약학석사학위논문

The Role of 14-3-3 eta in EGF-induced Skin Cell Transformation

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

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Abstract

The Role of 14-3-3 eta in EGF-induced Skin Cell Transformation

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14-3-3 proteins are evolutionarily conserved, acidic 28-30-kDa proteins of seven isoforms (β, γ, ε, ζ, η, σ, and τ) in mammals. 14-3-3s act as adaptor proteins that control the function of their target proteins through highly regulated protein-protein interactions. Recently, some of 14-3-3 isoforms were suggested to be tumor-suppressing or promoting, but the role of 14-3-3s in skin carcinogenesis has not yet been elucidated. Here, I suggest that 14-3-3 η is involved in epidermal growth factor (EGF)-induced skin cell transformation. First, it was shown that knockdown of 14-3-3 η efficiently suppressed anchorage-independent HaCaT cell transformation and proliferation induced by EGF compared with control si- or sh-mock cells. Moreover, knockdown of 14-3-3 η inhibited phosphorylation of CREB upon EGF stimulation followed by reduction of c-Fos expression. Furthermore, I investigated the molecular mechanisms underlying the involvement of 14-3-3 η in

EGF-induced skin cell transformation. I found direct physical interaction of 14-3-3 η with RSK2, a critical factor in EGF-induced skin cell transformation and direct kinase of CREB. In addition, I identified that four serine residues (Ser160, Ser386, Ser415, and Ser635) of RSK2 were crucial for the physical binding with 14-3-3 η. Taken together, I suggest that 14-3-3 η might have a role as a tumor enhancer in EGF-induced skin cell transformation, via direct interaction with RSK2.

Keywords: 14-3-3 eta, EGF, c-Fos, CREB, RSK2, skin cell transformation

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Introduction

14-3-3 proteins are acidic 28-30-kDa proteins and consist of seven family members (β, γ, ε, ζ, η, σ, and τ) in mammals. 14-3-3 proteins are highly conserved and widely expressed in all tissues and organs. 14-3-3 proteins represent the prototype of a phosphoserine/phosphothreonine (pSer/Thr)-recognition protein class. They selectively bind to two phosphorylation motifs: RSXpSXP and RXXXpSXP, where X represents any residue and pS respresents a phosphoserine. [1] This feature allows 14-3-3 proteins to bind with a multitude of functionally diverse signaling proteins, including kinases, phosphatases, transmembrane receptors and transcription factors. More than 50 signaling proteins have been reported as 14-3-3 binding partners. This broad range of partners suggests that 14-3-3 participates in the regulation of diverse biological processes, including neural development, cell cycle, cell proliferation, apoptosis, and cell motility. [2]

Recently, it was reported that some isoforms of 14-3-3 are involved in tumorigenesis. [3] Especially, 14-3-3 σ has been best understood for its tumor-suppressor activity. Decreased expression of 14-3-3 σ has been observed in several human cancers and its loss seems to happen in early stages of tumorigenesis. [4, 5] Other than 14-3-3 σ, 14-3-3 ζ has been investigated for its contribution to oncogenesis. [6] Whether other 14-3-3 isoforms are involved in human cancer is less clear and the role of 14-3-3s especially in skin carcinogenesis have not yet been

elucidated.

The mitogen-activated protein kinases (MAPKs) are important regulators of proliferation and oncogenesis. The MAPK extracellular signal-regulated kinases (ERKs) 1 and 2 mediate the phosphorylation of 90-kDa ribosomal S6 kinase (RSK), which is a family of serine/threonine kinases regulated by many growth factors, hormones and neurotransmitters. RSK2 is a member of p90RSK family and is directly activated by ERK1/2 in response to growth factors including epidermal growth factor (EGF). Activated RSK2 is translocated into nucleus, then phosphorylates various nuclear proteins, including cyclic AMP-responsive element binding protein (CREB) [7], c-Fos [8], activating transcription factor 4 [9]and histones [10]. Based on its broad substrate specificity, the RSK2 has role in various cellular processes including proliferation and transformation, as well we cell cycle. Recently, it was reported that RSK2 is crucial role in skin cell transformation induced by tumor promoters such as EGF and 12-O-tetradecanoylphorbol-13-acetate.[11]

In this study, I demonstrated the involvement of 14-3-3 protein, especially 14-3-3 η, in human skin cell transformation induced by EGF. Knockdown of 14-3-3 η efficiently suppressed cell proliferation and anchorage-independent HaCaT cell transformation induced by EGF. Moreover, I showed that 14-3-3 η was required for the phosphorylation of CREB and expression of c-Fos induced by EGF. Additionally, I identified RSK2, a novel binding partner of 14-3-3 η and verified the binding sites of 14-3-3 η in RSK2. Taken together, I suggest that 14-3-3 η may have

a role as a tumor enhancer in EGF-induced skin cell transformation, via direct interaction with RSK2.

Materials and Methods

Materials

Chemical reagents for molecular biology and buffer preparation were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture media and other supplements were purchased from Gibco BRL (Grand Island, NY, USA). 14-3-3 η small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies against 14-3-3 η, CREB, phosphorylated CREB (Ser 133), c-Fos, RSK2, ERK1/2, phosphorylated ERK1/2 and histone H3 were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated antibodies against FLAG-tag and HA-tag were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). HRP-conjugated antibody against His-tag was from Invitrogen (Carlsbad, California) and non-conjugated antibody against HA-tag was purchased from Covance, Inc. (Princeton, NJ, USA). Antibody against phosphorylated RSK1/2 (Thr 359/Ser 363) was purchased from Santa Cruz Biotechnology, Inc. and anti-phosphrylated histone H3 (Ser10) was purchased from Upstate Biotechnology, Inc. (Charlottesville, VA, USA). Anti-FLAG M2 affinity gel was purchased from Sigma-Aldrich and A/G agarose beads were purchased from Santa Cruz Biotechnology, Inc.

Cell culture and transfection

HaCaT and HEK 293T cells were maintained 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. The cells were maintained by splitting at 90% confluence and media were changed every 2 or 3 days. When cells reached 50% 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 24-36 h and then proteins were extracted for further analysis or starved for EGF treatment.

Expression vectors

The expression constructs including pcDNA4.0-His-RSK2, pCS2-3FLAG-RSK2, and pBabe.puro-HA-14-3-3 η were amplified and used for expression in HEK 293T cells. pcDNA4.0-His-RSK2-S160A, S325A, S386A, S415A, S635A, and S715A mutants were constructed from pcDNA4.0-His-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.

Lentiviral infection

To establish knockdown 14-3-3 η cells, the lentivirus plasmid shRNA of 14-3-3 η was transfected into HEK 293T cells together with PSPAX2 and PMD2-G. Viral supernatant fractions were collected at 48 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 16 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 assay

Cells were trypsinized when cells reached appropriate confluence or when 24 h was passed after siRNA transfection. 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 200 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. Absorbance at 490nm was measured with microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Anchorage-independent cell growth

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 three 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.

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

Knockdown of 14-3-3 η suppresses HaCaT cell proliferation.

To explore the role of 14-3-3 η in EGF-induced skin cell proliferation and transformation, I established HaCaT cells (human keratinocyte of skin) stably expressing 14-3-3 η shRNA. I generated HaCaT cells stably expressing five different sequences of 14-3-3 η shRNAs and confirmed the efficiency of shRNAs by western blotting. 14-3-3 η protein level was dramatically decreased in #2 and #5 of sh-14-3-3 η stably transfected HaCaT cells compared with sh-mock stably transfected cells (Fig. 1A). Thus, HaCaT cells stably expressing these sh-14-3-3 η were used for anchorage-independent cell transformation assay in soft agar. To examine the effect of 14-3-3 η on cell proliferation, we analyzed cell growth of sh-mock and sh-14-3-3 η stably transfected HaCaT cells. The results showed that cell proliferation of 14-3-3 η stably transfected cells was significantly suppressed compared with sh-mock stably transfected cells (Fig. 1B). These results suggested that 14-3-3 η was involved in HaCaT cell proliferation.

Knockdown of 14-3-3 η 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. [11] Therefore, this model was used to determine the role of 14-3-3 η in neoplastic cell transformation. sh-mock or sh-14-3-3 η stably transfected cells were stimulated with EGF (20 ng/mL) as described in Materials and Methods. The number of colonies formed after treatment with EGF was significantly decreased in the sh-14-3-3 η stably trasnfected cells compared with the sh-mock stably transfected cells (Fig. 2A).

To test the role of 14-3-3 η in EGF-induced HaCaT cell proliferation, 14-3-3 η or control siRNA was transiently transfected into HaCaT cells for 24 h. Control siRNA or si-14-3-3 η transfected HaCaT cells were incubated with EGF (100 ng/mL) for 24, 48, and 72 h. The proliferation of si-14-3-3 η transfected HaCaT cells was suppressed compared with control siRNA transfected HaCaT cells (Fig. 2B). This result was consistent with Fig. 1B. In addition, the cell proliferation enhanced by EGF treatment was reduced by knockdown of 14-3-3 η (Fig. 2B). This result was consistent with the data of anchorage-independent HaCaT cell transformation (Fig. 2A).

Taken together, these results indicated that 14-3-3 η have an important role in promoting cell transformation and proliferation of human skin cells exposed to EGF.

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

EGF receptor is a well-known tyrosine kinase receptor. Binding of EGF to its receptor results in activation of the Ras-dependent MAPK cascade. Coupled phosphorylation events induce activation of mitogen-activated protein kinase kinase (MEK), ERK1/2 and RSKs, especially RSK2. Nuclear translocation of RSK2 is thought to influence gene expression through phosphorylation of transcription factors. Especially, EGF signaling via Ras/ERKs/RSK2 pathway is critical in EGF-induced skin cell transformation and proliferation. [11]

To assess whether 14-3-3 η is required for MAPKs pathway activated by EGF, activation of ERKs/RSK2 pathway was tested in knockdown 14-3-3 η HaCaT cells following EGF treatment. To knockdown 14-3-3 η, si-14-3-3 η was transiently transfected into HaCaT cells for 24 h. Serum in growing medium was removed by washing with PBS, and cells were starved for additional 24 h in medium without serum. After that, 10 ng/ml of EGF was treated for 30 min and 60 min. The result showed that EGF-induced phosphorylation of ERK1/2 and RSK2, downstream molecule of ERK1/2, was not significantly suppressed by knockdown of 14-3-3 η (Fig. 3). This result suggests that 14-3-3 η may affect downstream of RSK2 through regulation of RSK2 activity but not the activation level of ERK or RSK2 in EGF-stimulated HaCaT cells.

To test whether 14-3-3 η could regulate downstream molecules of RSK2,

activation of CREB and histone H3 and expression of c-Fos were investigated in knockdown 14-3-3 η HaCaT cells following EGF treatment. Interestingly, phospho-CREB enhanced by EGF was significantly reduced by knockdown of 14-3-3 η (Fig. 3). However, phospho-histone H3 Ser10 was not affected by knockdown of 14-3-3 η (Fig. 3). Moreover, knockdown of 14-3-3 η inhibited EGF-induced c-Fos expression. Taken together, these results indicated that 14-3-3 η might regulate RSK2 activity to phosphorylate CREB and subsequently induce c-Fos transcription.

14-3-3 η directly interacts with RSK2.

As demonstrated in previous results, knockdown of 14-3-3 η impaired the phosphorylation of CREB and also inhibited EGF-induced c-Fos expression. According to current knowledge regarding RSK2 substrate, CREB was reported as a direct substrate of RSK2. [7] Moreover, RSK2-mediated CREB phosphorylation is a prerequisite for c-Fos inducibility by EGF. [8] As described in the introduction, 14-3-3 proteins associate with various intracellular signaling molecules to influence their signaling events. Based on these results and previous reports, I hypothesized that 14-3-3 η might associate with RSK2 to influence signaling events of RSK2, which phosphorylate CREB and subsequently induce c-Fos transcription.

To address the hypothesis, I tested whether 14-3-3 η could interact with RSK2. FLAG-RSK2 and HA-14-3-3 η were co-transfected into HEK 293T cells. HA-14-3-3 η was immunoprecipitated (IP) with anti-HA and FLAG-RSK2 was detected in the IP complex. The data showed that 14-3-3 η interacted with RSK2.

As mentioned in the introduction, 14-3-3 proteins selectively bind to two motifs: RSXpSXP and RXXXpSXP. The sequence of RSK2 was analyzed and six candidates of binding motifs on RSK2 were found. To further verify the specific sites of RSK2 for the binding, I generated six different mutants of His-tagged RSK2s in which serine 160, 325, 386, 415, 635 and 715, respectively, was replaced with alanine (S160A, S325A, S386A, S415A, S635A, and S715A). His-tagged wild-type or mutant RSK2 and HA-14-3-3 η were co-transfected into HEK 293T cells. HA-14-3-3 η was immunoprecipitated with anti-HA and His-RSK2s were detected in the IP complex. The results showed that 14-3-3 η strongly bound with wild-type RSK2. Interestingly, compared with wild-type RSK2, RSK2 S160A, S386A, S415A, and S635A showed reduced binding affinity with 14-3-3 η. Meanwhile, mutations of S325A and S715A in RSK2 sustained strong binding affinity with 14-3-3 η. Taken together, these results demonstrated that 14-3-3 η interact with RSK2, and serine 160, 386, 415, and 635 of RSK2 are important for physical binding with 14-3-3 η.

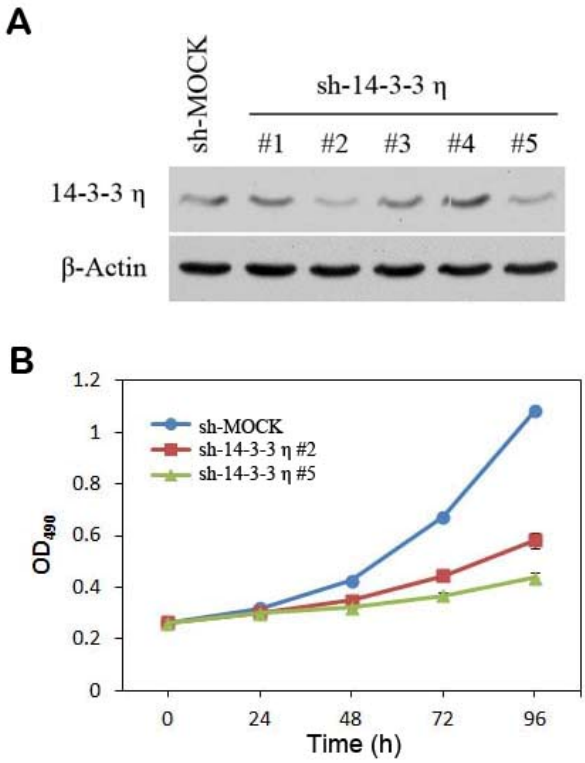
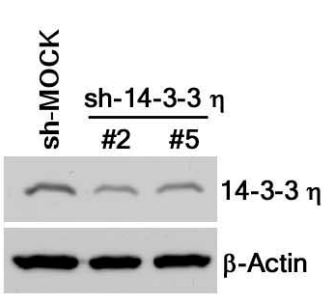
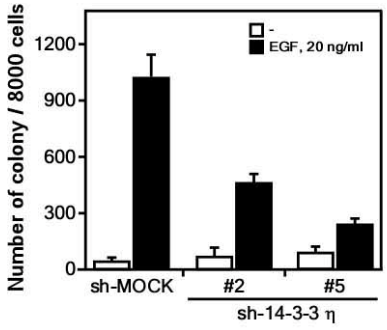


Figure 1. sh-14-3-3 η stable HaCaT cell lines and their cell proliferation assay. (A) To establish stable 14-3-3 η shRNA HaCaT cells, five different sequences of 14-3-3 η shRNAs were stably expressed. The efficiency of shRNAs was confirmed by western blotting. 14-3-3 η protein level was dramatically decreased in #2 and #5 of sh-14-3-3 η stably transfected HaCaT cells compared with sh-MOCK stably transfected cells. (B) sh-MOCK or sh-14-3-3 η stably transfected HaCaT cells were plated in 96-well plates at a density of 10^3 cells/100 μl/well and incubated for 24 h, 48 h, 72 h, and 96 h. The cell growth was determined by MTS assay. 20 μl of Cell proliferation assay solution was added to each well and cells were incubated under normal culture conditions for 1 h. Absorbance at 490 nm was measured by a microplate reader. Cell proliferation of 14-3-3 η stably transfected cells was significantly suppressed compared with sh-mock stably transfected cells.

A


sh-MOCK
sh-14-3-3 η
#2
#5
14-3-3 η
β-Actin


Number of colony / 8000 cells
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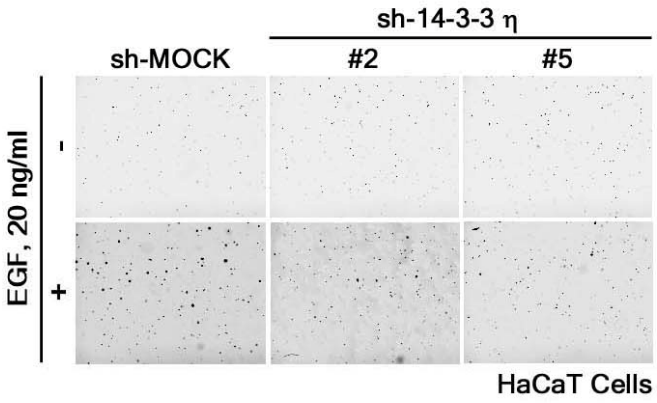
900
600
300
0
-
EGF, 20 ng/ml
sh-MOCK
#2
#5
sh-14-3-3 η


sh-14-3-3 η
sh-MOCK
#2
#5
EGF, 20 ng/ml
-
+
HaCaT Cells

B

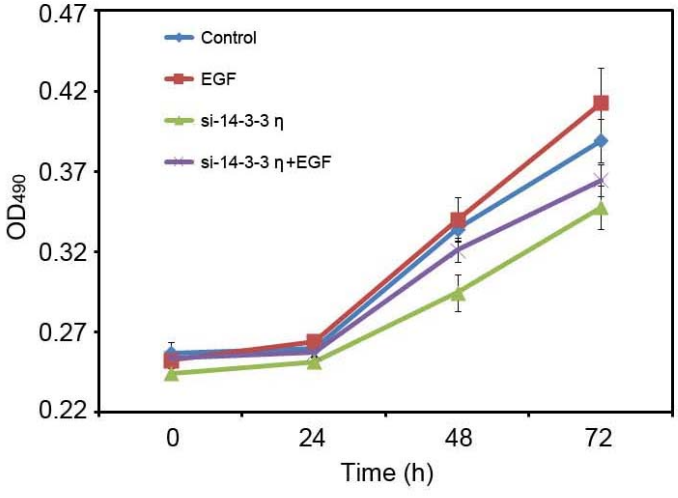

0.47
0.42
0.37
0.32
0.27
0.22
OD490
Control
EGF
si-14-3-3 η
si-14-3-3 η+EGF
0
24
48
72
Time (h)

Figure 2. Knockdown of 14-3-3 η inhibits anchorage-independent HaCaT cell transformation and proliferation induced by EGF. (A) Anchorage-independent growth assay was performed with sh-MOCK or sh-14-3-3 η stably trasnfected HaCaT cells. The number of colonies formed after treatment with EGF was significantly decreased in the sh-14-3-3 η stably trasnfected cells compared with the sh-MOCK stably transfected cells. (B) HaCaT cells were transiently transfected with control siRNA or si-14-3-3 η for 24 h, plated in 96-well plates at a density of 10^3 cells/50 μl/well, and 50 μl of media containing 0 ng/mL or 200 ng/mL EGF was added at 6 h after plating. From this point, cells were incubated for 24 h, 48 h, and 72 h. The cell growth was determined by MTS assay. Knockdown of 14-3-3 η suppressed HaCaT cell proliferation induced by EGF compared with control siRNA-transfected cells.

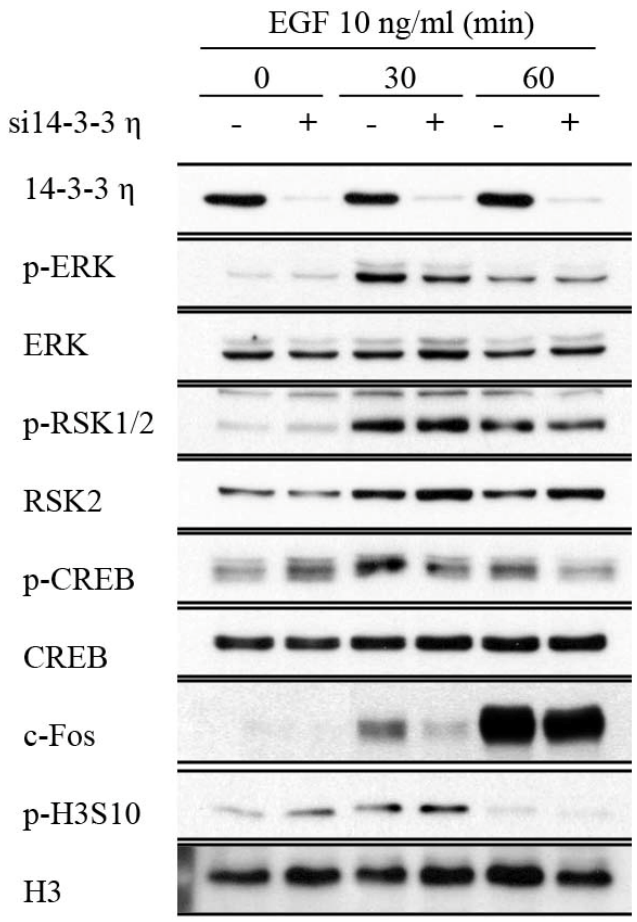
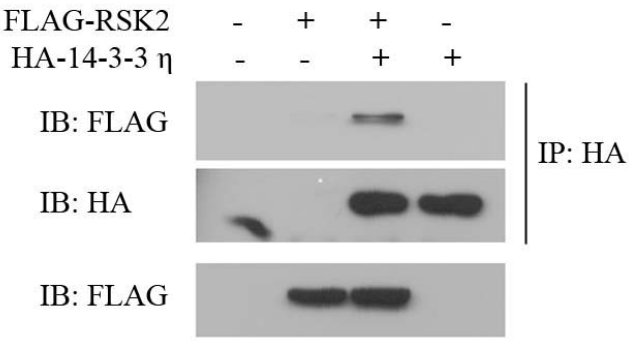


Figure 3. Effects of 14-3-3 η knockdown on EGF signal transduction. HaCaT cells were transiently transfected with control siRNA or si-14-3-3 η for 24 h, starved for additional 24 h, and exposed to EGF (10 ng/mL) for 30 or 60 min. While the of phosphorylation of ERK, RSK2, and histone H3 Ser10 were not affected by knockdown of 14-3-3 η, phosphorylation of CREB and expression of c-Fos (both induced by EGF) were reduced.

A



B

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GYEVKEDIGV GSYSVCKRCI HKATNMEFAV KIIDKSKRDP TEEIEILLRY GQHPNIITLK
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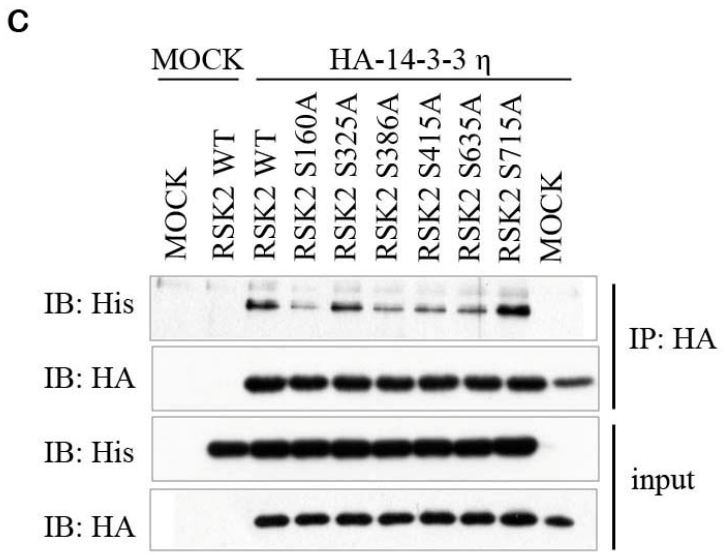

Figure 4. Physical binding between RSK2 and 14-3-3 η. (A) HEK 293T cells were transiently co-tranasfected with FLAG-tagged RSK2 and HA-tagged 14-3-3 η for 36 h. HA-14-3-3 η was immunoprecipitated with HA antibody and analyzed by western blotting. The blots indicated the interaction between RSK2 and 14-3-3 η in vitro (B) The amino acid sequence of RSK2 is marked by boxes which indicate potential 14-3-3 η-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. 4C); and these residues are Ser 160, 325, 386, 415, 635, and 715. (C) To specify the binding sites of 14-3-3 η in RSK2, His-tagged RSK2 mutants were prepared by site-directed mutagenesis as described in Materials and Methods. HEK 293T cells were transiently co-transfected with RSK2 WT or mutants (S160A, S325A, S386A, S415, S635A, and S715A), and HA-tagged 14-3-3 η expressing vectors for 36 h. HA-14-3-3 η was immunoprecipitated with HA antibody and analyzed by western blotting.

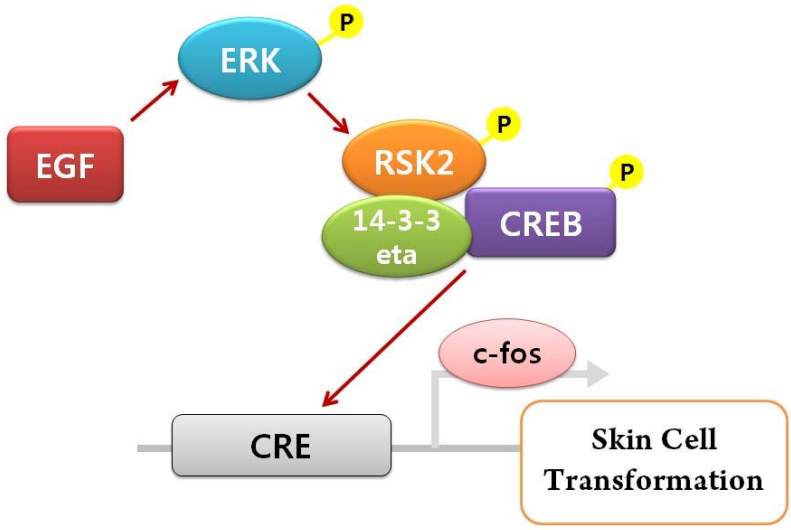

Figure 5. Proposed mechanism illustrating how 14-3-3 η is involved in EGF-induced skin cell transformation.

Discussion

In the current study, I provided pieces of evidence for a novel function of 14-3-3 protein, especially 14-3-3 η, in EGF-induced skin cell transformation. I showed that 14-3-3 η is required for phosphorylation of CREB and expression of c-Fos, which is mediated by RSK2 in EGF-stimulated HaCaT cell. Furthermore, I identified RSK2 as a novel binding partner of 14-3-3 η. Taken together, these results suggested that 14-3-3 η might regulate RSK2 activity for EGF-induced skin cell transformation through physical interaction between them.

Previously, 14-3-3 η has been mainly studied in the field of neuroscience because 14-3-3 proteins are abundantly expressed in the brain and are considered to serve various biological functions involved in neuronal development and cell fate. [12] For example, some reports 14-3-3 η as a susceptibility gene for schizophrenia [13]and Parkinson's disease [14]; however, the exact role of 14-3-3 η in diseases of nervous system remains unknown. While little is known about the specific role of 14-3-3 η, the present study suggests a novel function of 14-3-3 η in EGF-induced skin cell transformation. In detail, I showed that knockdown of 14-3-3 η inhibited anchorage-independent HaCaT cell transformation and proliferation induced by EGF. These results suggested 14-3-3 η as a tumorigenic factor.

14-3-3 proteins are phosphoserine or phosphothreonine binding protines, which are involved in a variety of cellular processes. Their mechanism of action is

typically to induce a conformational change of the target proteins, which results in altering their catalytic activity, to enhance localization within the cell, or to facilitate interactions with other proteins. In addition, 14-3-3 proteins can act as scaffolding proteins, leading to multiprotein complex formation on the target protein. Recently, a number of proteomics studies have characterized over 200 binding partners for 14-3-3s. Although there are a number of plausible binding partners for 14-3-3 proteins, the relationship between 14-3-3s and each binding partner has not yet been elucidated. In the present study, I found physical interaction between 14-3-3 η and RSK2. Moreover, I identified that serine 160, 386, 415, and 635 residues of RSK2 were important for the binding with 14-3-3 η.

Meanwhile, as shown in Fig. 3, knockdown of 14-3-3 η impaired the phosphorylation of CREB and inhibited expression of c-Fos in EGF-stimulated HaCaT cell, without affecting phosphorylation of ERK1/2 or RSK2. It is already known that CREB is one of target phosphoprotien of RSK2 and this phosphorylation is required for c-Fos induction by EGF stimulation. Based on our results and previous studies, I suggested that 14-3-3 η associated with RSK2 to influence signaling events of RSK2, which phosphorylate CREB. This activated CREB would bind to cAMP response elements (CRE) in the promoter region of c-Fos and subsequently induce c-Fos transcription. Elevated level of c-Fos would then contribute to transcriptional induction of genes involved in neopastic cell transformation (Fig.5). Although I have not yet studied the physiological significance of their binding in EGF-induced skin cell transformation, it was

assumed that 14-3-3 η might alter catalytic activity of RSK2 on CREB, or binding activity of RSK2 to CREB through their physical interaction. This hypothesis remains to be identified in future studies.

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국문 초록

EGF로 유도된 피부 세포의 transformation에서 14-3-3 eta의 역할

14-3-3 단백질은 진화적으로 보존된 산성의 작은 (28-30 kDa) 단백질로 포유류 동물에는 β, γ, ε, ζ, η, σ, 그리고 τ의 7 가지 이성체가 존재한다. 14-3-3은 adaptor 단백질로서 목적 단백질의 기능을 단백질-단백질 상호 작용을 통해 조절한다. 최근, 일부 14-3-3 이성체가 발암 과정 억제 혹은 촉진에 관여한다고 보고된 바 있으나, 14-3-3이 피부 암 발생에서 어떠한 역할을 하는지에 대한 연구는 아직 미비하다. 본 연구에서는 14-3-3 η이 EGF로 유도된 피부 세포의 transformation에 관여한다는 것을 밝히고자 하였다. 14-3-3 η이 knockdown 되면, HaCaT의 세포 증식과 EGF로 유도된 anchorage-independent transformation이 대조군과 비교하였을 때 줄어듦을 확인하였다. 그리고, 14-3-3 η 이 knockdown된 경우에 EGF에 의한 CREB의 인산화가 감소하였고, c-Fos의 발현도 역시 감소하였다. 이 결과에 더하여, 본 연구에서는 14-3-3 η가 EGF로 유도된 피부 세포의 transformation에 관여하는 분자적 기전도 조사하였다. 그 기전의 핵심

으로 생각되는 부분은 14-3-3 η와 RSK2의 direct physical interaction으로, 여기에서 RSK2는 EGF로 유도된 피부 세포의 transformation에 중요하다고 알려져 있다. 그리고 RSK2에 14-3-3 η이 결합하는 데에 있어서 RSK2의 네 가지 Serine 잔기 (Ser160, Ser386, Ser415, and Ser635)가 중요하다는 것 또한 밝혔다. 종합적으로, 본 연구는 14-3-3 η이 EGF로 유도된 피부 세포의 transformation에서 RSK2와의 결합을 통해 발암 촉진제로서 작용할 수 있다는 점을 시사한다.