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# Mac-2 결합단백질 발현에서의 STAT3의 역할

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# Role of STAT3 as a Negative Regulator in Mac2- Binding Protein Expression

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**Background :** Mac-2 binding protein (Mac-2BP) is a secreted glycoprotein from the culture fluid of several human cancer cells, especially breast, lung, and gastric cells. Mac-2BP plays a role in immune response and cell adhesion activity in patients with various cancer and infectious diseases. In this study, we attempted to identify the regulators of Mac-2BP expression at the transcriptional level.

**Methods :** To determine the effect of epidermal growth factor (EGF) to Mac-2BP expression in gastric cancers, we constructed the different lengths of Mac-2BP promoter plasmids and measured the promoter activity and Mac-2BP expression. In addition to investigating the role of signal transducer and activator of transcription3 (STAT3) or human telomerase reverse transcriptase (hTERT) as a regulator of Mac-2BP, we transfected the small interfering RNA (siRNA) specific for STAT3 or hTERT, and Mac-2BP level was observed by a quantitative ELISA.

**Results :** EGF treatment could suppress the Mac-2BP transcription in HEK293 or gastric cancer cell lines (SNU-638 or AGS). In 5′-deleted promoter experiment, pGL3-Mac Pro-2377 transfected cells showed a decreased luciferase activity compared to pGL3-Mac Pro-2277. We also identified that (-2,366/-2,356) on Mac-2BP promoter is a putative STAT3 binding site and suppression of STAT3 with STAT3 specific siRNA increased the Mac-2BP level, suggesting the role of STAT3 as a negative regulator, in contrast to hTERT, which is known as a positive regulator.

**Conclusions :** EGF signal is critical for the Mac-2BP expression, and more importantly, STAT3 could work as a negative regulator, while hTERT as a positive regulator in Mac-2BP transcription. *(Korean J Lab Med 2008;28:230-8)*

Key Words : *Mac-2BP, STAT3, EGF, hTERT*

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## INTRODUCTION

The signal transducer and activator of transcription (STAT) family of transcription factors are direct mediators of signaling from the cell membrane to the nucleus. These transcription factors are latent in the cytoplasm

until they are activated by extracellular signaling proteins, such as mainly cytokines and growth factors and some peptides. These extracellular-signaling proteins can activate various tyrosine kinases, including Src and Jak family kinases that phosphorylate STAT proteins and thereby acquire high affinity DNA-binding activity[1, 2]. Then STAT dimerizes via reciprocal phosphotyrosine-SH2 domain interactions. Within minutes, the dimers translocate to the nucleus where they interact with other transcriptional modulators bound to cytokine inducible promoter regions of genes containing gamma activated site (GAS) motif and induce gene expression, and the duration and degree of gene activation are under strict regulation by a series of negatively acting proteins[1-3]. However, tyrosine phosphorylation is required for STAT3 to bind to specific DNA target sites, and nuclear import takes place constitutively and independently of tyrosine phosphorylation[3].

In a number of tumor-derived cell lines, the STATs, particularly STAT3, are required to maintain transformed phenotypes. STAT5 is also commonly found to be consistently activated in certain malignancies, especially leukemias and lymphomas[4]. Cellular transformation by activated STAT3 and its relatives undoubtedly occurs through the transcriptional regulation of specific genes. Many STAT3 target genes are known, including those encoding the anti-apoptotic proteins Bcl-xL, Mcl-1, and Bcl-2, the proliferation-associated proteins cyclin D1/D2 and c-Myc, and the pro-angiogenic factor vascular endothelial growth factor (VEGF)[5-10].

Since VEGF antigen in gastric cancer cells can serve as a pertinent predictive factor for hematogenous invasion or metastasis, it means that STAT3 activation is an important factor in gastric cancer oncogenesis. Indeed, constitutive activation of STAT3 signaling conferred resistance to apoptosis in human U266 myeloma cells, while inhibition of STAT3 signaling led to apoptosis of leukemic large granular lymphocytes and decreased Mcl-2 expression[6]. Therefore, still other genes must be regulated directly or indirectly by STAT3, many of which may contribute to oncogenesis or tumor progression[11, 12].

Mac-2 binding protein (Mac-2BP) is a tumor-associated protein which has possible immuno-regulatory properties in CG-5 human breast cancer cells, and it is detected in the sera of normal as well as tumor-bearing patients[13-15]. The Mac-2BP belongs to the scavenger receptor cysteine-rich domain superfamily of proteins implicated in immune defense and immunoregulation[16]. Recently, we reported that the expression of Mac-2BP was up-regulated by human telomerase reverse transcriptase (hTERT) in gastric tumors, suggesting that Mac-2BP may be used as a diagnostic and/or prognostic marker for gastric tumor patients[15]. For several years, we have focused on the function of Mac-2BP in gastric tumorigenesis in various viewpoints. In the current study, we show that epidermal growth factor (EGF) signal induces the STAT3 protein and it has a role for down-regulated the Mac-2BP expression at transcriptional level using by a luciferase reporter assay or knock-down experiments. Therefore, it suggests that STAT3 is crucial as a negative regulator contrast to hTERT on Mac-2BP expression.

## MATERIALS AND METHODS

### 1. Cell culture and reagents

HEK293 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in MEM-alpha (Gibco-BRL, Grand Island, NY, USA) containing 10% FBS in a humidified 5% $CO_2$ incubator at 37°C. The human gastric cancer cell line, SNU-638, was obtained from the Korean Cell Line Bank (Seoul, Korea), and cultured in RPMI-1640 medium (Hyclone, Road Logan, UT, USA) containing 10% FBS and antibiotics gastric adenocarcinoma (AGS) was also purchased from ATCC and maintained in RPMI-1640 medium as well. Recombinant human EGF was from R&D Systems (Minneapolis, MN, USA), rabbit anti-STAT3 polyserum from Cell Signaling Technologies (Danvers, MA, USA), anti-hTERT polyserum from Calbiochem (La Jolla, CA, USA), and all the other reagents used in this study were purchased from Sigma (St. Louis, MO, USA), unless otherwise indicated.

### 2. ELISA for Mac-2BP

EGF treated HEK293 cells were lysed in a cell lysis buffer (Cell Signaling Technology) containing a 1 mM phenylmethylsulfonyl fluoride (PMSF) and incubated for 40 min on ice. The lysates were centrifuged for 20 min at 12,000 rpm, and the protein concentrations were measured by Bradford assay kit (Bio-Rad, Hercules, CA, USA). The quantities of Mac-2BP in the cytosol of the cells were measured via ELISA, using an s90k/Mac-2BP ELISA kit (Bender Med Systems GmbH, Vienna, Austria) in accordance with the manufacturer's instructions.

### 3. Western blot analysis

The proteins were separated via gel electrophoresis with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto polyvinylidene fluoride (PVDF) membranes (Hybond-P, Amersham Biosciences, Buckinghampshire, UK). The membranes were blocked with 5% non-fat dry milk in TBS-T (50 mM Tris-HCl, pH7.6, 150 mM NaCl, 0.1% Tween-20) and then incubated with the anti-STAT3 or alpha tubulin antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 hr. Following extensive washing, protein bands were visualized using an Immobilon Western Chemilumiscent HRP substrate (Millipore Corp, Billerica, MA, USA).

### 4. Introduction of siRNA for specific STAT3 or hTERT

The unmodified 21-nucleotide synthetic RNAs were chemically synthesized (Samchully Pharm, Seoul, Korea). The siRNA sequences were as followed: STAT3-siRNA sense, 5′-CUU CAG ACC CGU CAA ATT-3′; antisense, 5′-UUU GUU GAC GGG UCU GAA GTT-3′; hTERT-siRNA sense, 5′-GAA CGU UCC GCA GAG AAA ATT-3′; antisense, 5′-UUU UCU CUG CGG AAC GUU CTT-3′; siRNA-control (GL2) sense, 5′-CGU ACG CGG AAU ACU UCG ATT-3′; antisense, 5′-UCG AAG UAU UCC GCG UAC GTT-3′. The transient transfection of synthetic siRNA was accomplished using Lipofectamine Plus Reagent (Invitrogen Corp, Carlsbad, CA, USA).

### 5. Mac-2BP reporter plasmid construction

The −2,377 bp DNA fragment (nucleotide positions −2,377 to +61) including the 5′ flanking region was cloned by PCR using human genomic DNA as a template. The specific regions, designated by the nucleotides spanned, and the respective 5′ primers used for amplification were −1,008 sense and +61 antisense. The −1,008/+61 PCR product was synthesized as followed: −1,008 sense, 5′-A AC GCG TGG TGC AAG TGA CAG AA CAA-3′, +61 antisense, 5′-C CT CGA G CC AGT ATG GAG CGT GGT CAG G-3′.

PCR was run with Ex Taq DNA polymerase (Takara, Otsu, Shiga, Japan) (5 min at 94°C, 30 cycles: 1 min at 60°C, 1 min at 72°C, and 10 min at 72°C). The PCR products were cloned into pCR2.1 TOPO vector (Invitrogen), and then transferred into pGL3-basic luciferase vector (pGL3-Mac Pro-1008) (Promega Corp, Madison, WI, USA). For cloning of pGL3-Mac Pro-2,377, −2,377/−1,008 site in Mac-2BP promoter region was amplified by PCR using Ex Taq polymerase (5 min at 94°C, 35 cycles: 1 min at 94°C, 1 min at 60°C, 2 min at 72°C, and 10 min at 72°C). The primers used were as follows: −2,377 sense, 5′-ACG CGT AAA TAG AGG GAA TTC TAG GAA AGG-3′, −1,008 antisense, 5′-ACG CGT CGG GCT CTT GTT TCT GC-3′. Amplified products were cloned into pCR2.1 TOPO vector and transferred into Mlu I digested pGL3-Mac Pro-1,008 vector. A series of 5′ deletion mutants of pGL2-Mac Pro were constructed by PCR using internal specific primer sets with pGL3-Mac Pro-2,377 as a template. These mutants contained the region from nucleotide positions −2,277 to +61 (pGL3-Mac Pro-2,277), −1,732 to +61 (pGL3-Mac Pro-1,732), −1,008 to +61 (pGL3-Mac Pro-1,008), −597 to +61 (pGL3-Mac Pro-597) and −407 to +61 (pGL3-Mac Pro-407). All constructs were confirmed by DNA sequence analysis (Genotech, Daejeon, Korea).

### 6. Transient transfection and luciferase reporter assay

The cells were plated on day 1 at a density of $5\times10^5$ cells/12 well. One day later, 0.15 $\mu$g of pGL3-Mac pro-2,377 plasmid, 0.15 $\mu$g of pcDNA3-hTERT wild type plasmid, and 0.1 $\mu$g of Renillar plasmid as an internal control were co-transfected with lipofectamine Plus Reagent (Invitrogen). In addition, 0.3 $\mu$g of pGL3 promoter plasmids and 0.1 $\mu$g of Renillar plasmid were co-transfected, transfection media was changed with 1% FBS culture media after 3 hr and then EGF was treated for additional 24 hr. The cells were lysed in a lysis buffer and luciferase assay was performed using Dual-Luciferase$^R$ Reporter Assay System according to the instructions of the manufacturer (Promega). Luciferase activity was determined in Microlumat Plus luminometer (Berthold Technologies, Bad Wildbad, Germany) by measuring light emission for 10 sec. The results were normalized to the activity of Renilla expressed by co-transfected R*luc* gene under the control of a constitutive promoter. For luciferase assays, all experiments were repeated at least three times, and one representative experiment was shown.

### 7. Statistical analysis

Student's *t* test was used to assess differences between groups. A *P* value <0.05 was considered significant. The values in the graphs correspond to the mean of at least three samples. Error bars indicate SD.

## RESULTS

### 1. EGF treatment decreases the Mac-2BP expression

As shown in (Fig. 1), 50% of Mac-2BP was decreased by EGF treatment, but it didn't show dose dependent effects.

### 2. EGF treatment suppresses the Mac-2BP expression transcriptional level

hTERT and pGL3-MacPro-2,377 plasmid co-transfected into HEK293 cells showed that the activity was 25-fold increased in comparison with the control cells. However, the activity was adversely suppressed by EGF treatment (Fig. 2A).

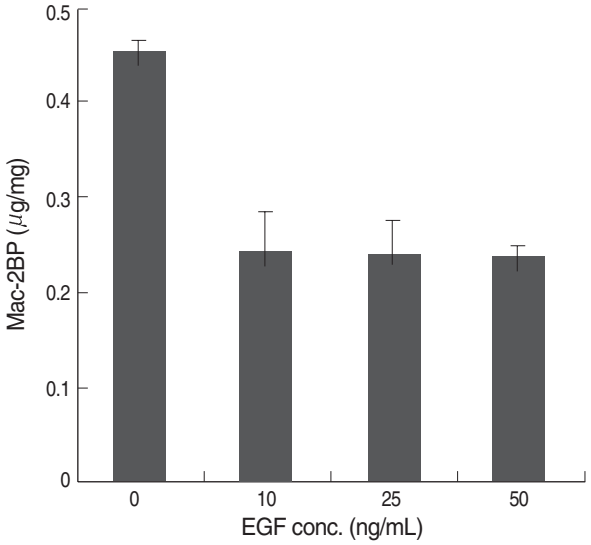


**Fig. 1.** The effect of EGF treatment in Mac-2BP expression. HEK293 cells were serum-starved for 18 hr and treated with the indicated concentrations of EGF. And the cells were lysed and cell lysates were used to measure Mac-2BP by an ELISA. EGF treatment decreased the Mac-2BP expression; however, it didn't show the dosage dependency.
Abbreviations: EGF, epidermal growth factor; Mac-2BP, Mac-2 binding protein.

Next, it was performed with gastric cancer cell lines, SNU-638 or AGS. As shown in (Fig. 2B, C), EGF signal also suppressed the transcriptional activity of Mac-2BP promoter in gastric cancer cells.

### 3. Comparative analysis of transcriptional activities using 5′ deletion mutants of Mac-2BP promoter

To further investigate the regulatory region of Mac-2BP promoter, a series of deleted mutants of the 5′ region were transiently transfected into SNU-638 or AGS cells (Fig. 3). It revealed that the regulatory mechanisms of Mac-2BP transcription could vary among cell lines: Mac-2BP promoter in the two gastric cell lines had several putative positive- and negative-regulatory regions. As shown in Fig. 3, deletion from -2,278 to -2,377 resulted in a loss of one half percent of activity, whereas deletions from

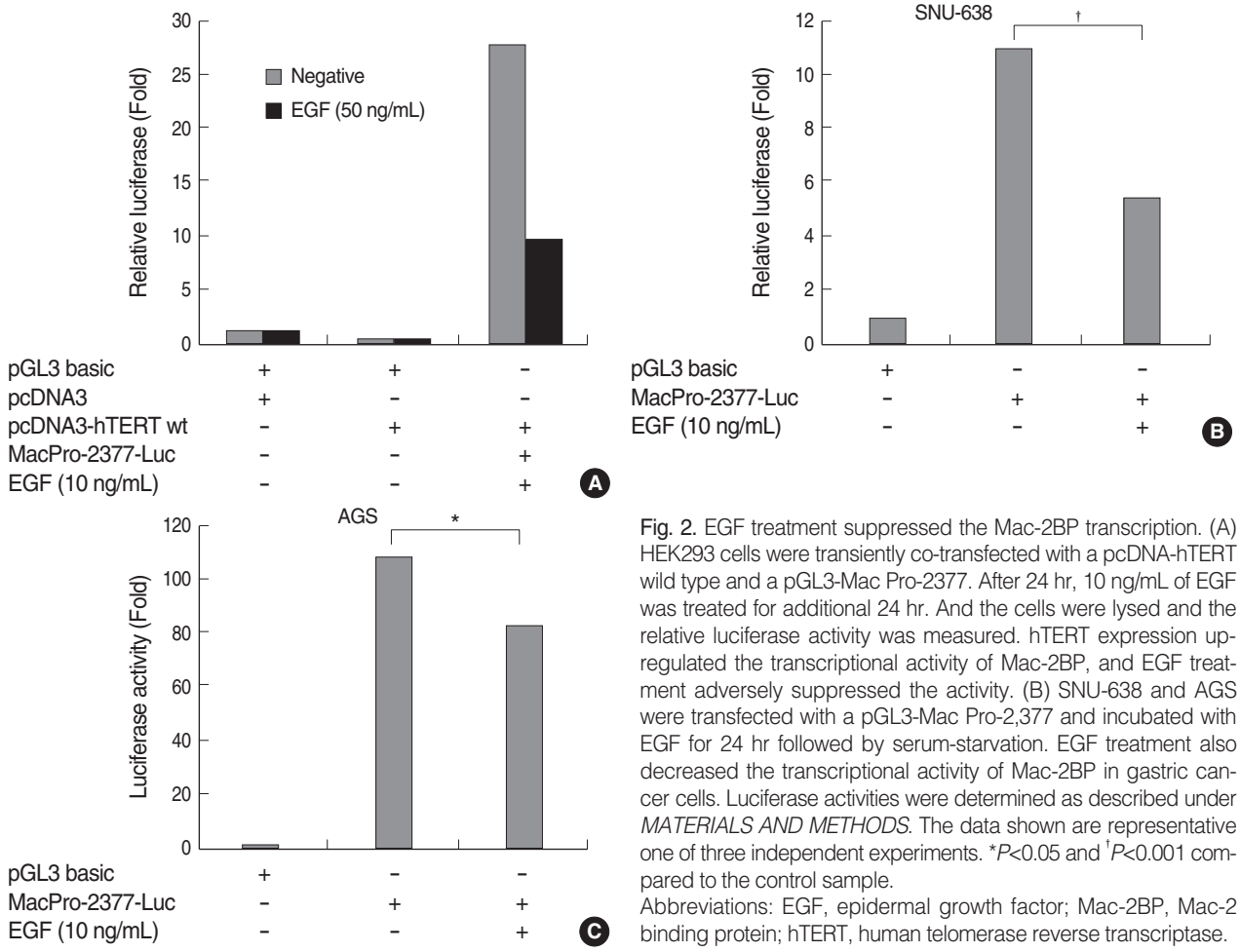


Fig. 2. EGF treatment suppressed the Mac-2BP transcription. (A) HEK293 cells were transiently co-transfected with a pcDNA-hTERT wild type and a pGL3-Mac Pro-2377. After 24 hr, 10 ng/mL of EGF was treated for additional 24 hr. And the cells were lysed and the relative luciferase activity was measured. hTERT expression up-regulated the transcriptional activity of Mac-2BP, and EGF treatment adversely suppressed the activity. (B) SNU-638 and AGS were transfected with a pGL3-Mac Pro-2,377 and incubated with EGF for 24 hr followed by serum-starvation. EGF treatment also decreased the transcriptional activity of Mac-2BP in gastric cancer cells. Luciferase activities were determined as described under *MATERIALS AND METHODS*. The data shown are representative one of three independent experiments. **P*<0.05 and †*P*<0.001 compared to the control sample.
Abbreviations: EGF, epidermal growth factor; Mac-2BP, Mac-2 binding protein; hTERT, human telomerase reverse transcriptase.

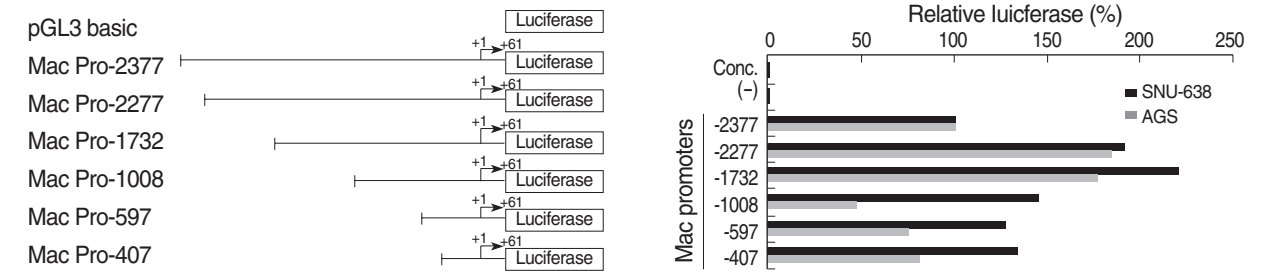


Fig. 3. Comparative analysis of transcriptional activity using 5′ deletion mutants of Mac-2BP promoter. In this schematic, the transcription start site is indicated as +1. Promoter activities of the deletion mutants of Mac-2BP were measured by transient transfection into SNU-638 or AGS cells. And luciferase activities of reporter were normalized with pRL-TK in each cell line. pGL3-Mac Pro-2,377 transfected sample showed 50% decreased activity compared to pGL-Mac Pro-2,277 transfected cells. There is a putative STAT3 binding site (-2,366/-2,356) of Mac-2BP promoter region. The data shown are representative one of three independent experiments; *bars*, SD.
Abbreviations: Mac-2BP, Mac-2 binding protein; hTERT, human telomerase reverse transcriptase; STAT, signal transducer and activator of transcription.

−1,009 to −1,732 enhanced the activity in both cell lines, supporting that there is a putative STAT3 binding site on (−2,366/−2,356) on Mac−2BP promoter. Taken together, it suggests that EGF has roles for suppressing the Mac−2BP expression at transcriptional level and STAT3 may have a possible role for putative negative *cis*−elements for the transcription of Mac−2BP.

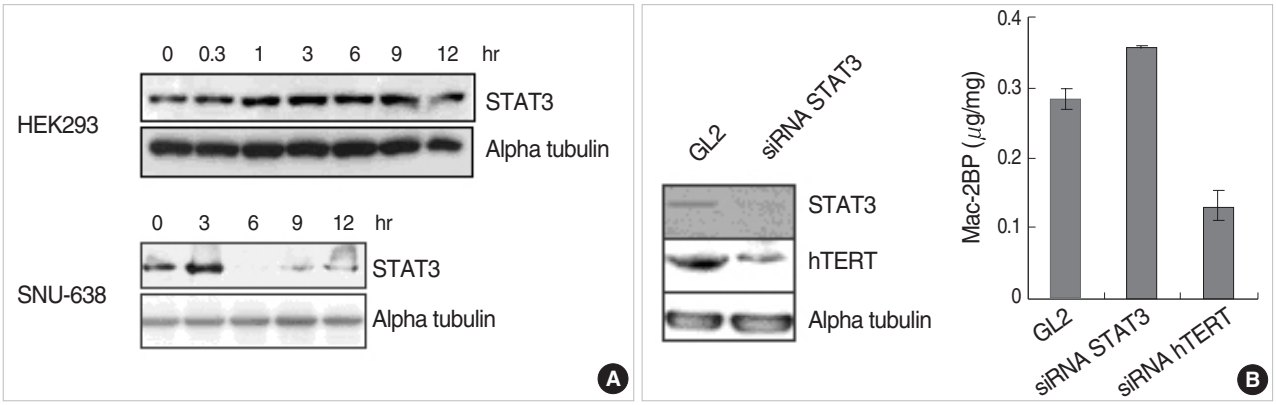


Fig. 4. STAT3 plays roles as a negative regulator of Mac-2BP expression. (A) HEK293 and SNU-638 cells were starved for 18 hr and was treated with EGF as time indicated. The cells were lysed and immunoblotted with STAT3 and alpha tubulin antibodies. STAT3 protein was induced by EGF signal in HEK293 or SNU-638 cells. (B) HEK293 cells were transfected with siRNA specific for STAT3 or hTERT. The cells were lysed and immunoblotted with STAT3, hTERT and alpha tubulin antibodies. The other cell lysates were used to measure the Mac-2BP by ELISA. STAT3 siRNA transfected cells showed to increase Mac-2BP level, but hTERT siRNA transfected cells decreased the Mac-2BP level.
Abbreviations: EGF, epidermal growth factor; Mac-2BP, Mac-2 binding protein; hTERT, human telomerase reverse transcriptase; STAT, signal transducer and activator of transcription.

### 4. The role of STAT3 as a negative regulator in Mac-2BP expression

As shown in Fig. 4A, EGF treatment induced the STAT3 protein within 1-3 hr of incubation, even though we didn't detect phosphorylated STAT3. As shown in Fig. 4B, knock-downed STAT3 or hTERT were observed by western blotting and we measured the Mac-2BP expression. Mac-2BP ELISA showed that hTERT deficiency decreased one third of the Mac-2BP, while STAT3 deficiency up-regulated the Mac-2BP expression. In summary, it suggests that hTERT may be working on a positive regulator, however, EGF induced STAT3 working on negative regulator on Mac-2BP expression.

## DISCUSSION

Here we have shown new evidence that EGF induced STAT3 is an important negative regulator of Mac-2BP expression in HEK293 or gastric cancer cell lines (AGS or SNU-638). The STAT3 was identified originally as a DNA-binding protein that responds to stimulation by EGF and interleukin-6 (IL-6) and critical for normal cellular processes, such as embryonic development, organ genesis and function, innate and adaptive immune function, and regulation of cell differentiation, growth and apoptosis[17-19]. Similar to other STATs, STAT3 is activated in response to cytokine stimulation, and dimerized and in turn translocates to the nucleus, where it binds to defined DNA elements within the promoter region of target genes and activates their transcription[20-22]. However, in many kinds of human tumors including several gastric cancer cell lines, STAT3 is constitutively activated and contributes to oncogenesis by both prohibiting apoptosis and enhancing cell proliferation[23-28].

In this study, we focused on the role of STAT3 by EGF signal in Mac-2BP expression. In order to elucidate whether Mac-2BP could be regulated by EGF signal, the recombinant EGF was treated into starved HEK293 cells in a dose dependent manner. As shown in Fig. 1, EGF signal apparently suppressed the Mac-2BP expression, but it didn't show the dose dependency. It was surprising that Mac-2BP was suppressed by growth factor signaling because nerve growth factor (NGF) could increase the Mac-2BP expression at transcriptional level in our previous study (data not shown). EGF is a 6-kDa polypeptide that binds to a 170-kDa transmembrane tyrosine kinase receptor (EGFR) expressed on a wide variety of normal and neoplastic cells. The ligation of EGF to EGFR induces and activates the number of downstream signal

transduction pathways, such as Ras/Raf/mitogen-activated protein kinase and PI3-kinase/Akt, which involve in cell proliferation, angiogenesis, and apoptosis[29].

Although EGFR appears to be amplified and activated in many cancers, its role in the malignant phenotype is not entirely clear[30]. Several studies showed that in most cells EGF is growth stimulatory and anti-apoptotic; however, a number of tumor cell lines have been shown to be killed by this peptide through unknown mechanisms[31-33]. Consistent with these studies, our results showed that EGF has a negative role to regulate the Mac-2BP expression, although it couldn't detect the apoptotic process.

In this study, we couldn't detect the tyrosin-phosphorylated STAT3 (data not shown) even though STAT3 was strongly induced by EGF signal (Fig. 4). Recent studies have determined that in unstimulated cells, STAT proteins can exist as stable unphosphorylated dimmers. For example, STAT1 and STAT3 homodimers can shuttle between the cytoplasm and nucleus independent of IFN stimulation[34, 35]. Although the function of these unphosphorylated STATs is uncovered, recent studies suggest that in the absence of ligand-induced phosphorylation, STATs may be able to regulate gene expression in a manner distinct from phosphorylated STAT dimers. Therefore, unphosphorylated STAT1 directly associates with IRF-1 to drive the constitutive expression of low molecular mass polypeptice 2 (LMP2)[36]. Unphosphorylated STAT3 also mediates the expression of several genes, including those encoding *Met* proto-oncogene, and serine/threonine kinase 6[37].

We suggest here that Mac-2BP is a new target gene of STAT3 induced by EGF and they are involved in gastric cancer progression via regulation of Mac-2BP transcription. Several reports have shown that Mac-2BP protein is released as a soluble factor from various tumors including breast, lung, and gastric[15, 38, 39], suggesting that Mac-2BP plays an important role in tumor progression. Although the exact biological function of Mac-2BP is still elusidated, the inhibition of Mac-2BP expression by controling STAT3 activity may attribute to tumor chemotherapy. Mac-2BP can also enhance the adhesive interactions between tumor cells and extracellular matrix, contributing the establishment of new tumor colonies. Additionally, increased adhesion to extracellular matrix may help tumor cells to evade apoptosis, a process which has been demonstrated for both Mac-2BP and galectin-3[40-42]. Moreover, when used as a immobilized substrate, Mac-2BP caused a significant reduction in chemotherapy-induced apoptosis of Jurkat T lymphoma cells, a finding that has been recalled to explain the lack of response to chemotherapeutic drugs in lymphoma patients displaying high circulating Mac-2BP level[42].

Our results reveal new lines of evidence which could have implications in therapeutic applications of targeting Mac-2BP expression in gastric tumors. Because it is highly expressed in gastric tumor cells, SNU-638, and also regulated by EGF-induced STAT3 activation.

## 요 약

**배경 :** Mac-2 결합단백질은 인간의 몇몇 암세포들, 특히 유방암, 폐암, 위암세포의 세포액으로 분비되는 당단백질로 면역반응과 다양한 암환자 및 전염성 질환환자에서의 세포부착 활동성의 기능을 담당하고 있다. 본 연구에서는 전사단계에서의 Mac-2 결합단백질의 조절자를 동정하고자 하였다.

**방법 :** 위암 세포주에서 Mac-2 결합단백질 발현에 대한 EGF의 효과를 결정하기 위하여, 서로 다른 길이의 Mac-2 결합단백질 프로모터 플라스미드를 제작하고 프로모터 활성과 Mac-2 결합단백질 발현을 측정하였다. 더욱이 STAT3와 hTERT가 Mac-2 결합단백질의 음성 혹은 양성 조절자인지 그 기능을 규명하기 위하여 STAT3와 hTERT에 특이적인 siRNA를 형질전환시킨 후, Mac-2 결합단백질 수준은 정량 ELISA로 측정하였다.

**결과 :** HEK293 또는 위암세포주인 SNU-638, AGS에 EGF를 처리하면 Mac-2 결합단백질전사를 억제할 수 있었다. 5′-절편 프로모터 실험에서 pGL3-Mac Pro-2,377을 형질전환한 세포가 pGL3-Mac Pro-2,277을 형질전환한 세포에 비해 감소된 루시퍼레이즈 활성을 나타냈다. 또한 Mac-2 결합단백질 프로모터부위 중 -2,366/-2,356이 잠정적인 STAT3 결합부위임을 동정하였고, 양성 조절자로 알려진 siRNA hTERT에 비해 siRNA STAT3가 형질전환된 세포는 Mac-2 결합단백질이 감소되었음을 증명하였다.

**결론 :** EGF 신호가 Mac-2 결합단백질 발현에 중요하며,

hTERT는 Mac-2 결합단백질의 양성 조절자인 반면 STAT3는 음성 조절자로 작용하였다.

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