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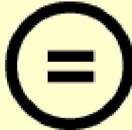

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약학석사학위논문

The Role of 14-3-3τ in EGF-induced Transformation of Human Keratinocytes

EGF 로 유도된 사람 피부 세포의 transformation 에서 14-3-3τ 의 역할

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Abstract

The Role of 14-3-3τ in EGF-induced Transformation of Human Keratinocytes.

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The 14-3-3 proteins, a family of conserved regulatory molecules, are expressed in all eukaryotic cells and have seven isoforms (β, γ, ε, ζ, η, σ, and τ) known in mammals. 14-3-3s are scaffold proteins that control of the signal transduction, direct other proteins to restricted specific pathway by interacting with many cellular proteins as a result of their specific phospho-serine/phospho-threonine binding activity. Recently, some of 14-3-3 isoforms were suggested tumor enhancers or suppressors. However, the roles of 14-3-3s in skin carcinogenesis has not been well studied yet. Here, I suggest that 14-3-3τ is involved in epidermal growth factor (EGF) - induced transformation of human keratinocytes. Knockdown of 14-3-3τ inhibited anchorage-independent cell transformation and proliferation of HaCaT induced by EGF. Moreover, phosphorylation of CREB (cAMP response element-

binding protein) and c-Fos expression induced by EGF was significantly suppressed by knockdown of 14-3-3τ. Furthermore, I revealed that 14-3-3τ directly binds with RSK2, a key regulator in EGF-induced skin cell transformation and direct kinase of CREB. Additionally I identified that Ser325 and Ser715 residues of RSK2 is important for interaction with 14-3-3τ. Taken together, these findings suggest that 14-3-3τ might have a role as a tumor inducer in EGF-induced transformation of human keratinocytes, through direct interaction with RSK2.

Keywords: 14-3-3τ, EGF, p-CREB, c-Fos, p-RSK2, human transformation of keratinocytes.

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Introduction

The highly conserved 14-3-3 protein family form a group of 28 – 33 kDa acidic polypeptides found in all eukaryotic organisms. There are seven mammalian 14-3-3 family members (β, γ, ε, ζ, η, σ, and τ) to date. With exception of sigma isoform, all 14-3-3 molecules exist as dimers in cells, forming both homo- and heterodimers. 14-3-3 proteins have risen to a position of importance in cell biology. The 14-3-3 proteins were the first phosphoserine/phosphothreonine (pSer/pThr) - recognition protein class. Two high-affinity 14-3-3 binding motifs have been described in 14-3-3 target proteins : RSXpSXP (mode 1) and RXXXpSXP (mode 2), where X represents any residue and pS represents a phosphoserine. (1) So, 14-3-3 proteins associates with many different molecules because of its specific phosphoserine/phosphothreonine-binding activity. Due to large number and diversity of interacting partners the 14-3-3 proteins have emerged as important components of essential biological processes including cell cycle control, apoptosis, cell adhesion, and neuronal plasticity, transcriptional regulation of gene expression and more. (2) Recently, a critical role of 14-3-3 proteins in cancer has been widely studied. Some isoforms of 14-3-3 are support the action of oncogenes. Especially, 14-3-3 ζ, play a pro-oncogenic role in multiple tumor types in head and neck cancer and lung cancer. Also ζ, β and τ isoforms of 14-3-3 have been reported to tumorigenesis in lung cancer. Other than 14-3-3 ζ, 14-3-3 σ, a suggested tumor suppressor, is silenced in cancer patient. (3-5) Whether other isoforms of 14-3-3 are

involved in human cancer is less clear and the involvement of 14-3-3s especially in skin carcinogenesis has not been well understood yet.

Also, there is a report showing that different 14-3-3s are detected in human skin. Among the isoforms, the level of 14-3-3 τ isoform which was not detectable in epidermis was easily detectable in keratinocytes. (6)

Cells recognize and respond to extracellular stimuli by specific growth factor, such as the signaling pathway that leads to activation of the mitogen-activated protein kinases. (MAPKs) The mitogen-activated protein kinases (MAPKs) are key regulators of proliferation, gene expression and tumorigenesis. The epidermal growth factor (EGF) binding to its tyrosine kinase receptor results in activation of the Ras-dependent mitogen-activated protein kinase (MAPK) cascade. (7) The MAPK extracellular signal-regulated kinases (ERKs) 1 and 2 mediate the phosphorylation of 90-kDa ribosomal S6 kinase (RSK), is a family of protein kinases involved in signal transduction. RSK2 is a member of the RSK (ribosomal S6 kinase) family that are growth-regulated serine/threonine kinases. Activated RSK2 is translocated into nucleus, then mediate growth factor signaling via RAS and MAPK leading to the induction of cyclic AMP-responsive element binding protein (CREB) serine-133 phosphorylation (8) ,activation of gene expression c-Fos (9), activating transcription factor 4 (ATF4) (10), p53 (11), NFAT3 (12) and ATF1 (13). Therefore, the RSK2 protein has important role in multiple cellular processes, such as proliferation and transformation as well as cell cycle regulation. Recently, it was reported that RSK2 is a key regulator in cell transformation induced by EGF

and 12-0-tetradecanoylphorbol-13-acetate (TPA) (14). These are well-known tumor promotion agents.

In the present study, I found the involvement of 14-3-3 protein, especially 14-3-3τ, in human skin cell transformation treated by EGF. Knockdown of 14-3-3τ effectively inhibited anchorage-independent HaCaT cell transformation and cell proliferation induced by EGF. Also, I demonstrated that 14-3-3τ was necessary for the activation of CREB and expression of c-Fos induced by EGF. In addition, I identified RSK2, a novel binding partner of 14-3-3τ and verified the binding sites of 14-3-3τ in RSK2.

Taken together, these findings suggest that 14-3-3τ might have a role as a tumor promotor in EGF-induced transformation of human keratinocytes through direct interaction with RSK2.

Materials and Methods

Materials

All culture media and other supplements were obtained from Gibco BRL (Grand Island, NY, USA). Chemical reagents for molecular biology and buffer preparation were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies against 14-3-3 τ, CREB, phosphorylated CREB (Ser 133), c-Fos, RSK2, ERK1/2, and phosphorylated ERK1/2 were obtained from Cell Signaling (Cell Signaling Technology, Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated antibodies against FLAG-tag and HA-tag were obtained from Cell Signaling (Cell Signaling Technology, Beverly, MA, USA). Antibody against phosphorylated RSK1/2 (Thr 359/Ser 363) was purchased from Santa Cruz Biotechnologies Co. (Santa Cruz, CA, USA). Anti-FLAG M2 affinity gel was purchased from Sigma-Aldrich and A/G agarose beads were purchased from Santa Cruz Biotechnologies Co. (Santa Cruz, CA, USA).

Cell Culture and Transfection

HaCaT and HEK 293T cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 mg/mL streptomycin, and cultured at 37 ℃ in a humidified incubator with 5% CO2/95% air. The cells were maintained by splitting at 80% confluence and media

were changed every 3 days. The cells were plated at an appropriate density according to each experimental scale. When cells were reached 70% confluence, transfection was performed using JetPEI (Polyplus-transfection Inc,. New York, NY, USA) or Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's suggested protocol. The cells were cultured for 24 h and then proteins were extracted for further studies or starved for EGF treatment.

Lentiviral Infection

To establish knockdown 14-3-3 τ cells, the lentivirus plasmid shRNA of 14-3-3 τ (1ug) was transfected into HEK 293T cells together with PSPAX2 (Packaging Vector, 750ng) and PMD2-G (Envelope Vector, 250ng). At 5h after transfection, the medium was replaced with normal fresh medium. Viral supernatant fractions were collected at 72 h after transfection and filtered through a 0.45-μm filter. The viral supernatant fractions were infected into the HaCaT cells together with 10 μg/ml polybrene. At 24 h after infection, the medium was replaced with fresh medium containing the appropriate concentration of puromycin. The cells were maintained, until the control cells (without infection) completely died (usually 2-3 days) in the puromycin medium. At 3-4 days after infection, the appropriate experiments were performed using these cells.

Cell Proliferation (MTS) Assay

To estimate proliferation, cells were trypsinized when cells reached

appropriate confluence using the sh-mock cells and sh-14-3-3 τ cells. Cells were then counted and plated in 96-well plates (10^3 cells/50 μl/well). When cells were attached to the plate bottom at 6 h after plating, 50 μl of medium containing 0 ng/ml or 10 ng/ml EGF was added to each well. The cell viability was determined by using CellTiter 96®Aqueous One Solution (Promega, Madison, WI, USA). 20 μl of cell proliferation assay solution was added to each well and cells were incubated under normal culture conditions for 1 h. And absorbance was measured at 492nm with microplate reader (Molecular Devices, Sunnyvale, CA, USA)

Anchorage-independent Cell Transformation Assay

For EGF-induced cell transformation, bottom agar (3 mL/well) was plated in 6-well plates. Bottom agar was made by adding EGF (final concentration of 0 or 20 ng/mL) to agar mixture. Agar mixture contained 70 mL of 2X basal medium Eagle (BME) (Sigma), 2 mL of 200 mM L-glutamine (GIBCO), 200 μl of 10 mg/mL gentamicin (Biowhittacker, Inc, MD, USA), 18 mL of phosphate-buffered saline (PBS), pH 7.4, 18 mL of FBS and 72 mL of 1.25 % Bacto Agar (BD Biosciences, San Jose, CA, USA) in 180 mL. Cells were counted and diluted to $8x10^3$ cells/mL and 1.2 mL of cell suspension was mixed with 2.4 mL of agar mixture and EGF (final concentration of 0 ng/mL or 20 ng/mL). This top agar mixture was placed ($8x10^3$ cells/mL/well) on bottom agar layer after bottom agar was all set. The cultures were maintained in a 37℃ 5% CO2 incubator for the appropriate number of days, and cell colonies were scored using a microscope and

the Image-Pro PLUS (v. 6) computer software (Media Cybernetics).

Immunoblotting

Cells were harvested and exposed to RIPA buffer (50 mM Tris-C,l pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, and 1X protease inhibitor cocktail) to extract protein. After centrifugation at 13,000 g for 15 minutes, supernatant was separated and the protein concentration was determined by using the BCA protein assay kit (Pierce, Rockford, IL, USA). 15-30 μg of each protein sample was electrophoresed in 8-12% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membrane. Blots were incubated in fresh blocking buffer (0.1% Tween-20 in Tris-buffered saline (TBS) containing 5% nonfat dry milk, pH 7.4) for 1h followed by incubation with appropriate primary antibodies in TBST. After washing with TBST two times, blots were incubated with HRP-conjugated secondary antibody in TBST for 1 h at room temperature. Blots were washed again three times in TBST buffer, and transferred proteins were detected with appropriate substrate of HRP on X-ray films.

AP-1 Luciferase Reporter Gene Assay

The AP-1-luciferase reporter plasmid construct containing the -73 to + 63 collagenase promoter sequence was used. Sh mock and sh 14-3-3 τ cells were transfected with AP-1 luc vector (500ng) using JetPEI (Polyplus-transfection Inc,. New York, NY, USA). Renilla plasmid vector (50ng) was co-transfected with AP-1 reporter plasmids to serve as the control for transfection

efficiency. At 24 h after transfection, the cells were starved for EGF treatment. At 24 h after starvation, the cells were left untreated or were treated with 20ng/ml of EGF for 12h. The luciferase assay (for AP-1) and enzyme assay (for Renilla) were then performed according to the instructions of the Promega kit, Luciferase Assay System and Renilla Luciferase Assay System. (Madison, WI, USA) The AP-1-luciferase activity was normalized against Renilla luciferase activity.

Expression Vectors

The expression constructs including pCS2-3FLAG-RSK2, and pBabe.puro-HA-14-3-3 τ were amplified and used for expression in HEK 293T cells. pCS2-3FLAG-RSK2 – Tetra Mutant(S160A, S386A, S415A and S635A) and Hexa Mutant(S160A, S325A, S386A, S415A, S635A, and S715A) were constructed from pCS2-3FLAG-RSK2 -WT using a site-directed mutagenesis kit (iNTRON, Seoul, South Korea) following the manufacturer's suggested protocol. The following oligonucleotides and their complementary were used to design the mutations in RSK2: 5'- ATT TGT TTA CAC GCT TAG CCA AAG AGG TGA TGT TC -3' (Ser160→Ala160), 5'- GTT GAA GAA ATT AAA AGA CAT GCA TTT TTC TCA ACA ATA GAC T -3' (Ser325→Ala325), 5'- GCT TTT TCG GGG GTT TGC TTT TGT TGC TAT TAC C -3' (Ser386→Ala386), 5'- GCA GTT ACA CAG GAA CGC TAT TCA GTT TAC TGA T -3' (Ser415→Ala415), 5'- ATT GGC ACG AAT AGG TGC CGG AAA ATT CTC ACT C -3' (Ser635→Ala635), 5'- TTT AAA CCG CAA TCA GGC CCC AGT CTT GGA ACC -3' (Ser715→Ala715). The

presence of site-directed mutations was confirmed by complete sequencing of RSK2 gene.

Immunoprecipitation

Protein samples from cells were extracted with RIPA buffer or NP-40 cell lysis buffer (50mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.5% NP-40, 10 mM NaF and 2 mM beta-glycerophosphate). For immunoprecipitation, the protein in cell extraction was quantified, exact amount (250-400 μg) of protein was combined with 1-2 μg of HA antibody or 30 μl of FLAG M2 affinity gel, and incubated at 4℃ overnight by rocking. When HA antibody was used, 30 μl of agarose A/G beads were added after 12 h and incubated for additional 6 h. When FLAG M2 affinity gel was used, no bead was additionally added. The beads were washed three times by 1 mL of RIPA or NP-40 cell lysis buffer, mixed with 20-30 μl of 2X SDS-sample buffer, boiled and then resolved by SDS-PAGE. The proteins were visualized by immunoblotting.

Results

14-3-3τ is highly expressed in Cancer cell lines.

I asked whether 14-3-3τ is related to skin carcinogenesis. This data shows that 14-3-3τ is overexpressed in skin carcinoma cell line A431 and skin melanoma cell line SK-MEL-5. However, In HaCaT cell lines, 14-3-3τ is slightly expressed.

(Fig. 1). HaCaT cell line has been known to retain a capacity for normal differentiation up to multiple passages similar to normal human epidermal keratinocytes. Therefore, this result indicates that 14-3-3τ is related to skin carcinogenesis.

Knockdown of 14-3-3τ inhibits HaCaT cell proliferation.

To examine whether 14-3-3τ regulates in EGF-induced human skin cell transformation and proliferation, I established HaCaT cell (immortalized human adult keratinocytes of skin) stably expressing 14-3-3τ sh RNA. I generated HaCaT cells stably expressing three different amount of 14-3-3τ sh RNAs and checked efficiency of sh RNAs by western blotting. 14-3-3τ protein level was significantly suppressed #1, #2 and #3 of sh 14-3-3τ stably transfected HaCaT cells compared with sh-mock stably transfected cells (Fig. 2A). To test the knockdown effect of 14-3-3τ in the cell proliferation, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium (MTS) assay used to examine the proliferation in cells stably transfected with sh-14-3-3τ stably transfected cells showed a marked decrease in the rate of proliferation compared with sh-mock stably transfected cells (Fig. 2B). Thus, using these cell lines, we studied differences in EGF-promoted cell transformation in soft agar assay. These results showed that 14-3-3τ expression increased in HaCaT cell proliferation.

Knockdown of 14-3-3τ inhibits anchorage-independent HaCaT cell

transformation and proliferation induced by EGF.

The keratinocyte of human skin, HaCaT cell system is a well-developed model for studying tumor promotion under anchorage-independent growth condition. Therefore, sh-mock or sh-14-3-3τ stable cell lines were subjected to the soft agar assay with stimulation by EGF (20ng/mL) to assess neoplastic cell transformation. The number of colonies formed after treatment with EGF was significantly decreased in the sh-14-3-3τ stably trasnfected cells compared with the sh-mock stably transfected cells (Fig. 3A).

To confirm the role of 14-3-3τ in EGF-induced HaCaT cell proliferation, we analyzed proliferation by MTS assay and results indicated that the proliferation of sh-14-3-3τ transfected HaCaT cells was suppressed compared with control sh-mock transfected HaCaT cells. In addition, the cell proliferation enhanced by EGF treatment was also suppressed by knockdown of 14-3-3τ (Fig. 3B). This data correspond to the data of anchorage-independent HaCaT neoplastic cell transformation (Fig. 3A).

Taken together, these results demonstrated that 14-3-3τ have a significant role in promoting cell transformation and proliferation of human skin cells treatment by EGF.

14-3-3τ is required for EGF-induced phosphorylation of CREB and gene expression of c-Fos.

EGF receptor is a representative receptor tyrosine kinase. Binding of EGF

to its tyrosine kinase receptor activates the Ras-dependent MAPK cascade. MAPK cascades regulate many cellular responses induced by various external stimuli. Coupled phosphorylation events lead to activation of mitogen-activated protein kinase (MEK), RAS/extracellular signal-regulated kinase (ERK1/2) and RSK2. When a cell is stimulated by EGF, RSK2 translocates into nucleus, then phosphorylates Ser-133 of cyclic AMP-responsive element binding protein (CREB). Moreover, RSK2 is required for growth factor-stimulated expression of c-Fos. Especially, EGF signaling through Ras/ERKs/RSK2 pathway is crucial in EGF-induced human skin cell transformation and proliferation.

To determine whether 14-3-3τ is required for MAPKs signaling pathway treatment by EGF, we assessed the ability of ERKs/RSK2 to trigger phosphorylation of endogenous in knockdown 14-3-3τ HaCaT cells following EGF treatement. To do this, the sh-14-3-3τ transfected HaCaT cells were starved for 24h with DMEM medium without serum and then 10ng/mL of EGF was treated for 30min. Results indicated that phosphorylation and activation of ERK1/2 and RSK2, downstream molecule of ERK1.2, was not significantly suppressed by knockdown of 14-3-3τ after EGF treatment (Fig. 4). This result suggest that 14-3-3τ enhanced by the tumor promoters EGF may affect downstream of RSK2 through regulation of RSK2 activity but not the phosphorylation level of ERK or RSK2 in HaCaT cells.

To check whether 14-3-3τ could control downstream molecules of RSK2, activation of CREB and expression of c-Fos were examined in sh-14-3-3τ transfected HaCaT cells following EGF treatment. Interestingly, phospho-CREB

induced by EGF was significantly suppressed by knockdown of 14-3-3τ (Fig. 4). Moreover, knockdown of 14-3-3τ suppressed the c-Fos expression induced by EGF (Fig. 4).

Taken together, these results indicated that 14-3-3τ might regulate RSK2 activity to phosphorylate CREB and subsequently induce c-Fos transcriptional expression.

14-3-3τ regulates AP-1 activity.

AP-1 (Activator Protein-1) is an important mediator of EGF-induced anchorage-independent cell transformation and tumor development. (15) And it comprises a dimeric complex that include Jun, Fos, ATF1, and other family members. (16) To determine whether 14-3-3 τ could regulate AP-1 activity, the AP-1 luciferase reporter gene, Renilla luciferase gene were co-transfected into sh-mock or sh-14-3-3τ transfected HaCaT cells. The data showed that AP-1 transcriptional activity was inhibited in sh-14-3-3τ transfected HaCaT cells (Fig 5).

Consistently the western blotting data, we demonstrated that 14-3-3τ regulates AP-1 activity.

14-3-3τ directly interacts with RSK2.

The above results strongly suggested that knockdown of 14-3-3τ suppressed the phosphorylation of CREB and also inhibited expression of c-Fos induced by EGF. Recent evidence indicates that when the MEK-MAPK pathway is

activated, RSK2-mediated phosphorylation of CREB Ser-133 contributes to the activation of gene expression. Also, RSK-2-mediated CREB phosphorylation is a prerequisite for c-Fos inducibility by EGF. So, the c-Fos, CRE is sufficient to direct the transcriptional response to RSK2. As I mentioned in the introduction, 14-3-3 proteins associates with many different molecules because of its specific phosphoserine/phosphothreonine-binding activity. In particular, 14-3-3τ mediates signal transduction by binding to phosphoserine-containing proteins. Based on these results and previous reports, I hypothesized that 14-3-3τ could interact with RSK2 to influence a series of signaling cascade of RSK2, which phosphorylates CREB and subsequently induce expression of c-Fos transcription.

To address the hypothesis, we wished to assess whether the 14-3-3τ could interact with RSK2. FLAG-RSK2 and HA-14-3-3τ were co-transfected into HEK 293T cells. FLAG-RSK2 was immunoprecipitated (IP) with anti-FLAG M2 affinity gel and HA-14-3-3τ was detected in the IP complex. The data showed that 14-3-3τ interacted with RSK2 (Fig. 6). As I described in the introduction, 14-3-3 proteins have two high 14-3-3 binding affinity motifs : RSXpSXP (mode 1) and RXXXpSXP (mode 2). So, we checked the 14-3-3 binding motifs in RSK2 amino acid sequence.

The sequence of RSK2 was analyzed and I found six candidates of binding motifs on RSK2. To further verify the specific sites of RSK2 for the binding, I generated two different mutants of FLAG-tagged RSK2s. The one is the Tetra Mutant(TM) in which serine 160, 386, 415 and 635 respectively was replaced with alanine (S160A,

S386A, S415A, S635A). The other is the Hexa Mutant(HM) in which serine 160, 325, 386, 415, 635 and 715 respectively was replaced with alanine (S160A, S325A, S386A, S415A, S635A, S715A). It was demonstrated in the previous study that 14-3-3η interact with RSK2, and serine 160, 386, 415 and 635 of RSK2 are important for physical binding with 14-3-3η. That's why I generated Tetra Mutant. FLAG-tagged wild-type or mutant RSK2 and HA-14-3-3τ were co-transfected into HEK 293T cells. FLAG-RSK2 was immunoprecipitated (IP) with anti-FLAG M2 affinity gel and HA-14-3-3τ was detected in the IP complex. The results indicated that 14-3-3τ strongly bound with wild-type RSK2. Also, 14-3-3τ interacted with Tetra Mutant RSK2 as almost same binding affinity. Therefore, these four mutated site (S160, S386, S415, S635) are not important for physical binding with 14-3-3τ. Interestingly, the result has a discrimination previous result 14-3-3η. Meanwhile, Hexa Mutant RSK2 showed reduced binding affinity with 14-3-3τ.

Taken together, these results demonstrated that 14-3-3τ interact with RSK2, and serine 325 and 715 of RSK2 are may be important for physical binding with 14-3-3τ.

Serine 325 and serine 715 of RSK2 is required for EGF-induced phosphorylation of CREB and gene expression of c-Fos

It was demonstrated in the previous study, I found the two specific sites of RSK2 for the binding with 14-3-3τ. If so, I wondered that the physiological meaning of the interaction between RSK2 and 14-3-3τ in EGF-induced human skin

cell transformation is. To examine this idea, I overexpressed FLAG-mock vector, wild-type RSK2 and Hexa Mutant RSK2 in HaCaT cells induced by EGF. I confirmed that the protein levels of related to Ras-dependent MAPK pathway. This result showed that when Hexa Mutant RSK2 was over-expressed in HaCaT cell lines induced by EGF, phosphorylation of CREB and expression of c-Fos significantly suppressed (Fig. 7). This result clearly showed that serine 325 and 715 of RSK2 are necessary to bind with 14-3-3τ that regulates cell proliferation pathway treatment of EGF.

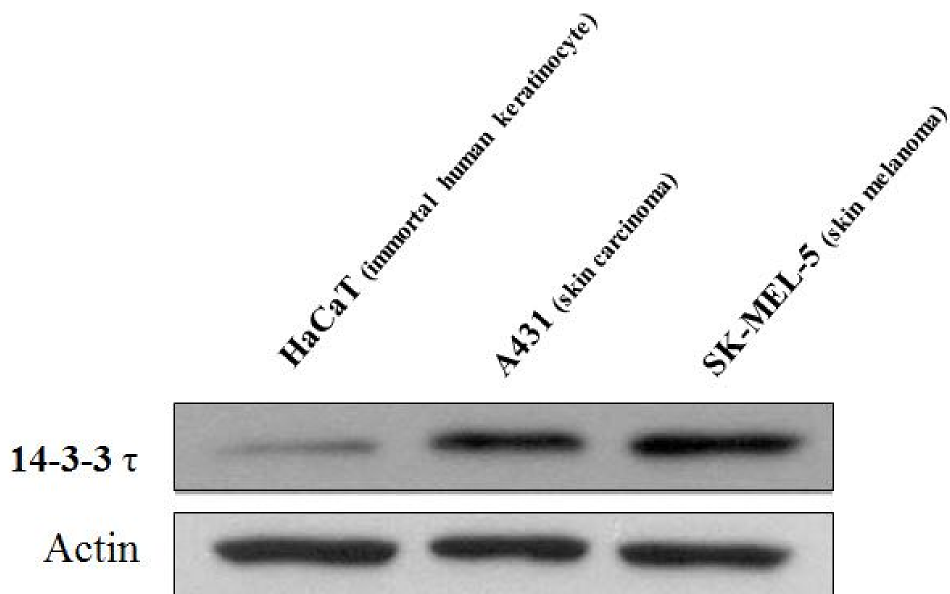


Figure 1. 14-3-3τ is highly expressed in skin cancer cell lines. HaCaT cells (5 x 10^5) were seeded into 6-cm dishes and then cultured overnight. Cells were subsequently starved for 24h with DMEM medium without serum and stimulated with 10ng/mL EGF and harvested after the 30min. The proteins were extracted with RIPA lysis buffer and Western blotting was conducted as described in Materials and Methods using the 14-3-3τ specific antibody as indicated. Equal protein loading was checked using the actin antibody on the membrane.

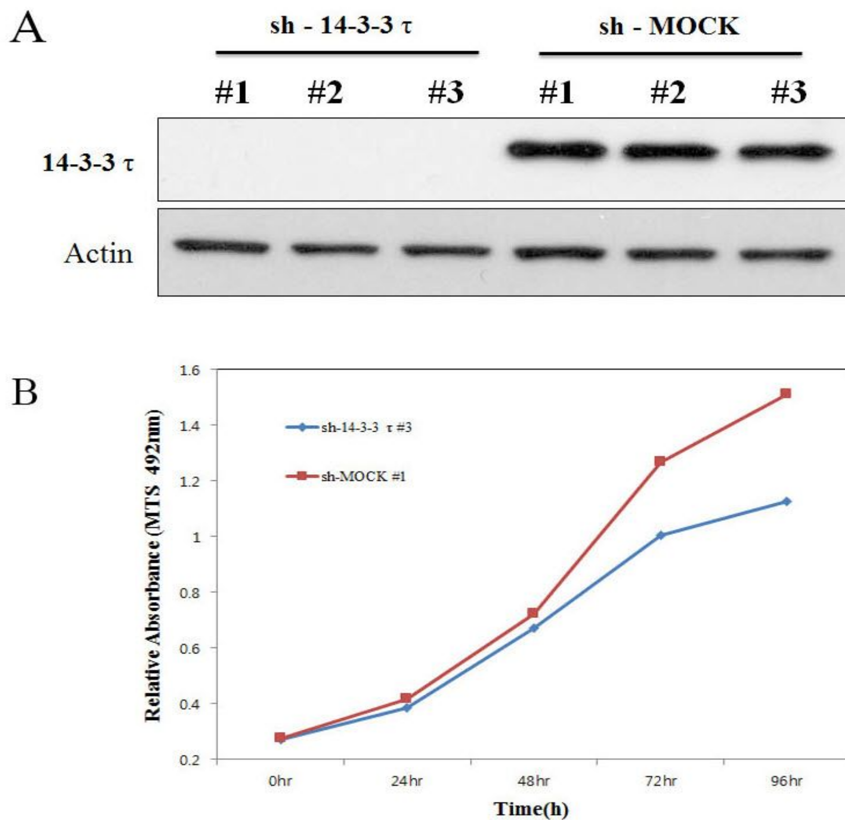
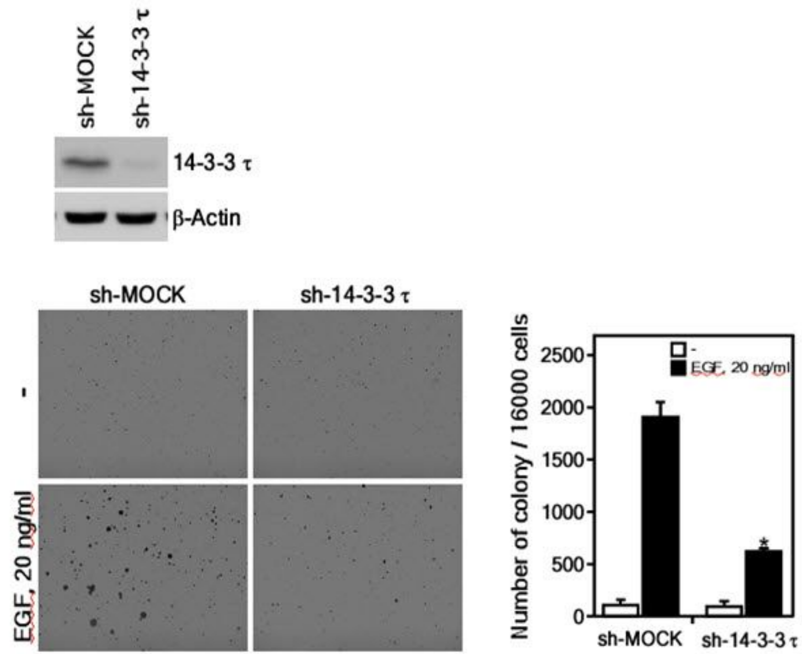


Figure 2. Knock-down of 14-3-3τ inhibited cell growth of HaCaT cell lines. (A) To establish stable 14-3-3τ sh-RNA HaCaT cell lines, three different amounts of 14-3-3τ sh-RNAs were stably expressed. The efficiency of sh-RNAs was examined by western blotting. 14-3-3τ protein level was significantly suppressed in #1, #2 and #3 of sh-14-3-3τ stably transfected HaCaT cell lines compared with sh-MOCK stably

transfected cells. (B) sh-MOCK or sh-14-3-3τ stably transfected HaCaT cells were seeded in 96-well plates at a density of 2 x 10^3 cells/100 μl/well and incubated for 24 h, 48 h, 72 h, and 96 h. 14-3-3τ suppresses the proliferation of HaCaT cells in a time-dependent manner as measured by MTS assay as described in Materials and Methods. 20 μl of Cell proliferation assay solution was added to each well and cells were incubated under normal culture conditions for 1h. Absorbance at 492 nm was measured by a micro-plate reader. Cell proliferation of 14-3-3τ stably transfected cells was markedly suppressed compared with sh-MOCK stably transfected cells.

A



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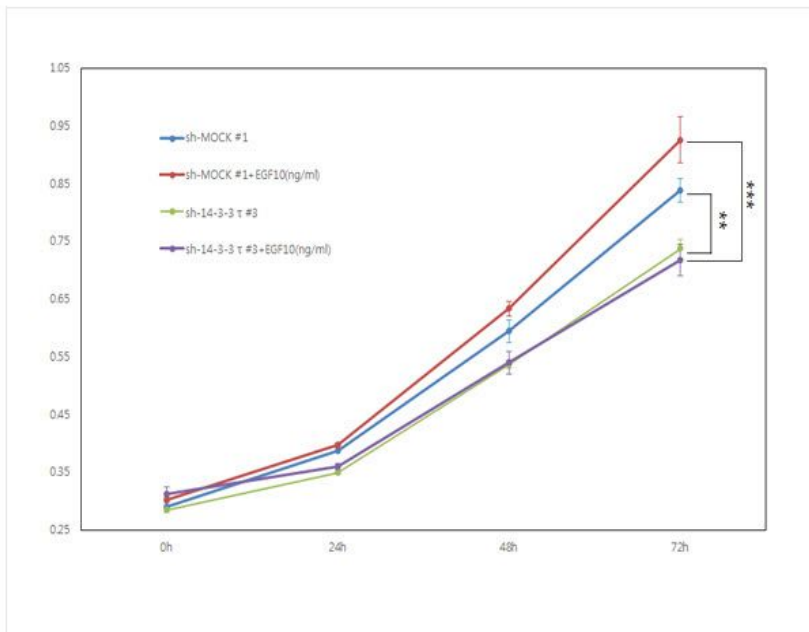

Figure 3. Knockdown of 14-3-3τ inhibits anchorage-independent HaCaT cell transformation and proliferation induced by EGF. (A) Knockdown of 14-3-3τ blocks EGF-induced cell transformation. Knockdown of 14-3-3τ in HaCaT cell was performed as described under Materials and Methods, and cells were exposed to EGF (20ng/mL) in 0.3% basal medium Eagle agar containing 20% FBS. The number of colonies formed after treatment with EGF was significantly decreased in the sh-14-3-3τ stably transfected cells compared with the sh-MOCK stably transfected cells.

(B) sh-MOCK or sh-14-3-3τ stably transfected HaCaT cells were seeded in 96-well plates at a density of 2 x 10^3 cells/50ul/well, and 50ul of media with serum containing 0ng/mL or 10ng/mL EGF was added at 6h after plating. From this point, cells were incubated for 24h, 48h, and 72h. The cell proliferation was determined by MTS assay. Knockdown of 14-3-3τ inhibited HaCaT cell proliferation treatment of EGF compared with control sh-MOCK transfected HaCaT cells.

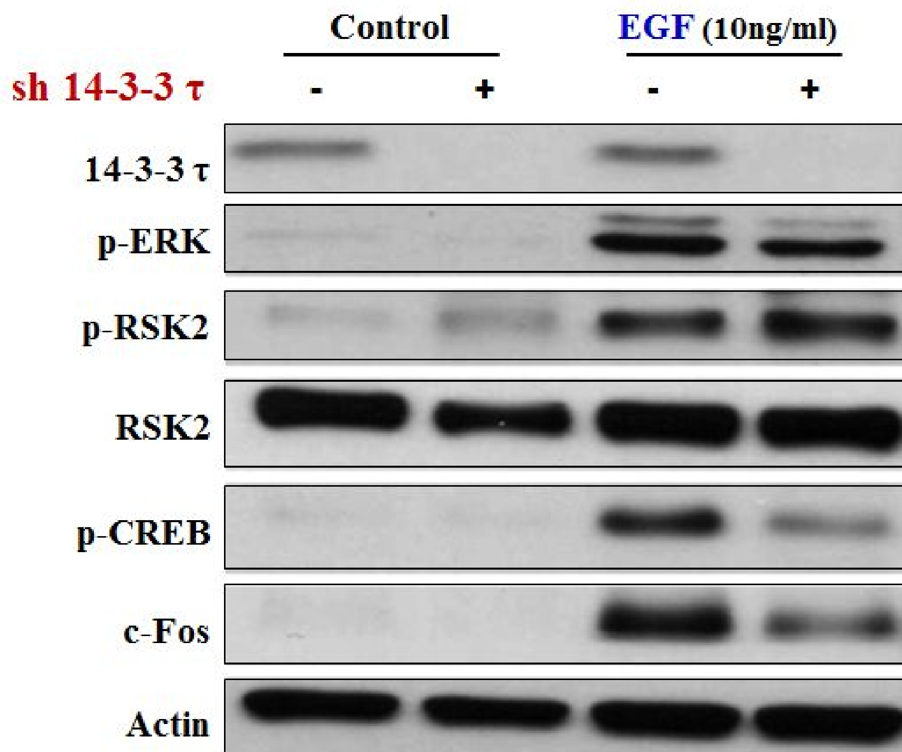

Figure 4. Effects of 14-3-3τ knockdown on EGF signal transduction. Knockdown of 14-3-3τ HaCaT cells induced by sh-14-3-3τ were starved for 24h and then treated with EGF(10ng/mL) for 30 min. Immunoblotting was used to detect phosphorylation of ERK, phosphorylation of RSK2, RSK2, phosphorylation of CREB, and c-Fos expression as indicated. Actin was used to demonstrate equal loading of protein. The phosphorylation of ERK and RSK2 were not changed by knockdown of 14-3-3τ, but phosphorylation of CREB and expression of c-Fos (both treatment of EGF) were significantly suppressed.

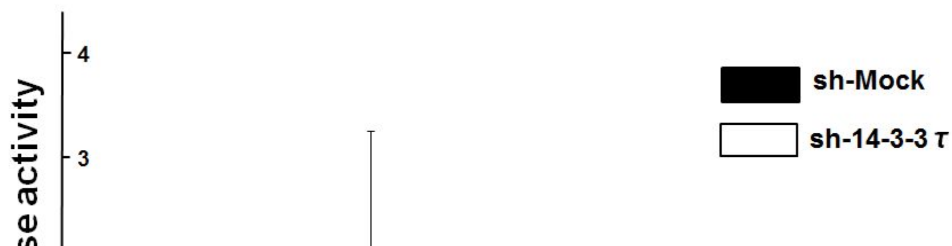

Figure 5. 14-3-3τ induces AP-1 activity. AP-1 activity is inhibited in 14-3-3τ knockdown HaCaT cells. sh-MOCK or sh-14-3-3τ stably transfected HaCaT cells were co-transfected with a plasmid mixture containing the AP-1 luciferase reporter gene (500ng) and the Renilla luciferase gene (50ng) for normalization. At 24h after transfection, cells were starved for 24h by incubating in serum-deprived DMEM and then treated with EGF (20ng/mL). After 12h, AP-1 luciferase activity was determined in cell lysates and normalized against Renilla luciferase activity.

A

MPLAQLADPW QKMAVESPSD SAENGQQIMD EPMGEEEINP QTEEVSIKEI AITHHVKEGH
EKADPSQFEL LKVLGQGSFG KVFLVKKISG SDARQLYAMK VLKKATLKVR DRVRTKMERD
ILVEVNHPFI VKLHYAFQTE GKLYLILDFL RGGDLFTRLS KEVMFTEEDV KFYLAELALA
LDHLHSLGII YRDLKPENIL LDEEGHIKLT DFGLSKESID HEKKAYSFCG TVEYMAPEVV
NRRGHTQSAD WWSFGVLMFE MLTGTLPFQG KDRKETMTMI LKAKLGMPQF LSPEAQSLLR
MLFKRNPANR LGAGPDGVEE IKRHSFFSTI DWNKLYRREI HPPFKPATGR PEDTFYFDPE
FTAKTPKDSP GIPPSANAHQ LFRGFSFVAI TSDDESQAMQ TVGVHSIVQQ LHRNSIQFTD
GYEVKEDIGV GSYSVCKRCI HKATNMEFAV KIIDKSKRDP TEEIEILLRY GQHPNIITLK
DVYDDGKYVY VVTELMKGGE LLDKILRQKF FSEREASAVL FTITKTVEYL HAQGVVHRDL
KPSNILYVDE SGNPESIRIC DFGFAKQLRA ENGLLMTPCY TANFVAPEVL KRQGYDAACD
IWSLGVLLYT MLTGYTPFAN GPDDTPEEIL ARIGSGKFSL SGGYWNSVSD TAKDLVSKML
HVDPHQRLTA ALVLRHPWIV HWDQLPQYQL NRQDAPHLVK GAMAATYSAL NRNQSPVLEP
VGRSTLAQRR GIKKITSTAL

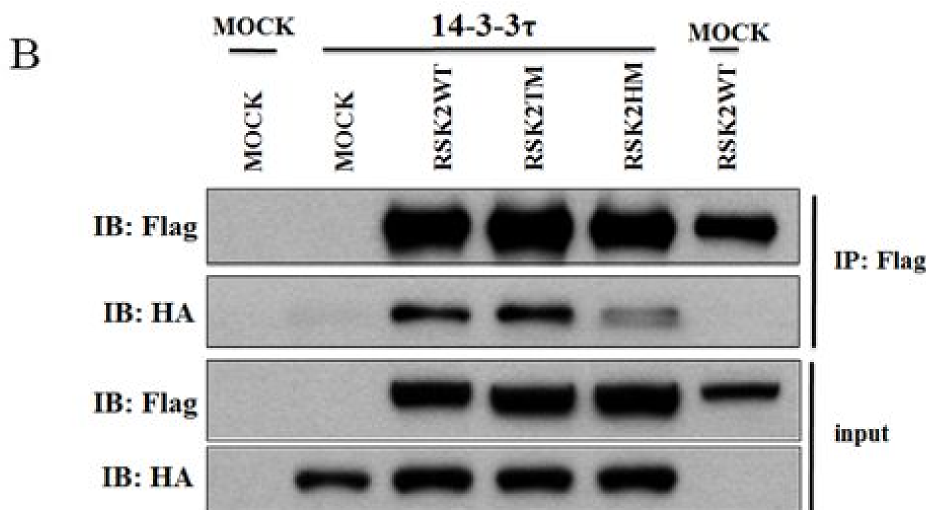

Figure 6. Physical binding between RSK2 and 14-3-3τ. (A) The amino acid sequence of RSK2 is marked by boxes 6which indicate potential 14-3-3τ-binding motifs. Bold-faced letters (S) in the boxes show serine residues which were replaced by alanine through site-directed mutagenesis for immunoprecipitation assay (Fig. 6B); and these residues are Ser 160, 325, 386, 415, 635 and 715. (B) Flag-tagged RSK2TM and Flag-tagged RSK2HM were prepared by site-directed mutagenesis as described in Materials and Methods. HEK 293T cells were transiently co-

tranasfected with FLAG-tagged RSK2WT or FLAG-tagged RSK2TM or FLAG-tagged RSK2HM and HA-tagged 14-3-3τ for 24 h. The immunoprecipitated complex was detected by Western blotting with an FLAG-tagged or HA-tagged antibody. The blots indicated the interaction between RSK2 and 14-3-3τ in vitro.

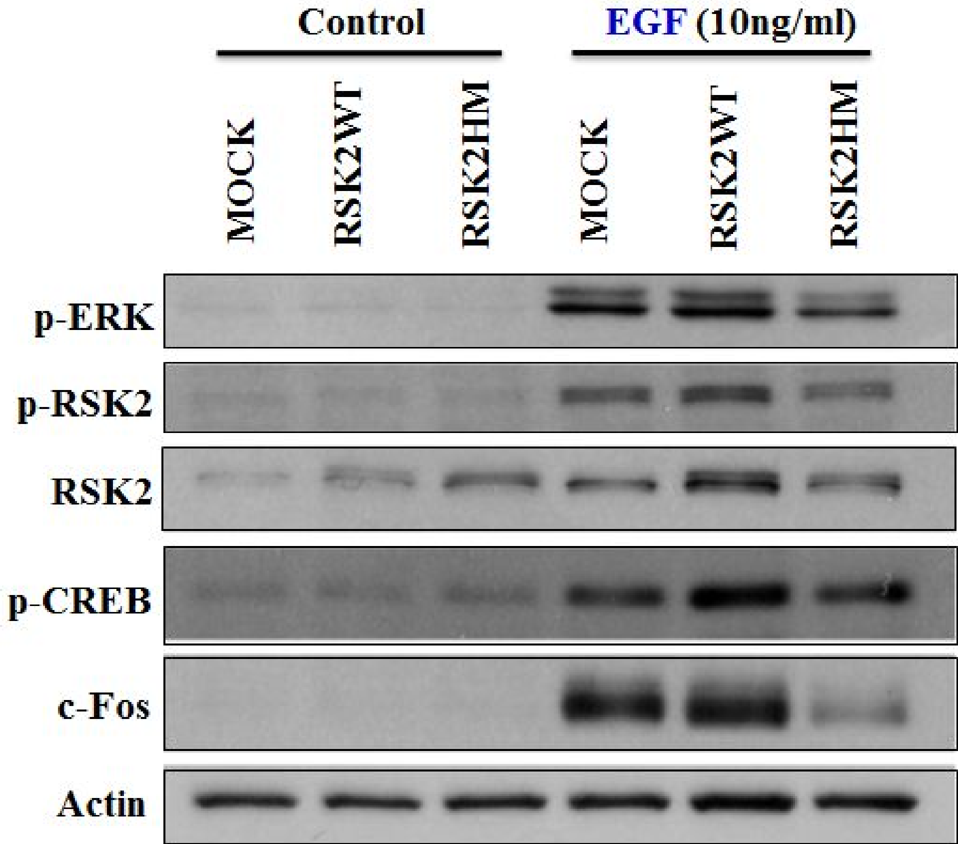

Figure 7. Physiological significance of 14-3-3τ binding sites on RSK2. MOCK vector, wild-type RSK2 (RSK2WT) and Hexa Mutant RSK2 (RSK2HM) were overexpressed in HaCaT cells. These cells were starved 24hr before induction with EGF(10ng/mL) and then harvested after the 30min. When RSK2HM was overexpressed in HaCaT cell lines induced by EGF, phosphorylation of CREB and expression of c-Fos was markedly inhibited.

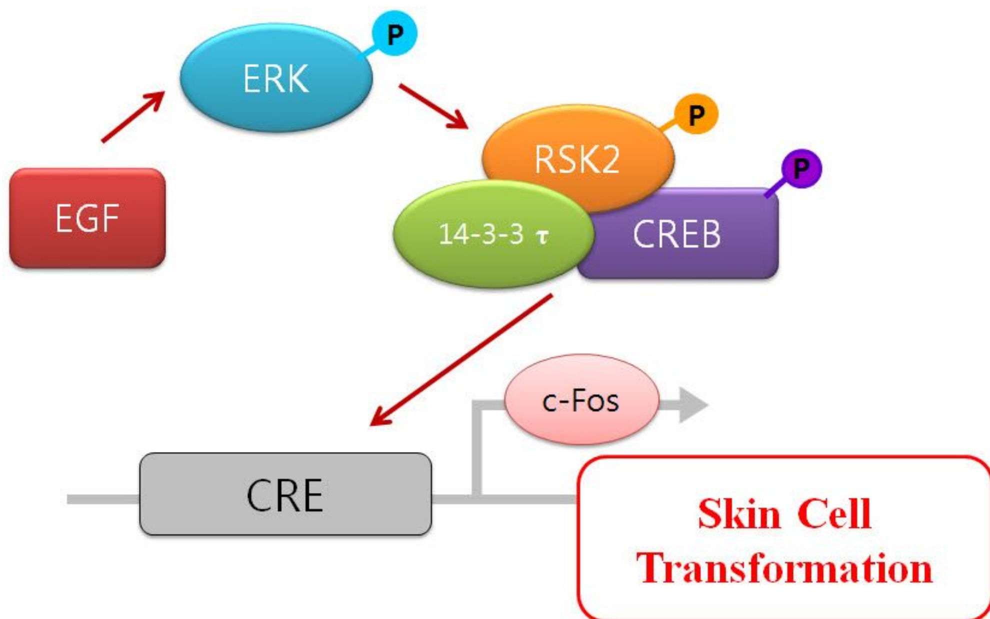

Figure 8. The proposed mechanisms by which 14-3-3τ is involved in EGF-induced transformation of keratinocytes.

Discussion

Most recently, the ability of 14-3-3 proteins to bind and control multiple oncogenic gene products as well as multiple tumor suppressor gene products points to a potential role in cancer. Like this, 14-3-3 proteins may serve as a novel molecular target for cancer therapy. Among the 14-3-3 isoforms, I focused on the 14-3-3τ, in EGF-induced transformation of human keratinocytes. Results shown in the present study demonstrate for the first time that 14-3-3τ is required for phosphorylation of CREB and expression of c-Fos. These proteins are related to Ras-Raf-mitogen activated protein kinase (MAPK) pathway which regulates cell proliferation by transmitting signals from membrane-bound receptors to nuclear and cytoplasmic targets, coordinating cellular response to various growth promoting factors (17). Furthermore, we identified RSK2 as a novel binding partner of 14-3-3τ.

Taken together, these results suggested that 14-3-3τ might control RSK2 activation for EGF-induced transformation of human keratinocytes via physical interaction between them.

Previously, in humans, 14-3-3τ is widely expressed in brain neurons and T cells (18), which is induced by DNA damage and is required for regulation of G2-M-transition and G1/S-transition in cells (19, 20). For example, it was reported that 14-3-3τ is shown to be required for the expression and induction of important E2F1 targets. So, it is involved in DNA damage-induced apoptosis (21). Also, 14-3-3τ controls the cell cycle participating in trophoblast differentiation. Therefore, 14-3-3τ might be arrest the cell cycle and thereby induced trophoblast differentiation (22). But there are no ideas about the potential role of 14-3-3τ in skin carcinogenesis. To further investigate in potential role, we demonstrated the effects of 14-3-3τ in EGF-induced transformation of human keratinocytes. To be specific, I showed that knockdown of 14-3-3τ suppressed anchorage-independent HaCaT cell transformation and cell growth treatment of EGF. These results suggested that 14-3-3τ may play a role as a tumor promotor.

The 14-3-3 proteins represent the prototype of a class of phosphoserine / phosphothreonine (pSer/Thr)-recognition proteins. 14-3-3s can directly binding pSer or pThr residues of cellular proteins, also can variably modify the function of their targets, altering either their catalytic activity, cellular localization. . In addition, 14-3-3 proteins are known to act as a scaffold for several proteins, leading to multiple protein complex formation on the target protein. Over 200 proteins have

been reported to interact with 14-3-3 proteins. Although there are numerous binding proteins for 14-3-3 proteins, the relationship between 14-3-3s and each binding proteins have not well understood yet. In my current study, I demonstrated physical interaction between 14-3-3τ and RSK2. Furthermore, I found that serine 325 and 715 residues of RSK2 were crucial for the binding with 14-3-3τ.

It is already known that RSK2 was able to CREB phosphorylation in vitro and this phosphorylation is a prerequisite for c-Fos inducibility by EGF. Our results, however, demonstrate a specifically knockdown of 14-3-3τ impaired the phosphorylation of CREB and suppressed expression of c-Fos in EGF-stimulated HaCaT cell, whereas phosphorylation of ERK1/2 and RSK2 were not changed. As proposed in the schematic diagram (Fig. 8), 14-3-3τ associated with RSK2 to stimulate signaling pathway of RSK2, which phosphorylate CREB. This phosphorylation of CREB would bind to cAMP response elements (CRE) in the promoter region of c-Fos and subsequently induce c-Fos transcription. The higher level of c-Fos would then contribute to transcriptional induction of genes involved in neoplastic cell transformation.

Reference

1. D.K. Morrison. The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development. Trends in cell biology. 19:16-23 (2009).

2. T. Obsiland V. Obsilova. Structural basis of 14-3-3 protein functions. Seminars in cell & developmental biology. 22:663-672 (2011).
3. J. Zhao, C.L. Meyerkord, Y. Du, F.R. Khuri, and H. Fu. 14-3-3 proteins as potential therapeutic targets. Seminars in cell & developmental biology. 22:705-712 (2011).
4. M. Gasco, A.K. Bell, V. Heath, A. Sullivan, P. Smith, L. Hiller, I. Yulug, G. Numico, M. Merlano, P.J. Farrell, M. Tavassoli, B. Gusterson, and T. Crook. Epigenetic inactivation of 14-3-3 sigma in oral carcinoma: association with p16(INK4a) silencing and human papillomavirus negativity. Cancer research. 62:2072-2076 (2002).
5. C.B. Umbricht, E. Evron, E. Gabrielson, A. Ferguson, J. Marks, and S. Sukumar. Hypermethylation of 14-3-3 sigma (stratifin) is an early event in breast cancer. Oncogene. 20:3348-3353 (2001).
6. R.T. Kilani, A. Medina, A. Aitken, R.B. Jalili, M. Carr, and A. Ghahary. Identification of different isoforms of 14-3-3 protein family in human dermal and epidermal layers. Molecular and cellular biochemistry. 314:161-169 (2008).
7. P.P. Rouxand J. Blenis. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiology and molecular biology reviews : MMBR. 68:320-344 (2004).
8. J. Xing, D.D. Ginty, and M.E. Greenberg. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science. 273:959-963 (1996).
9. D. De Cesare, S. Jacquot, A. Hanauer, and P. Sassone-Corsi. Rsk-2 activity is necessary for epidermal growth factor-induced phosphorylation of CREB protein and transcription of c-fos gene. Proceedings of the National Academy of Sciences of the United States of America. 95:12202-12207 (1998).
10. X. Yang, K. Matsuda, P. Bialek, S. Jacquot, H.C. Masuoka, T. Schinke, L. Li, S. Brancorsini, P. Sassone-Corsi, T.M. Townes, A. Hanauer, and G. Karsenty. ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. Cell. 117:387-398 (2004).

11. Y.Y. Cho, Z. He, Y. Zhang, H.S. Choi, F. Zhu, B.Y. Choi, B.S. Kang, W.Y. Ma, A.M. Bode, and Z. Dong. The p53 protein is a novel substrate of ribosomal S6 kinase 2 and a critical intermediary for ribosomal S6 kinase 2 and histone H3 interaction. Cancer research. 65:3596-3603 (2005).
12. Y.Y. Cho, K. Yao, A.M. Bode, H.R. Bergen, 3rd, B.J. Madden, S.M. Oh, S. Ermakova, B.S. Kang, H.S. Choi, J.H. Shim, and Z. Dong. RSK2 mediates muscle cell differentiation through regulation of NFAT3. The Journal of biological chemistry. 282:8380-8392 (2007).
13. K. Liu, Y.Y. Cho, K. Yao, J. Nadas, D.J. Kim, E.J. Cho, M.H. Lee, A. Pugliese, J. Zhang, A.M. Bode, Z. Dong, and Z. Dong. Eriodictyol inhibits RSK2-ATF1 signaling and suppresses EGF-induced neoplastic cell transformation. The Journal of biological chemistry. 286:2057-2066 (2011).
14. Y.Y. Cho, K. Yao, H.G. Kim, B.S. Kang, D. Zheng, A.M. Bode, and Z. Dong. Ribosomal S6 kinase 2 is a key regulator in tumor promoter induced cell transformation. Cancer research. 67:8104-8112 (2007).
15. Z. Dong, M.J. Birrer, R.G. Watts, L.M. Matrisian, and N.H. Colburn. Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells. Proceedings of the National Academy of Sciences of the United States of America. 91:609-613 (1994).
16. C. Peng, F. Zhu, W. Wen, K. Yao, S. Li, T. Zykova, K. Liu, X. Li, W.Y. Ma, A.M. Bode, and Z. Dong. Tumor necrosis factor receptor-associated factor family protein 2 is a key mediator of the epidermal growth factor-induced ribosomal S6 kinase 2/cAMP-responsive element-binding protein/Fos protein signaling pathway. The Journal of biological chemistry. 287:25881-25892 (2012).
17. G. Tzivion, V.S. Gupta, L. Kaplun, and V. Balan. 14-3-3 proteins as potential oncogenes. Seminars in cancer biology. 16:203-213 (2006).
18. Y.C. Liu, C. Elly, H. Yoshida, N. Bonnefoy-Berard, and A. Altman. Activation-modulated association of 14-3-3 proteins with Cbl in T cells. The Journal of biological chemistry. 271:14591-14595 (1996).
19. K. Katayama, N. Fujita, and T. Tsuruo. Akt/protein kinase B-dependent phosphorylation and inactivation of WEE1Hu promote cell cycle

progression at G2/M transition. Molecular and cellular biology. 25:5725-5737 (2005).

20. C. Charvet, A.J. Canonigo, S. Becart, U. Maurer, A.V. Miletic, W. Swat, M. Deckert, and A. Altman. Vav1 promotes T cell cycle progression by linking TCR/CD28 costimulation to FOXO1 and p27kip1 expression. Journal of immunology. 177:5024-5031 (2006).

21. B. Wang, K. Liu, F.T. Lin, and W.C. Lin. A role for 14-3-3 tau in E2F1 stabilization and DNA damage-induced apoptosis. The Journal of biological chemistry. 279:54140-54152 (2004).

22. Y. Cheng, R. Hu, H. Jin, K. Ma, S. Zhou, H. Cheng, D. Ma, and X. Li. Effect of 14-3-3 tau protein on differentiation in BeWo choriocarcinoma cells. Placenta. 31:60-66 (2010).

국문 초록

EGF 로 유도된 사람 피부 세포의 transformation 에서 14-3-3τ 의 역할

14−3−3 단백질은 진화적으로 보존된 작은 단백질로서, 모든 진핵세포에서 발현되며, 다양한 세포 과정에 관여를 한다. 지금까지, 포유류 동물에서 β, γ, ε, ζ, η, σ, 그리고 τ 의 7 가지 이성체가 존재함이 알려졌다. 14−3−3은 신호 전달을 조절하며 신호 전달의 단백질들의 이동을 도와주는 scaffold 단백질로서 작용을 한다. 그래서 14−3−3은 단백질−단백질 상호작용을 조절할 수 있다. 또한 14−3−3은 인산화된 Serine 또는 Threonine에 결합하려고 하는 성질을 가지고 있기 때문에 많은 세포내의 단백질들과 상호작용을 할 수가 있다. 최근에, 일부 14−3−3 이성체가 발암 과정 촉진 또는 억제 기능에 관여한다고 보고된 바

있으나, 14-3-3이 피부 암 발생에서 어떠한 역할을 하는지에 대한 연구는 아직 미비하다. 따라서 본 연구에서는 14-3-3τ 가 EGF로 유도된 사람 피부 세포의 transformation에 관여한다는 것을 밝히고자 하였다. Physiological 의미로서, 14-3-3τ가 knockdown 되면, HaCaT의 세포 증식과 EGF로 유도된 anchorage-independent transformation이 대조군과 비교하였을 때 억제됨을 확인하였다. 게다가, 14-3-3τ가 knockdown 된 경우에 EGF에 의한 CREB의 인산화가 감소하였고, c-Fos의 발현도 역시 억제되었다. 이 결과에 더하여, 본 연구에서는 14-3-3τ가 EGF로 유도된 사람 피부 세포의 transformation에 관여하는 분자적 기전도 조사하였다. 그 기전의 핵심으로 생각되는 부분은 14-3-3τ와 RSK2의 direct physical interaction으로, 여기에서 RSK2는 EGF로 유도된 사람 피부세포의 transformation에 중요하다고 알려져 있으며, CREB의 인산화에 직접적으로 관여하는 단백질이다. 그리고 RSK2에 14-3-3τ가 결합하는 데에 있어서 RSK2의 두 가지 Serine 잔기 (Ser325, Ser715)가 중요하다는 것 또한 증명하였다. 마지막으로는, EGF로 유도된 사람 피부세포의 transformation에서 14-3-3τ와 RSK2 상호작용의 physiological 의미도 확인하였다. 종합적으로, 본 연구는 14-3-3τ가 EGF로 유도된 사람 피부 세포의 transformation에서 RSK2와의 결합을 통해 발암 촉진제로서 작용할 수 있다는 점을 시사한다.