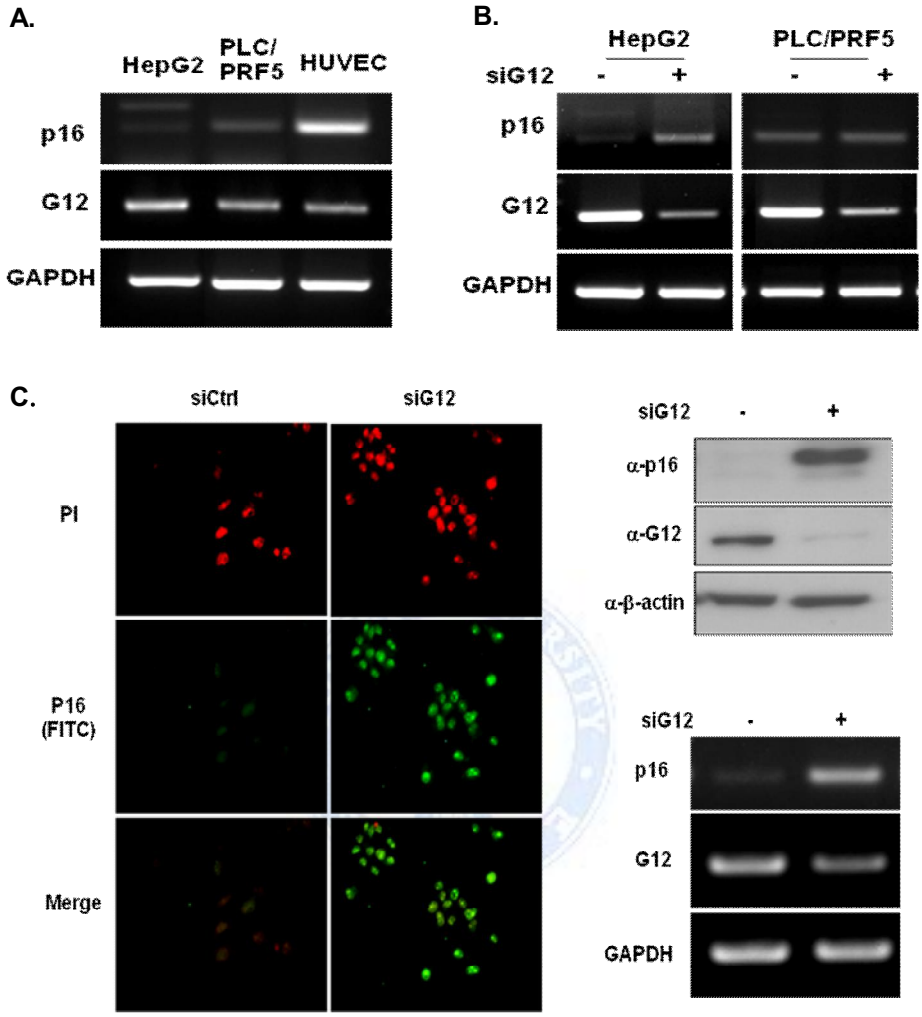


Figure 8. Restoration of p16 expression by siGα12 in HCCs. (A) Total RNA isolated from 2 HCCs and HUVEC normal control cells. Gα12 and p16 mRNA basal level detected by RT-PCR (B) HepG2 and PLC/PRF5 cells were transfected with Gα12 siRNA for 48h. p16 mRNA expression levels were measured by RT-PCR (C) Immunofluorscence, western blot and RT-PCR for Gα12 in HepG2 cells reveal restoration of p16 protein and mRNA levels by Gα12 siRNA.

C. DNA methylation by Gα12 in HepG2 cells

HepG2 cells were treated with 5-Aza-dC or transfected siGα12. 5-Aza-dC treated cells induced p16 expression and we obtained similar result by siGα12 (Figure 9.A). P16 is also frequently inactivated in several cancers via DNA methylation. Therefore, we examined whether Gα12 induces p16 promoter demethylation. Methylation of the p16 promoter CpG island was assessed by MeDIP analysis and MSP on genomic DNA isolated from HepG2 cells. According to data from MSP, down regulation of Gα12 induced demethylation of p16, suggesting that expression of p16 is up-regulation by siGα12 via promoter demethylation. The same effect was obtained when cells were treated 5'-Aza-dC (Figure 9.B). MeDIP assay enabled the purification of enriched methylated-DNA by direct immunoprecipitation of the 5′-methylcytosine modification related to the CpG sites of the p16 promoter. As shown in here, siGα12 decreased 5’-methylcytosine on the promoters of p16 (Figure 9.C).

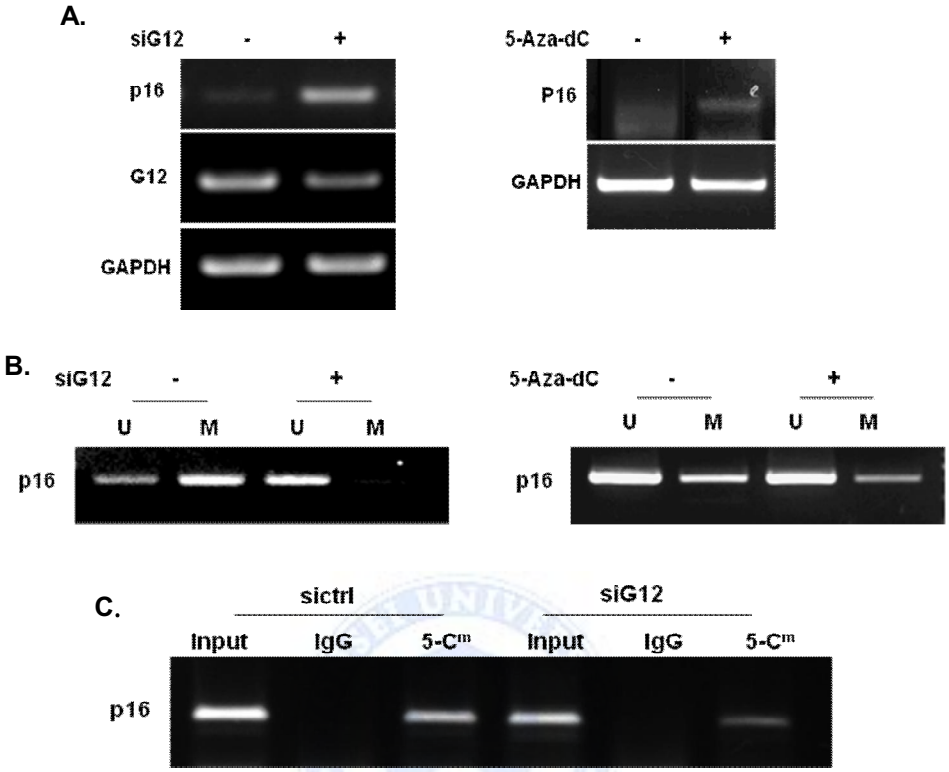


Figure 9. Methylation of p16 promoter was controlled by Gα12 in HepG2 cells. (A) Effects of Gα12 and 5-Aza-dC on promoter methylation and expression of p16 in HepG2 cells. HepG2 cells were transfected with siGα12 RNA for 48h or treated 5-Aza-dC (10 μM) for 5days. P16 expression was evaluated by RT-PCR. (B) Methylation of p16 promoter analyzed by MSP. MSP analysis of p16 promoter compared between siCtrl and siGα12 transfected cells (left). P16 methylation in response to exposure to 5-Aza-dC for 5 days (right). U; unmethylated, M; methylated DNA. (C) MeDIP assay was performed to promoter methylation status. Immunoprecipitation of genomic DNA from HepG2 cells with antibody against 5-C^m followed by RT-PCR analysis of MeDIP. Input as DNA control and IgG as negative control.

D. Down-regulation of Gα12 decreases DNMT1-mediated p16 methylation

We next aimed to gain insights into the mechanisms that underlie hypermethylation in HepG2 cells. A crucial step in DNA methylation involves DNA methyltransferases that catalyze methylation of CpG dinucleotides in genomic DNA. To gain insights into the possible involvement of DNMTs, we performed RT-PCR on total RNA samples isolated from siGα12 transfected HepG2 cells. DNMT1 mRNA was decreased by Gα12 knockdown. The same effect was obtained when cells were treated 5'-Aza-dC (Figure 10.A). In addition, the DNMT activity in the nuclear protein extract of siGα12 cells decreased compared with sictrl (Figure 10.B), similar to the mRNA and protein pattern of DNMT1 by 5'-Aza-dC. From these results, we can show that Gα12 regulates p16 expression by epigenetic modification mechanism by inducing DNMT1 expression and activation.

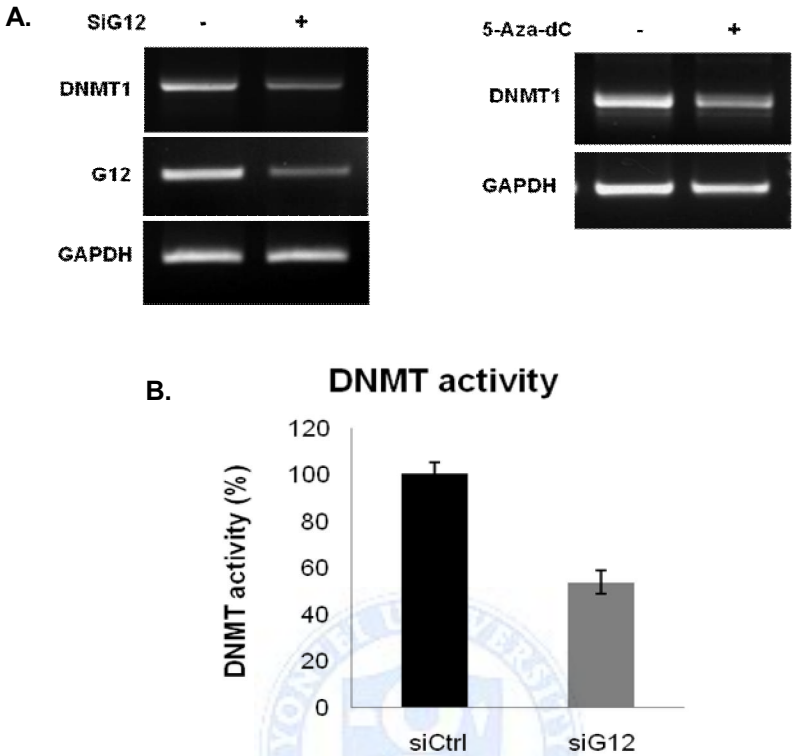


Figure 10. DNMT1 mediates epigenetic modification by Gα12 knock down. (A) HepG2 cells were transfected with DNMT1 siRNA for 48h or treated 5-Aza-dC for 5days. DNMT1 and Gα12 mRNA levels were examined in G12-knocked down cells by RT–PCR. (B) Quantification of enzyme activity of DNMT1. HepG2 cells were transfected with siGα12 RNA. DNMT activity was determined in the nuclear extracts, and the methylated DNA can be recognized with an anti-5-methylcytosine antibody. The ratio or amount of methylated DNA, which is proportional to enzyme activity, can then be measured using an ELISA-like reaction by reading the absorbance at 450 nm.

E. **Regulation of Histone modification by Gα12**

To investigate the histone modification by siGα12, we performed Modified Histone peptide array. Histone peptide array is experiment for the assessment of change in various histone modifications, such as methylation, acetylation, and phosphorylation modification. We obtained outstanding result that siGα12 increased Histone H3K27 acetylation. This result indicated that Gα12 alter chromatin structure by modifying histone tails.

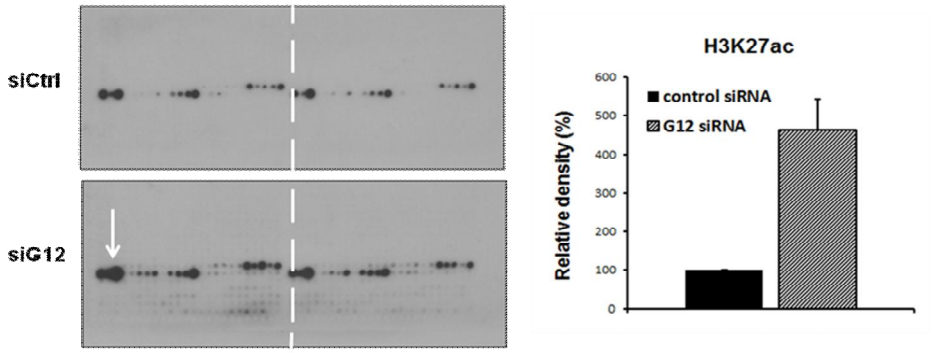


Figure 11. Regulation of histone modification by Gα12. Protein extracts were prepared from cells 48 h after Gα12 siRNA transfection on the Modified Histone Peptide Array. P16 primary antibody was used at a 1:2000 dilution for overnight at 4℃. Anti-rabbit HRP secondary antibody was used at a 1:2500 dilution, followed by ECL detection. Active motif's Array Analysis Software was used to analysis spot intensity.

F. Effect of Gα12 knock down on HepG2 cells proliferation

Next, to determined physiological roles of p16 in liver cancer, we performed functional studies. Flow cytometry was performed to observe the effects of Gα12 down regulation on cell cycle progression. The proportion of cells in the G0/G1 phase was significantly higher in the siGα12 transfected cells compared to the proportion of cells in the sictrl cells (Figure 12.A). And we were obtained result from cell counting assay that cell number was decreased in Gα12 knockdown (Figure 12.B). The levels of cell cycle regulatory proteins, such as phospho-Rb, E2F and CDK4, were also determined after siGα12 transfection. Cell cycle marker proteins were reduced by siGα12 (Figure 12.C). And we were obtained result from cell counting assay that cell number was decreased in Gα12 knockdown cells. We next examined the effect of Gα12 depletion in liver cancer cells on *in vitro* tumorigenicity using the soft agar colony formation assay. Consistent with the proliferation assays, siGα12 cells approximately decreased two fold in colony formation numbers. Colony size was reduced in Gα12 knockdown cells (Figure 12.D).

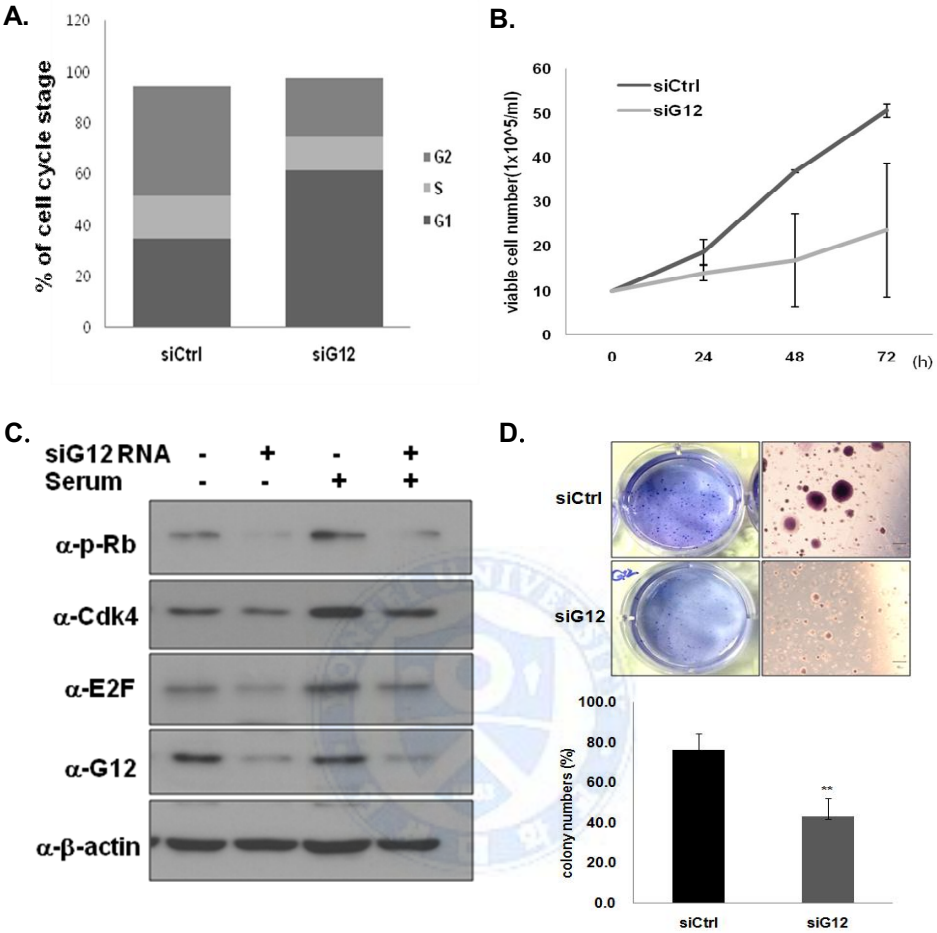


Figure 12. Gα12 promotes hepatoma carcinoma cell proliferation and tumorigenicity in HepG2 cells. (A) Cell cycle analysis of Gα12 knockdown HepG2 cell by flowcytometry. (B) Cell growth was determined by cell count assay. HepG2 cells were transfected with Gα12 siRNA. Cell numbers were counted using a hemocytometer for 3 days at 24h intervals. (C) Expression of cell cycle regulatory proteins E2F, Cdk4 and phospho-Rb in Gα12 knockdown cells. Western blots were performed on lysates from siGα12 cells. Comparable density of β-actin levels confirms equal loading of total protein. (D) Analysis of *in vitro* tumorigenicity by soft agar assay. HepG2 cells transfected with siGα12

were seeded in soft agar and maintained for 2 weeks. Colonies were stained with crystal violet. Results are representative of at least three independent experiments performed in triplicates. Data are expressed as average ± SD *$p<0.05$;**$p<0.01$.

4. DISCUSSION

The G12 class includes Gα12 and Gα13 and is ubiquitously expressed in mammalian tissues and cells. The last of the four families to be identified, the G12 family has been of particular interest to cancer researchers, since its members were found to promote the growth and oncogenic transformation of murine fibroblasts. Many studies have suggested a role for the members of the Gα12 family of heterotrimeric G proteins in oncogenesis and tumor cell growth.[35,36]

In breast and prostate cancer cells, previous studies showed a role of Gα12 and of Gα13 in invasiveness, but not in tumor cell proliferation.[16,17] In contrast, Grzelinski M et al. reported that in SCLC cells, the downregulation of either Gα12 or Gα13 leads to a clear inhibition of proliferation in vitro as well as in vivo.[37] Our observations indicate that in hepatoma cells, the downregulation of Gα12 leads to a clear inhibition of proliferation *in vitro* and the anchorage-independent colony formation. This finding further supports the concept that intact Gα12 signaling is a prerequisite for tumor growth in hepatoma cancer cells. Moreover, Gα12 siRNA restored the expression of $p16^{Ink4}$ known to be silenced in many cancer cells and arrested the cell cycle progression of HepG2 cells.

Currently, p16 is considered a tumor suppressor protein because of its physiological role and downregulated expression in a large number of tumors. Inactivation of p16 by aberrant methylation of CpG islands is a frequent event in carcinomas and precancerous lesions of various organs.

Precancerous conditions with aberrant DNA methylation appear to generate HCCs rapidly. Liu, L. H. et al HepG2 cells treated with 5-Aza-dC, showed demethylation of the p16 gene. The p16 mRNA and protein were all increased dramatically, cell cycle was arrested in G1, apoptototic rate increased and implanted tumor grew more slowly.[38] We obtained similar result by siGα12. We

show that p16 hypermethylation is associated with HCC cell line and downregulation of Gα12 induces p16 promoter demethylation. According to data from MSP and MeDIP, we suggest that Gα12 expression is correlation with hepatocarcinogenesis through aberrant p16 hypermethylation.

Increased DNMT1 mRNA expression in a number of human cancers has been reported. Moreover, Sun *et al*. found that DNA methyltransferase mRNA levels were significantly higher in HCC and HCC cell lines. We confirmed that DNMT1 was increased in HepG2 cell, and DNMT1 mRNA was decreased by Gα12 knockdown. In addition, the DNMT activity in the nuclear protein extract of siGα12 cells decreased. From these results, we can show that Gα12 regulates p16 expression by epigenetic modification mechanism by inhibiting the activities and expression of DNMT. Sarkar et al reported that HDACi inhibited cell cycle progression, and reversed promoter methylation and silencing of three tumor suppressor genes: RARB2, p16 and p21. HDACi repressed MAP kinase I (ERK) activation and down-regulated DNMT1 levels. Recent studies suggest that DNA methylation in colon cancer cells and in NIH 3T3 cells may be regulated in some circumstances by ERK (MAP kinase1) activity.[39] c-JUN is also involved in protecting the promoter region of the tumor suppressor p16(INK4a), which is consistently methylated over time in c-JUN deficient cells.[40] The association of JNK1 with the epigenetic alternations, mainly the elevation of H3K4me3, H3K9me3, and the expression of EZH2 that is involved in both histone and DNA methylation indicates a pivotal role of JNK on the development of the human HCC.[41] Mitsui, H. indicated that important roles of both Ras/MAPK and Ras/Rac/JNK cascades in activated Gα12-induced G1/S cell cycle progression.[42] Gα12 stimulates cell proliferation and neoplastic transformation of NIH3T3 cells by attenuating p38MAPK-associated apoptotic responses, while activating the mitogenic responses through the stimulation of ERK- and JNK-mediated signaling pathway. These observations expected that Gα12 is controlling the activity and expression of DNMT through the

stimulation of ERK- and JNK-mediated signaling pathway and this is expected to affect the methylation of p16 promoter.

p16 is a protein involved in regulation of the cell cycle. The p16-cyclin D1-CDK4-Rb pathway regulates cell cycle transition from the G1 to the S phase; point mutations and/or epigenetic modifications in this pathway are observed in almost all human cancers. Recent investigations have elucidated that G_1 to S phase progression requires activation of E2F family transcription factors, a process dependent on cyclin dependent kinase-mediated phosphor rylation and inactivation of pRb family proteins. It is also known that S phase entry is associated with transcriptional activation of the cyclin A gene.[43] The activated transcription factor up-regulates p16 expression. p16 forms a complex with Cdk4, leading to the redistribution of p21 and p27 from Cdk4 to Cdk2 and then revealed hypophosphorylation of pRb. The hypophosphorylated pRb eventually causes cell cycle arrest at G1.[44] From figure 6 data, the proportion of cells in the G0/G1 phase was significantly higher in the siGα12 transfected cells compared to the proportion of cells in the sicontrol cells. The levels of cell cycle regulatory proteins, such as phospho-Rb, E2F and CDK4, were also determined after siGα12 transfection. Cell cycle marker proteins were reduced by siGα12. And we were obtained result from cell counting assay that cell number was decreased in Gα12 knockdown. Therefore, reactivated p16 by Gα12 knockdown may affect the growth of the HCC cell line.

In conclusion, Gα12 siRNA inhibits the proliferation of HepG2 cells by upregulating p16 expression, suggesting that the abnormal proliferation of HepG2 cells might be resulted from Gα12 signaling to suppress p16 expression of HepG2 hepatoma cells. And this study finds, for the first time, which Gα12 protein regulate cellular signaling pathway via epigenetic modification of target gene promoter.

Part Ⅲ. Galpha12 inhibits serum deprivation induced-apoptosis of endothelial cells by miRNA regulation

1. INTRODUCTION

Vascular complications remain a leading cause of morbidity and mortality in subjects with diabetes, and experimental evidence suggests that progression of diabetes is associated with profound endothelial dysfunction. The endothelial cells play a major role in securing body homeostasis. Under pathological conditions, endothelial dysfunction is a driving force in the initiation and development of atherosclerosis. Apoptosis is one of the central mechanisms leading to endothelial dysfunction and results in inflammatory cell infiltration, lipid transport and neointima formation.[42] These alterations induce atherosclerotic lesion rupture and later clinical complications. Therefore, inhibiting ECs apoptosis is a promising novel therapeutic option against atherosclerosis in diabetes.[45]

MicroRNAs (miRNAs) are a family of highly conserved, small non-coding RNAs that posttranscriptionally repress gene expression via degradation or translational inhibition of their target mRNAs.[46] There is mounting evidence suggesting that miRNAs are involved in nearly all physiological and pathological processes. Early studies have revealed a significant role of miRNAs in ECs inflammation, migration, senescence, proliferation, and apoptosis [47]. miR-155 represents a typical multifunctional miRNA. miR-155 is involved in numerous biological processes including haematopoiesis, inflammation, immunity, cancer, and cardiovascular diseases. miR-155 was increased expression of heme oxygenase 1, a stress-inducible enzyme that exerts anti-inflammatory effects in the endothelium, and plays a protective role in cardiovascular diseases, including atherosclerosis. In HUVECs, miR-155 targeted the 3′ UTR of eNOS. miR-155 overexpression decreased whereas miR-155 inhibition increased eNOS expression and NO production in ECs.[48]

And miR-155 is upregulated by unidirectional shear stress and targets the angiotensin-II type-1 receptor and Ets-1, which reduces the proinflammatory activity of angiotensin II on ECs.[47] Gα12 is reported to be involved in cell proliferation, transformation, tight junction assembly and cell migration. However, the mechanism by which Gα12 regulates apoptosis of vascular endothelial cells is poorly understood. . Thus, we aimed to investigate the role of Gα12 in serum withdrawal-induced apoptosis of HUVECs and its underlying mechanisms.

2. MATERIALS AND METHODS

A. Cell culture

Human umbilical vein endothelial cells (HUVECs) (Invitrogen Life Technology, CA, USA) were grown in endothelial cell growth medium-2 supplemented with 2% fetal bovine serum (FBS), 0.04% hydrocortisone, 0.4% human epidermal growth factor (hEGF)-B, 0.1% vascular endothelial growth factor (VEGF), 0.1% R3-insulin like growth factor (IGF)-1, 0.1% ascorbic acid, 0.1% hFGF, 0.1% GA-1000, and 0.1% heparin (Lonza, Basel, Switzerland). Cells were used between passages 4 and 9 in this study. Cells were cultured in a 5% CO2 incubator at 37℃.

B. Small interfering RNA transfection

Gα12 was knocked down using ON-Target plus-SMARTpool pooled siRNAs. A nonspecific control pool was used for the negative control. Cells were transfected with siRNA using Lipofectamine 2000 reagents according to the manufacturer's guidelines. Briefly, 100 pmol of siRNA and Lipofectamine 2000 were added separately to OPTI MEM medium. After 5 min, the two solutions were mixed and incubated for 20 min at room temperature. The mixture was added to monolayer of cells seeded in 10cm tissue culture plates. The media were replaced with complete cell culture medium after 6 h and whole-cell lysates were prepared 48 h later for detection of protein expression.

C. Western blot analysis

The whole cell lysates were prepared with lysis buffer (50mM Tris-HCl, 150mM Sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.5, and 2mM EDTA, 1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, protease inhibitor cocktail.) The

protein concentration was determined by the bicinchoninic acid assay (BCA protein assay kit, Pierce) with bovine serum albumin as the standard. Equal aliquots of total cell lysates (40ug) were solubilized in sample buffer and electrophoresed on denaturing SDS-polyacrylamide gel (10% and 15% separating gel). The proteins were transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in TBS containing 0.05% Tween 20 and incubated with primary antibodies overnight at 4℃ and then with horseradish peroxidase-conjugated secondary antibodies for 2hr at RT. Antigen-antibody complexes were detected with WEST-SAVE Up luminol-based ECL reagent.

D. TUNEL assay

HUVEC cells were seeded on confocal dish and transfected with Gα12 siRNA. After Gα12 siRNA transfection for 48h, apoptosis was determined by terminal TUNEL assay using a kit methodology. Briefly, cells were washed once with PBS and fixed in 4% paraformaldehyde for 1 h at 25℃. Cells were washed with PBS and incubated in permeabilization solution (0.1% triton X-100 in 0.1% sodium citrate) for 2 min on ice. They were then washed twice with PBS and treated with TUNEL reaction mixture for 60 min at 37℃ in the dark in a humidified atmosphere. Finally, the cells were washed three times with PBS ananalyzed under fluorescence microscope, using an excitation wavelength in the range of 450 - 500 nm and a detection wavelength in the range of 515 - 565 nm (green).

E. miRNA qRT-PCR detection and quantification

miRNAs were isolated using the mirVana kit and protocol (Ambion, Austin Texas, USA). miRNAs were reverse transcribed into cDNAs with a TaqMan MicroRNA reverse transcription kit using microRNA-specific primers (Applied

Biosystems, Foster City, CA, USA). Briefly, for reverse transcription reactions, 30 ng miRNA was used in each reaction and mixed with the RT primer. RT reaction was carried out at 16◦C for 30 minutes, 42◦C for 30 minutes, and 85◦C for 5 minutes. The quantitation of miRNA levels was performed by real-time PCR (StepOnePlus; Applied Biosystems, Foster City, CA, USA) using TaqMan gene expression assays and TaqMan MicroRNA assays, respectively. The amplification steps consisted of denaturation at 95◦C, followed by 40 cycles of denaturation at 95◦C for 15 s and then annealing at 60◦C for 1 min. All reactions, including controls were performed in triplicate. Relative expression of miRNAs was analyzed using $2^{-\Delta\Delta}$Ct method and was normalized by RNU6B expression for all samples.

F. miRNA mimic, inhibitor transfection

Cells were transfected with miR-155 mimic and inhibitor by Lipofectamine 2000 according to the manufacturer's protocol. After transfection, cells were used in subsequent assays. The level of transfected miR present in HUVECs was determined by quantitative real-time -PCR. Transfection of HUVEC was performed with stability-enhanced mature miR-155 mimics (miR-155, sense: 5'-CUCCUACAUAUUAGCAUUAACA-3'; antisense: 5'-UGUUAAUGCUAA UAUGUAGGAG-3') and the negative control miR (miR-NC, sense: 5'-CCUCG UGCCGUUCCAUCAGGUAG-3'; antisense: 5'-CUACCUGAUGGAACGGA CGAGG-3'). Both miR-155 mimics and miR-NC were double-stranded RNA oligonucleotides, while miR-155 inhibitors were single-stranded RNA oligon-ucleotides conjugated with 2' - *O* -methoxyethyl phoshorothioate (Genolution, Seoul, Korea).

G. Statistical analysis

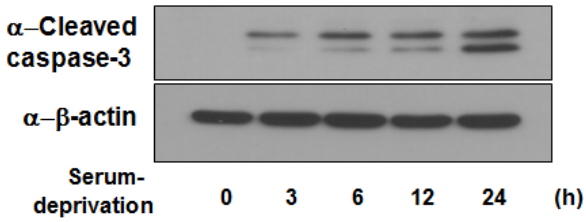
Results are expressed average ± S.D. Statistical analysis are as performed by student's t-test. Relationships were considered statistically significant when p- value was less than 0.05.

3. RESULTS

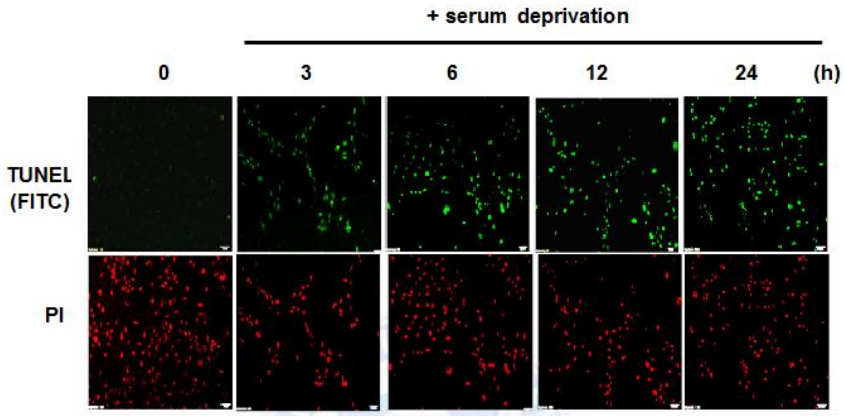
A. Gα12 siRNA augments serum withdrawal-induced apoptosis of HUVECs

To examine the effects of serum withdrawal on apoptosis of HUVECs, HUVECs were deprived from serum for indicated time and assessed for apoptosis by Western blotting using antibody against cleaved caspase-3 and by TUNEL assay to label apoptotic cells that were viewed under fluorescent microscopy. Serum withdrawal induced the apoptosis in time-dependent manner and 3h after serum free condition HUVECs were undergoing and proceeding apoptosis until 24 h after serum withdrawal (Figure 13.A & B). To investigate the role of Gα12 in serum withdrawal-induced apoptosis, HUVECs were transfected with Gα12 siRNA to knock down expression of endogenous Gα12, and then serum-deprived for 6 h to induce apoptosis. The downregulation of Gα12 expression was confirmed at the levels of both mRNA and protein by RT-PCR and Western blotting, respectively. As shown in Figure 13C & D, Gα12 siRNA markedly increased the serum withdrawal-induced apoptosis of HUVECs up to 367% compared to control siRNA-transfected cells in the level of cleaved caspase-3, which was confirmed by TUNEL assay. The knockdown of Gα12 expression also augmented the serum withdrawal-induced p38 activation that has been known to mediate the apoptosis of numerous cells including endothelial cells. These results show that serum-deprivation induced the apoptosis of HUVECs, which was augmented when Gα12 expression was knockdown in HUVECs, suggesting that Gα12 might protect vascular endothelial cells from endothelial damages such as apoptosis.

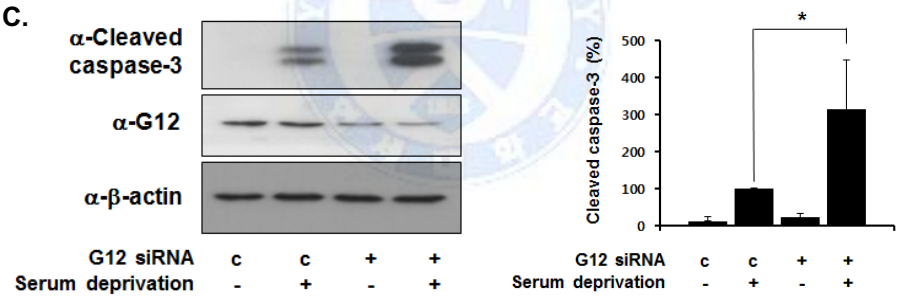
A.


α–Cleaved caspase-3
α–β-actin
Serum-deprivation
0
3
6
12
24
(h)

B.


+ serum deprivation
0
3
6
12
24
(h)
TUNEL (FITC)
PI

C.


α-Cleaved caspase-3
α-G12
α-β-actin
G12 siRNA c c + +
Serum deprivation - + - +
Cleaved caspase-3 (%)
500
400
300
200
100
0
*
G12 siRNA c c + +
Serum deprivation - + - +

D.

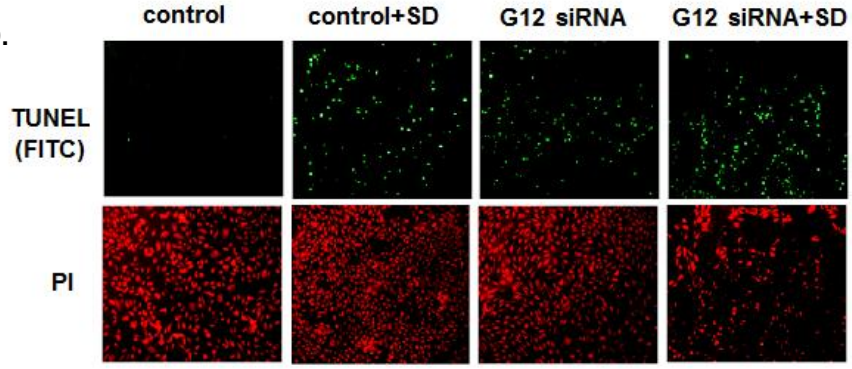

control
control+SD
G12 siRNA
G12 siRNA+SD
TUNEL (FITC)
PI

Figure 13. Effect of Gα12 siRNA on serum deprivation-induced apoptosis of HUVECs. (A) HUVECs were cultured in serum-free EBM or 2% fetal bovine serum-containing EGM as a control. After the indicated time points, these cells were analyzed by immunoblot using an antibody against cleaved caspase-3.(B) Microscopic detection of apoptotic cells by TUNEL assay at 0, 3, 6, 12, or 24h, respectively, after serum deprivation. (C) HUVEC cell transfected with Gα12 siRNA. After 48h, these cells were deprived serum for 6 h, and then harvest and lysised for immunoblot analysis against cleaved-caspase3. (D) Microscopic detection of apoptotic cells by TUNEL assay after siGα12 RNA transfection for 48h and then serum-deprived for 6 h to induce apoptosis.

B. Gα12 is related to the regulation of microRNAs expression

To investigate how Gα12 regulate serum withdrawal-induced apoptosis, we examined whether Gα12 is related to the regulation of miRNAs expression. HUVECs were transfected with Gα12 siRNA, and these cells were assessed for the expression of miRNAs, miR-17-3p, miR-31, miR-155 and miR-191 that have been reported to be involved in the regulation of endothelial damages including inflammation and apoptosis. Gα12 siRNA reduced the expression of miR-17-3p, miR-31and miR-191 and remarkably suppressed the miR-155 expression that has been known to regulate apoptosis of endothelial cells.

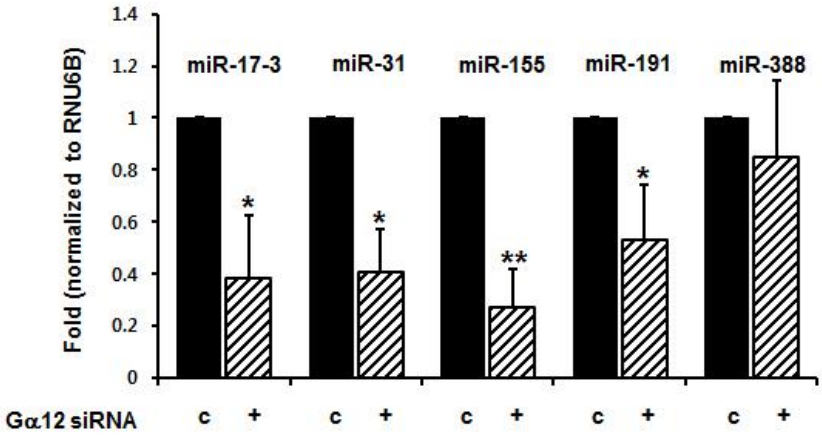
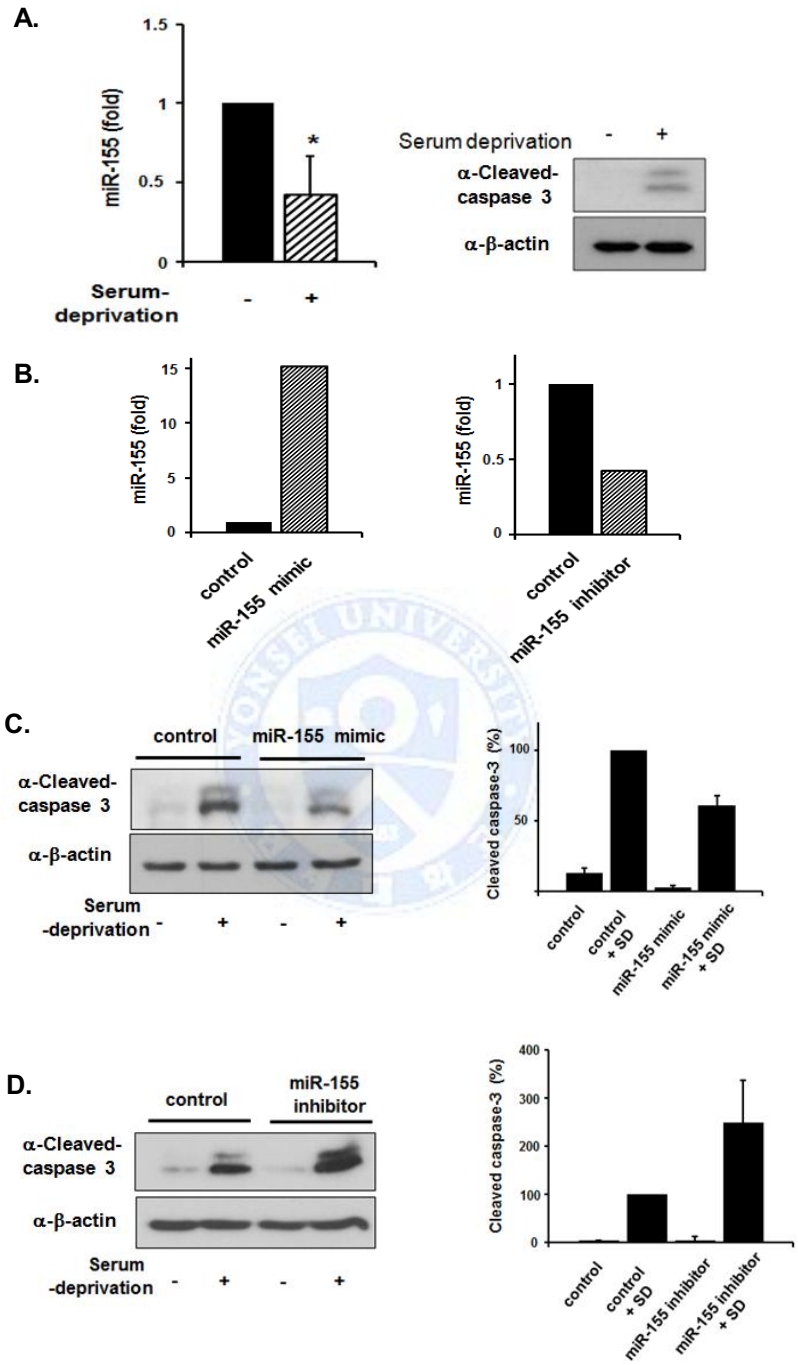


Figure 14. Gα12 is related to the regulation of miRNAs expression. HUVEC cell transfected with G12 siRNA for 48 h. Relative expression levels of miR-17-3p, -31, -155, -191 and -338 were analyzed using qRT-PCR. Data are expressed as average ±SD, $*p<0.05$, $**p<0.01$, $***$ $p<0.005$ compared to control group.

C. Gα12 siRNA augments apoptosis of HUVECs by suppressing the expression of miR-155

To examine the relationship between miR-155 and apoptosis, HUVECs were cultured in serum free conditions for 6 h, and then analyzed for expression of miR-155, which showed that serum withdrawal significantly suppressed the expression of miR-155 by 2 fold (Figure 15.A). To confirm the effects of miR-155 on apoptosis of HUVECs, HUVECs were transfected with miR-155 mimic or miR-155 inhibitor, which were confirmed by analyzing the elevated or diminished level of miR-155 compared to control-transfected cells, respectively (Figure 15.B). The overexpression of miR-155 by miR-155 mimic protected HUVECs against serum withdrawal-induced apoptosis and the inhibition of miR-155 expression by miR-155 inhibitor enhanced the apoptosis of HUVECs, showing that miR-155 is involved in the regulation of apoptosis of HUVECs (Figure 15.C & -D). To examine the role of miR-155 in the regulation of Gα12 siRNA-mediated apoptosis, HUVECs were cotransfected with Gα12 siRNA and miR-155 mimic, which showed that miR-155 mimic significantly abolished the serum withdrawal-induced, Gα12 siRNA-mediated apoptosis and p38 phosphorylation of HUVECs (Figure 15. E), indicating that Gα12 regulates the apoptosis of vascular endothelial cells by regulating the expression of miR-155.


A.
miR-155 (fold)
1.5
1
0.5
0
*
Serum-deprivation
-
+
Serum deprivation
-
+
α-Cleaved-caspase 3
α-β-actin
B.
miR-155 (fold)
15
10
5
0
control
miR-155 mimic
miR-155 (fold)
1
0.5
0
control
miR-155 inhibitor
C.
control
miR-155 mimic
α-Cleaved-caspase 3
α-β-actin
Serum -deprivation
-
+
-
+
Cleaved caspase-3 (%)
100
50
control
control + SD
miR-155 mimic
miR-155 mimic + SD
D.
control
miR-155 inhibitor
α-Cleaved-caspase 3
α-β-actin
Serum -deprivation
-
+
-
+
Cleaved caspase-3 (%)
400
300
200
100
0
control
control + SD
miR-155 inhibitor
miR-155 inhibitor + SD

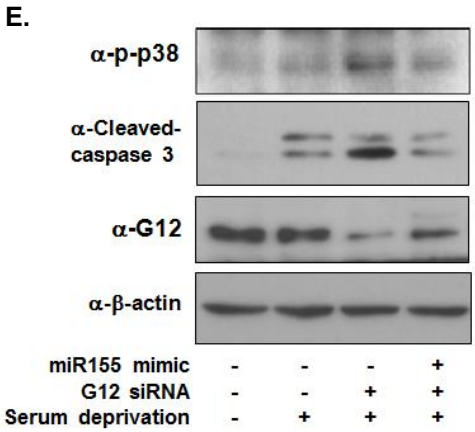


Figure 15. Effect of miR-155 on serum deprivation-induced Gα12 siRNA-mediated apoptosis in HUVECs. (A)Effect of serum starvation on miR-155 miRNA expression. (B, C and D) Cleaved caspase-3 expression in HUVECs after serum deprivation, miR-155 mimics or miR-155 inhibitor transfection. (C&D) Effect of Gα12 on miR-155 expression under condition of serum withdrawal. HUVECs were transfected with miR-155 mimic/inhibitor. After 24h, these cells were incubated serum starved condition for 6h. And then harvested and lysed for immunoblot analysis against cleaved caspase-3 (E) Relevance of Gα12 and miR-155 on serum deprivation–induced apoptosis through activation of caspase-3 and phosphorylation of p38 MAPK. HUVECs were cotransfected with Gα12 siRNA and miR-155 mimic for 48 h, and then incubated in serum deprived condition for 6 h. After serum starvation, HUVECs were harvest and lysised for immunoblot analysis against cleaved caspase-3 and phospho-p38 MAPK.

4. DISCUSSION

In this study, we investigated the role of Gα12 in the regulation apoptosis of vascular endothelial cells and its underlying mechanisms, and we found that Gα12 protects HUVECs against serum withdrawal-induced apoptosis by inhibiting the suppression of miR-155 expression. This finding is supported by the results that Gα12 siRNA increased the serum withdrawal-induced apoptosis of HUVECs. Additionally, Gα12 siRNA markedly suppressed the expression of miR-155 and the restoration of miR-155 by transfection of miR-155 mimic abolished the enhanced apoptosis mediated by Gα12 siRNA, indicating that Gα12 could inhibit the apoptosis of HUVECs by retaining the expression of miR-155 against cell death signal in vascular endothelial cells.

G proteins, as a signal transducer, have known to be involved in the modulation of cellular responses, including proliferation, differentiation, migration and apoptosis. Several studies have shown that Gα12 is involved in the regulation of proliferation and migration. Gα12 has found to mediate the lysophosphatidic acid-induced proliferation of ovarian cancer cells and to simulate the expression of genes promoting cell growth in NIH3T3 cells.[49,50] Also, overexpression of constitutively active mutant of Gα12 promoted cell invasion of breast and prostate cancer cells[16,17] and migration of vascular smooth muscle cells (VSMCs) by inducing CYR61 expression.[51] Gα12 has also reported to exert pro-apoptotic effect in epithelial cells. Overexpression of constitutively active mutant of Gα12 stimulated the apoptosis of Madin-Darby canine kidney cells through JNK activation and also induced apoptosis of COS-7 cells by MAP kinase pathway including apoptosis-signal regulating kinase 1 (ASK1) and MAP kinase kinase 1 (MEKK1).[52,53] On the other hand, we showed that the blockade of endogenous Gα12 expression augmented the serum withdrawal-induced apoptosis of HUVECs, which indicated that Gα12

inhibits the apoptosis of HUVECs and protects them against cellular stress evoking endothelial dysfunctions. Thus it is suggested that the role of Gα12 in modulation of apoptosis might be dependent on cell type specificity. Vascular endothelial cells (ECs) are one of cells that composed blood vessel, which form the lining of inner surface of blood vessel. Injury or damage of ECs by inflammatory cytokines, oxidative stress, modified lipoproteins and apoptosis has been known to initiate the pathogenesis of vascular diseases such as atherosclerosis. Apoptosis of ECs has been regarded as an initial step to develop atherosclerosis and found in atherosclerotic plaque and also reported to be responsible for the formation of coronary thrombotic atherosclerotic plaque. Thus apoptosis of ECs could represent the ECs damage causing endothelial dysfunction and it is important to regulate the apoptosis of ECs to prevent and inhibit the pathogenesis of atherosclerosis. Thus we revealed the role of Gα12 in modulation of apoptosis of vascular endothelial cells and suggested that Gα12 might play a crucial role in vascular system by regulating damage of ECs.

This study also demonstrated the mechanisms by which Gα12 regulates the apoptosis of vascular endothelial cells. Serum-withdrawal resulted in the apoptosis with the repression of miR-155 and Gα12 siRNA remarkably suppressed miR-155 expression, which makes HUVECs more susceptible to apoptosis induced by serum deprivation. Using miR-155 mimic, we showed that the elevation of miR-155 expression inhibited the serum deprivation-induced apoptosis of HUVECs and furthermore abrogated the enhanced apoptosis that was resulted from blockade of Gα12 expression, indicating that the repression of miR-155 might be responsible for increased susceptibility to apoptosis of HUVECs. Taken together, our study supports the roles of Gα12 and miR-155 in protecting vascular endothelial cells from apoptotic cell death. The role of miR-155 has been found to be involved in the development of cancer and the pathogenesis of cardiovascular disease including atherosclerosis. miR-155 stimulated cellular proliferation and migration, and inhibited apoptosis of renal

cancer cells, indicating that miR-155 contributes to the oncogenesis of tumor cells.[54] However, the role of miR-155 in the pathogenesis of atherosclerosis has been in conflict. Silencing of miR-155 resulted in the enhanced inflammation and lipid uptake in macrophages, indicating that miR-155 is anti-atherogenic and other study showed that miR-155 exerts pro-atherogenic effects by repressing Bcl6 in macrophages.[55,56] Whereas, miR-155 is enriched in ECs and the effects of miR-155 have been thought to be anti-atherogenic in ECs, supported by finding that miR-155 inhibited angiotensin II (Ang II)-induced inflammation, migration and apoptosis of HUVECs by targeting Ang II type 1 receptor (ATR1).[57-59] In agreement with that, we found that overexpression of miR-155 inhibited serum withdrawal-induced apoptosis of HUVECs and prevented the enhanced apoptosis mediated by Gα12 knockdown, indicating that miR-155 acts downstream of Gα12 playing a protective role against apoptotic cell death. Although Gα12 siRNA markedly reduced the expression of miR-155, Gα12 siRNA alone did not induce the detectable apoptosis. Moreover, only the inhibition of miR-155 expression also could not induce apoptosis. miR-155 inhibitor induced the apoptosis when HUVECs were exposed to apoptotic stimulus, indicating that only the repression of miR-155 is not sufficient to induce apoptosis and makes HUVECs more susceptible to apoptosis, which resulted in the enhanced apoptosis compared to HUVECs that retain the expression of miR-155.

From these results, we conclude that Gα12 protects HUVECs against serum withdrawal-induced apoptosis by regulating the expression of miR-155, suggesting that endothelial apoptosis can be modulated by Gα12 protein signaling through regulation of miRNAs and the novel strategies to protect endothelial cells from endothelial dysfunctions by regulating Gα12 protein signaling.

Ⅳ. CONCLUSION

In part Ⅰ, XAF1 undergoes epigenetic silencing in a considerable proportion of lung cancer cell lines by aberrant CpG site hypermethylation of the gene promoter. In our data shown that siGα12 was enhanced demethylation of XAF1 promoter and was induced apoptosis. Therefore, our data presented here demonstrate that Gα12 was caused cancer cell progression and maintenance by controlling the methylation of XAF1.

Next, Gα12 siRNA inhibits the proliferation of HepG2 cells by upregulating p16 expression, suggesting that the abnormal proliferation of HepG2 cells might be resulted from Gα12 signaling to suppress p16 expression of HepG2 hepatoma cells.

Finally, Gα12 protects HUVECs against serum withdrawal-induced apoptosis by regulating the expression of miR-155. We suggest that novel strategies to protect endothelial cells from endothelial dysfunctions by regulating Gα12 protein signaling.

These results indicate that Gα12 regulates epigenetic modification in cancer and vascular cells.

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ABSTRACT (IN KOREAN)

G단백질 알파 12에 의한 암세포와 혈관내피세포의 epigenetic 조절

< 지도교수 이은직 >

연세대학교 대학원 의과학과

이현정

G 단백질(Guanine nucleotide binding protein)은 세포의 외부에서 들어오는 신호를 변화시켜 세포 내로 전달시켜주는 단백질 family 중의 하나이다. G 단백질은 metabolic enzyme과 ion channel, transporter 등을 조절한다. 또한 세포내의 전사 조절과 cell motility, contractility, 그리고 secretion 등을 조절하여 embryonic development, learning, memory 그리고 homeostasis 등을 변화시킨다. G12 family는 Gα12와 Gα13의 2개의 멤버로 구성되고 이들은 세포의 증식과 분화 그리고 세포의 사멸에 관련되어 있을 뿐만 아니라 세포 형태에도 영향을 준다. 또한 Gα12가 활성화되면 DNA 합성, 세포 증식과 악성으로의 변성 등이 촉진된다.

Epigenetic 변형은 DNA의 염기서열이 변화하지 않는 상태에서 세포가 분열되는 동안 DNA 또는 크로마틴의 변형을 통하여 유전자의 발현 양상이 다음 세대로 그대로 전달되는 현상을 말하며, 여기에는 methylation, histone modification, non-coding RNA, genomic imprinting 등이 속한다. 유전자의 promoter 에서 일어나는 methylation은 유전자의 발현을 억제하는 기능을 한다. Histone modification은 다양한 chromatin remodeling에 의해서 유전자의 발현 조절, 세포 사멸 조절, DNA 복제

및 repair 그리고 염색체의 응축 및 분열 등에 관여한다. miRNA는 target mRNA에 결합하여 해당 mRNA를 파괴하거나 translation을 억제하여 유전자 발현을 억제시키는 작용을 한다. 따라서 epigenetic modification이 정상적으로 이루어지지 않으면 암과 같은 질병들이 유발된다.

본 연구에서는 Gα12 단백질의 신호 전달이 epigenetic 변형을 조절하는지 살펴보고, 이러한 epigenetic 변형에 의한 생리학적 의미를 관찰하고자 하였다.

Part Ⅰ에서는 폐암세포주에서 Gα12가 세포의 apoptosis에 영향을 주는지 살펴보고, 이런 작용이 XAF1 프로모터 부분의 epigenetic 변형을 조절함으로써 일어나는 것인지 관찰하고자 하였다. 폐암세포주에서는 XAF1 mRNA가 거의 발현이 되지 않았다. 그러나 A549 세포주에 Gα12의 신호를 차단하였더니 XAF1의 발현이 회복되는 것을 확인할 수 있었다. 또한 MSP와 MeDIP 실험을 통해서 siGα12 에 의해 XAF1 프로모터의 메틸화가 감소되는 것을 확인하였다. 이러한 실험을 통해 siGα12에 의해서 XAF1 프로모터의 탈메틸화가 증가되고, 이로 인해 세포의 apoptosis 가 유도되는 것을 확인할 수 있었다.

Part Ⅱ 에서는 Gα12가 간암세포주인 HepG2 세포주의 세포증식에 영향을 주는지 살펴보고 그것이 일어나는 기전을 알아보고자 하였다. HepG2 세포주에서 거의 발현이 되지 않았던 p16의 발현이 Gα12의 knockdown에 의해 발현이 확연하게 증가되는 것을 확인하였다. MSP와 MeDIP 그리고 DNMT1의 발현을 보는 실험을 통해, Gα12가 p16 프로모터 부분의 메틸화를 조절함으로써 p16의 발현을 조절하고, 이러한 작용이 DNMT1을 통해서 일어나는 현상임을 알 수 있었다. 이러한 결과를 통해서 HepG2에서 Gα12 신호가 p16의 발현을 억제함으로써 HepG2 세포의 비정상적이 증식을 일으키는 것을 확인하였다.

Part Ⅲ 에서는 혈관내피세포인 HUVECs 에서 무혈청에 의해 유도된 apoptosis 에 Gα12가 미치는 영향을 살펴보고 그것이 어떠한 기전을 통해서 일어나는지 연구를 진행하였다. Gα12가 downregulation이 되면 HUVECs의 apoptosis가 증가되는 것을 확인할 수 있었다. miRNA 중에 miR-155가 HUVECs 의 apoptosis를 유도한다고 알려져 있었기 때문에 Gα12가 miR-155의 발현에 영향을 주는지 관찰하였다. 그 결과 siGα12가 miR-155의 발현을 억제함으로써 혈관내피세포의 apoptosis를 유도하는 것을 확인하였다. 이러한 결과들로 보아 Gα12가 miR-155 발현의 조절을 통해서 내피세포 기능장애의 원인이 되는 혈관 injuries로부터 혈관내피세포를 보호한다는 결과를 얻을 수 있었다.

이러한 실험 결과들을 통해서 Gα12가 암세포와 혈관내피세포에서 epigenetic 변형을 조절함으로써 다양한 생리학적 작용을 일으킨다는 결론을 얻을 수 있었고, 이로 인해 Gα12의 새로운 신호전달 기전을 제시하고자 한다.

핵심되는 말 : Gα12, epigenetic 변형, 암, 혈관질환, XAF1, p16, miRNA