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The Study on the relationship between STAT3 and β-Catenin

STAT3 와 β-Catenin 의 연관성에 관한 연구

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ABSTRACT

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β-catenin is known to function as an adhesion molecule, but also as a component of Wnt signaling pathway. In the absence of Wnt ligand, β-catenin is constantly phosphorylated and these designated β-catenin is degraded by APC complex, which is a key regulation mechanism of β-catenin. The amounts of β-catenin are also controlled by E3 ubiquitin protein ligase SIAH-1,

committed to phosphorylation-independent degradation pathway. Similar to β-catenin, STAT3 is responsible for various cellular processes such as survival, proliferation, and differentiation. However, little is known about how these molecules balance each other to regulate diverse cellular processes.

In the present study, I investigated regulatory relationship of activation signaling between STAT3 and β-catenin in HEK293T cells. β-catenin-TCF-4 transcriptional activity was suppressed by STAT3 activation, which could be restored by STAT3 inactivation, by which assessed dominant negative STAT3. STAT3 knockdown and inhibition by its siRNA and inhibitors elevated protein levels of nonphosphorylated mutant form of β-catenin, which is same as active β-catenin. SIAH-1, the regulator of active β-catenin through its degradation, showed similar expression pattern of STAT3. Interaction between STAT3 and SIAH-1 stabilizes protein levels of SIAH-1, which increases interaction between SIAH-1 and β-catenin. These results suggest that activated STAT3 may regulate active β-catenin protein levels through stabilization of SIAH-1 that leads to ubiquitin-dependent proteasomal degradation of β-catenin in HEK293T

cells.

Keywords : STAT3, β-catenin, SIAH-1

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CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

STAT3: Signal Transducer and Activator of Transcription 3

SIAH-1: Seven In Absentina Homolog 1

TCF: T Cell Factor 4

LEF: Lymphoid Enhancer Factor

HCC: HepatoCellular Carcinoma

PBS: Phosphate Buffered Saline

SDS: Sodium Dodecyl Sulfate

PAGE : Poly-Acrylamide Gel Electrophoresis

TBS-T: Tris Buffered Saline with Tween-20

CA-STAT3: Constitutively Activated-STAT3

STAT3wt: STAT3 wild-type

LIF: Leukemia Inhibiting Factor

JAK: Janus Kinase

INTRODUCTION

β-catenin is known to function as an adhesion molecule associated with E-cadherin and actin filaments at the cell membrane, but also functions as a component of Wnt canonical pathway [1]. In the absence of Wnt ligand, cytoplasmic β-catenin is constantly degraded by the 'destruction complex', which consists of the scaffolding protein Axin, tumor suppressor adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK3β). CK1 and GSK3 sequentially phosphorylate β-catenin, resulting in recognition by E3 ubiquitin protein ligase β-Trcp and ubiquitin-dependent proteasomal degradation [2]. This continual elimination of β-catenin leads to repression of Wnt target genes, such as c-Myc, c-jun, and cyclin D1, by preventing translocation of β-catenin to the nucleus and formation of complexes with TCF/LEF family of proteins [3, 4]. Wnt/β-catenin signaling has been known to regulate diverse cellular processes, including

organ development, cellular proliferation, morphology, motility, stemness maintenance, and fate determination [5, 6].

Control of the amounts of β-catenin by constant degradation is a major regulation mechanism of β-catenin, as reduced levels of β-catenin prevent nuclear translocation and activation of target genes of β-catenin. As mentioned above, phosphorylated β-catenin is recognized by β-Trcp and targeted ubiquitin-dependent proteasomal degradation. In response to induction of p53, β-catenin also undergoes phosphorylation-independent degradation pathway by interacting with E3 ubiquitin protein ligase SIAH-1, which encourages binding of the ubiquitin-conjugating enzymes (E2s) and proteasomal degradation [7, 8]. Human contains two *SIAH* genes encoding Sina-like proteins, SIAH-1 and SIAH-2 [9], which was first revealed as *Drosophila* protein Sina for requirement of R7 photoreceptor cell differentiation [10]. SIAH-1 protein plays a key role in biological processes such as the cell cycle, programmed cell death, and oncogenesis [11].

STAT3 is one of the STAT family members, which is latent transcription factor that mediates cytokine- and growth factor-directed transcription [12]. In response to binding of

extracellular ligands, the receptor and non-receptor protein tyrosine kinases lead to phosphorylation of STAT3 at the site of tyrosine 705 residue within the transactivation domain close to the carboxy-terminus [13]. The phosphorylated STAT3, active form of STAT3, in the cytoplasm dimerizes with other activated STAT proteins, forming homo- or hetero-dimers and translocates to the nucleus, where binds to the DNA and stimulates target genes [14]. STAT3 participates in a wide variety of cellular processes, including proliferation, postnatal survival, differentiation in the context of growth and development, and invasion, angiogenesis, and metastasis in the context of cancer progression [15, 16].

Despite of common cellular functions, such as proliferation, and fate determination, the relationship of activity between STAT3 and β-catenin has been rarely investigated. It has been reported that STAT3 cooperates with β-catenin to exert their oncogenic effects in breast cancer cells [17]. By contrast, other report showed that treatment of siSTAT3 in HCC leads to increase the levels of β-catenin [18]. To study the precise regulatory relationship between STAT3 and β-catenin, I introduced

artificial systems by transfecting STAT3 and β-catenin in HEK293T cells. Here I report that the STAT3 activation regulates protein levels of β-catenin. Specifically, STAT3 stabilizes SIAH-1 protein that enhances interaction between SIAH-1 and active β-catenin, resulting in ubiquitin-dependent proteasomal degradation of β-catenin in HEK293T cells.

MATERIALS AND METHODS

Cell lines

Human embryonic kidney cells, HEK293T was maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% FBS (Life Technologies, Inc.) and 1% antibiotics (Life Technologies, Inc.). The cells were maintained in a humidified incubator at 37℃ in the presence of 5% CO_2.

Plasmids

To generate expression vector for STAT3 cDNA (GenBank™ accession number NM_213662, purchased from Origene Technologies Inc.), the corresponding STAT3 cDNA was cloned in-frame into pLL3.7 vector (Enzynomics Inc.). A point mutant plasmids of the tyrosine 705 residue, the serine 727 residue, and the tyrosine 705 and serine 727 residues of STAT3 was made

using pLL3.7-STAT3 (Enzynomics Inc.). The plasmid encoding constitutively activated STAT3 used in the present study, namely pCMV-CA-STAT3, was purchased from Addgene (Cambridge, MA, USA). HA-β-catenin was kindly provided by Dr. Jong-Wan Park.

Transfection, siRNA and MG132 treatment

The transient plasmid DNA transfection was done using Lipofectamine 2000 (Invitrogen, Paisley, UK) according to the manufacturer's instructions. siRNAs against STAT3 and negative control were used at 100 nM on HEK293T cells transfected using Qiagen HiPerfect according to the manufacturer's instructions (Catalog No. 301705). MG132 (10 μM) (PeproTech) was used to treat HEK293T cells for 10 h before harvest.

Western blot analysis

Cells were washed with cold PBS and lysed in a cold Radio ImmunoPrecipitation Assay (RIPA) buffer containing protease inhibitor (2 mM PhenylMethylSulphonylFluoried (PMSF), 10 μg/ml leupeptin and 2 mM EthyleneDiamineTetraAcetic acid (EDTA)). The lysates were collected and centrifuged for 20 min at 13,000 rpm at 4℃, and the supernatants were collected. Equal amounts of proteins from the supernatants were separated by SDS-PAGE and transferred on to nitrocellulose (NC) membrane. The membranes were blocked in a TBS-T containing 5% non-fat dried milk for at least one hour and subsequently incubated with specific primary antibodies overnight at 4℃. After washing with TBS-T for 30 min at room temperature, the membranes were further incubated with a HRP-conjugated secondary antibody for 1 h. After washing with TBS-T, the signals were detected using SuperSignal West Femto (Thermoscientific, IL, U.S.A.). The following antibodies were used: phosphotyrosine STAT3, total STAT3, HA-tag, and ubiquitin (Cell signaling technology, MA, U.S.A.), and active β-catenin, Myc-tag (Millipore), and total β-

catenin (Santa Cruz Biotechnologies, Santa Cruz, CA) and α-tubulin (Thermoscientific).

Immunoprecipitation

Cell extracts were precleared with anti-Myc antibody or anti-HA antibody overnight at 4℃ and incubated with protein G sepharose beads for 1 h at 4℃. The beads were then washed five times with ice-cold lysis buffer and suspended in SDS sample loading buffer. Western blotting was then performed.

Luciferase assay

Cells were cotransfected with both 0.25 to 2 μg each of reporter construct and CMV-β-gal plasmid. Luciferase activity was measured using Lumat-LB960 luminometer (Berthold), and divided by β-gal activity to normalize the transfection efficiency.

Statistical analysis

Data are presented as average ± Standard Deviation (SD) and statistical significance was determined by unpaired two-tailed Student's *t* test implemented in Microsoft Excel software. A single asterisk indicates $P < 0.001$.

RESULTS

STAT3 activation suppresses β-catenin-TCF-4 transcriptional activity.

To determine whether CA-STAT3 influences β-catenin transcriptional activity, I analyzed TOPflash reporter activities when overexpressing both CA-STAT3 and β-catenin in HEK293T cells. The protein levels of tyrosine 705 phosphorylated STAT3 and total STAT3 were elevated in a concentration-dependent manner as the amounts of transfected DNA (Figure 1A). According to the amounts of transfected CA-STAT3, the β-catenin/TCF-4-dependent transcriptional activities were decreased in a concentration-dependent manner. To confirm STAT3 activation is associated with decreased transcriptional activity of β-catenin, I performed co-transfection of several STAT3 mutants with β-catenin (Figure 1B). The decreased TOPflash activity by STAT3 was restored similar to the control activity, which was assessed by transfection with STAT3 mutants. These findings indicate that STAT3 regulates β-catenin transcriptional activity of β-catenin.

STAT3 inhibition leads to elevation of active β-catenin levels.

To examine the role of STAT3 on protein levels of β-catenin, I performed siRNA-mediated STAT3 knockdown with or without GSK inhibitor treatment in HEK293T cells. Western blot analysis showed that GSK inhibitor increased the protein levels of endogenous β-catenin. Furthermore STAT3 knockdown with GSK inhibitor treatment more enhanced protein levels of active β-catenin (Figure 2A). To confirm whether inhibition of STAT3 activity can also increase nonphosphorylatable mutant β-catenin, I incubated AG490 (40 μM) or STAT3 inhibitor (10 μM) for overnight. HA-β-catenin is a mutant form of β-catenin, also termed active β-catenin since serine 33 phosphorylation site of β-catenin is mutated so that cannot be degraded by E3 ubiquitin protein ligase β-Trcp-dependent degradation pathway. As shown in Fig. 2B, treatment with either AG490 or STAT3 inhibitor increased protein levels of active β-catenin. These results suggest that STAT3 is one of important regulators of active β-catenin levels.

STAT3 increases the protein levels of SIAH-1

The increasing nonphosphorylatable mutant β-catenin by STAT3 knockdown suggests the possibility the involvement of SIAH-1, which leads to degradation of active β-catenin [8]. To study whether STAT3 can regulate the protein levels of SIAH-1, I first examined the effect of STAT3 on SIAH-1 protein levels by western blotting in HEK293T cells. As shown in Fig. 3A, expression patterns of SIAH-1 is depended on the activation of STAT3, whereas the active β-catenin showed opposite expression aspects of STAT3. In accordance with these findings, the expression of c-Myc, one of β-catenin target gene, also decreased when STAT3 was activated (Figure 3B). These data clearly indicate that the activated STAT3 upregulates the expression levels of SIAH-1 protein, which may decrease active β-catenin in HEK293T cells.

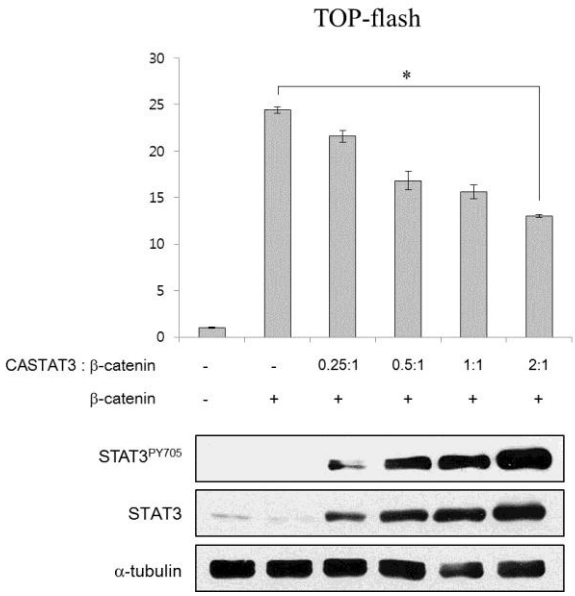
STAT3 interacts with SIAH-1.

The ability that STAT3 inhibits β-catenin-dependent transcriptional activity, decreases HA-β-catenin, and slightly increases SIAH-1 may suppose the possibility of the interaction between STAT3 and SIAH-1, which reduces active β-catenin. Either wild-type Myc-STAT3 or tyrosine 705 residue mutated Myc-STAT3 was transfected in HEK293T cells, and immunoprecipitation was performed with anti-Myc antibody (Figure 4A). Ectopic STAT3 co-precipitated with endogenous SIAH-1, but the interaction between STAT3 and SIAH-1 was reduced when STAT3 activation was inhibited by mutation at tyrosine 705 residue of STAT3. These results indicate that STAT3 activation is essential to interact with SIAH-1. To evaluated whether β-catenin affects the interaction of STAT3 and SIAH-1, Myc-STAT3 and HA-β-catenin was co-transfected in HEK293T cells and performed western blotting, followed by co-immunoprecipitation with anti-Myc antibody (Figure 4B). The data show that STAT3 interacted with SIAH-1, although HA-β-catenin was overexpressed. These results suggest that STAT3 interacts with SIAH-1 and active β-catenin does not influence the interaction between STAT3 and SIAH-1.

STAT3 enhances proteasomal degradation of β-catenin

Since activated STAT3 increases protein levels of SIAH-1 and interacted with this protein, it may possible that STAT3 could promote interaction between SIAH-1 and active β-catenin to increase proteasomal degradation of β-catenin. The results showed that co-expression of STAT3 and HA-β-catenin increased the binding of endogenous SIAH-1 and HA-β-catenin (Figure 5A). These findings indicate that HA-β-catenin is easy to undergo proteasomal degradation through SIAH-1. To examine whether STAT3 enhances polyubiquitination of HA-β-catenin, STAT3 and HA-β-catenin were co-expressed in HEK293T cells in the presence or absence of proteasome inhibitor MG132 (10 μM) to detect ubiquitination of HA-β-catenin. As shown in Fig. 5B, the polyubiquitination of the HA-β-catenin was increased in the presence of both MG132 and STAT3. Taken together, these results suggest that downregulation of active β-catenin by STAT3 is achieved by SIAH-1 that leads to proteasomal degradation of β-catenin.

(A)


TOP-flash
30
25
20
15
10
5
0
*
CASTAT3 : β-catenin - - 0.25:1 0.5:1 1:1 2:1
β-catenin - + + + + +
STAT3PY705
STAT3
α-tubulin

(B)

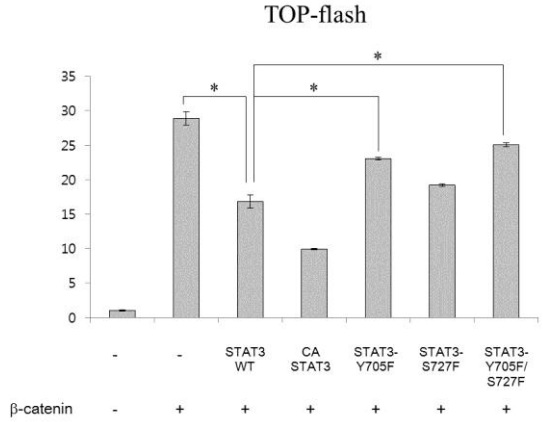
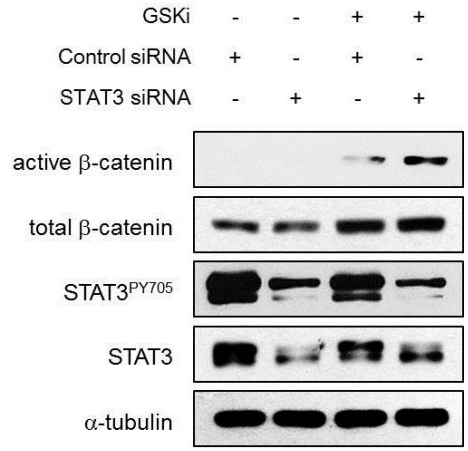

TOP-flash
35
30
25
20
15
10
5
0
*
*
*
- - STAT3 WT CA STAT3 STAT3-Y705F STAT3-S727F STAT3-Y705F/S727F
β-catenin - + + + + + +

Figure 1. STAT3 activation suppresses β-catenin-TCF-4 transcriptional activity.

(**A**) HEK293T cells were transfected with CA-STAT3 and β-catenin. After 24 h of incubation, cells were subjected to luciferase reporter assay. HEK293T cells transfected with CA-STAT3 and β-catenin for 24 h were harvested and western blot analysis was performed. (**B**) HEK293T cells were transfected with STAT3wt, CA-STAT3, several STAT3 mutants, and β-catenin, and then luciferase reporter activity was measured. (**A-B**) β-gal activity was used to normalize transfection efficiency. Data are the mean ± SD, $*P < 0.001$.

(A)


GSKi - - + +
Control siRNA + - + -
STAT3 siRNA - + - +
active β-catenin
total β-catenin
STAT3PY705
STAT3
α-tubulin

(B)

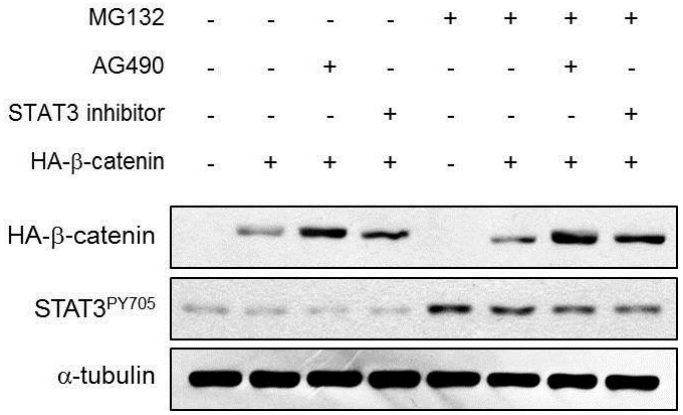
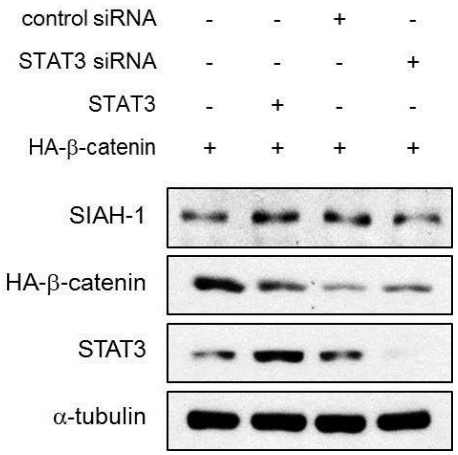

MG132 - - - - + + + +
AG490 - - + - - - + -
STAT3 inhibitor - - - + - - - +
HA-β-catenin - + + + - + + +
HA-β-catenin
STAT3PY705
α-tubulin

Figure 2. STAT3 inhibition leads to elevation of active β-catenin levels.

(**A**) HEK293T cells were transfected with siSTAT3 (100 nM) and scrambled siRNA (100 nM, nonspecific siRNA used as a negative control). After treatment with GSK inhibitor 1 μM for 4 h, cells were harvested and proteins were extracted in RIPA buffer for performing western blot assay. Anti-α-tubulin was used as a loading control. (**B**) HEK293T cells were transfected with β-catenin, and then MG132 10 μM was treated 10 h before harvest. AG490 40 μM and STAT3 inhibitor 10 μM was treated overnight before harvest. Cells were harvested and proteins were extracted in RIPA buffer for performing western blot assay. Anti-α-tubulin was used as a loading control.

(A)


control siRNA - - + -
STAT3 siRNA - - - +
STAT3 - + - -
HA-β-catenin + + + +
SIAH-1
HA-β-catenin
STAT3
α-tubulin

(B)

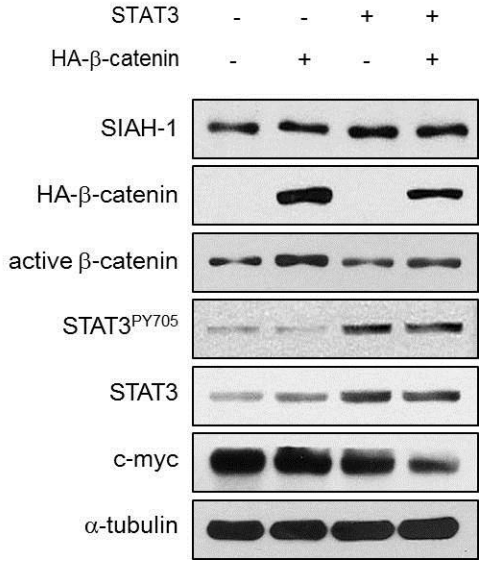
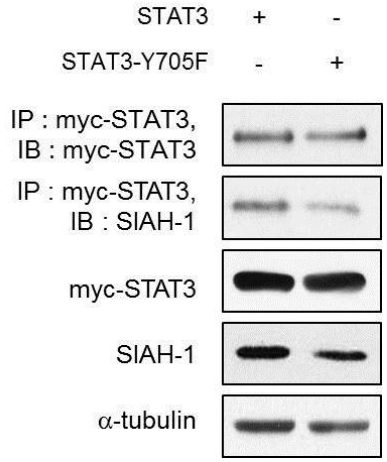

STAT3 - - + +
HA-β-catenin - + - +
SIAH-1
HA-β-catenin
active β-catenin
STAT3PY705
STAT3
c-myc
α-tubulin

Figure 3. STAT3 increases the protein levels of SIAH-1.

(**A**) HEK293T cells were transfected with siSTAT3 (100 nM) and scrambled siRNA (100 nM, nonspecific siRNA used as a negative control) and also with STAT3wt, β-catenin. HEK293T cells were transfected with CA-STAT3 and control pCMV vector. Following 24 h of culture, cells were subjected to western blot analysis. Anti-α-tubulin was used as a loading control. (**B**) HEK293T cells were transfected with STAT3wt and β-catenin. After 24 h later, cells were harvested and proteins were extracted in RIPA buffer for performing western blot assay. Anti-α-tubulin was used as a loading control.

(A)


STAT3 + -
STAT3-Y705F - +
IP : myc-STAT3,
IB : myc-STAT3
IP : myc-STAT3,
IB : SIAH-1
myc-STAT3
SIAH-1
α-tubulin

(B)

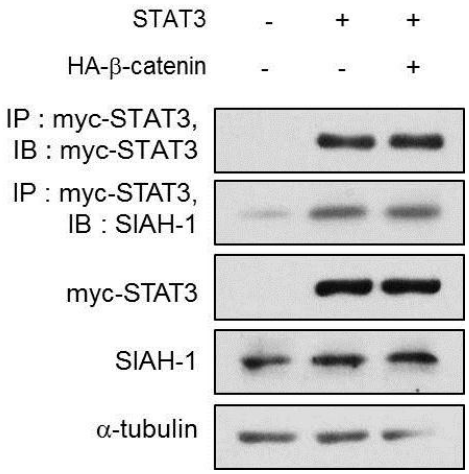
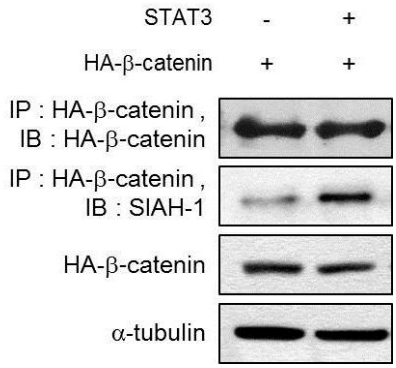

STAT3 - + +
HA-β-catenin - - +
IP : myc-STAT3,
IB : myc-STAT3
IP : myc-STAT3,
IB : SIAH-1
myc-STAT3
SIAH-1
α-tubulin

Figure 4. STAT3 interacts with SIAH-1.

(**A-B**) HEK293T cells were transfected with STAT3wt and STAT3-Y705F. After 24 h later, immunoprecipitation was performed with anti-Myc-antibody and western blotting was done with Myc, SIAH-1 antibody.

(A)


STAT3 - +
HA-β-catenin + +
IP : HA-β-catenin ,
IB : HA-β-catenin
IP : HA-β-catenin ,
IB : SIAH-1
HA-β-catenin
α-tubulin

(B)

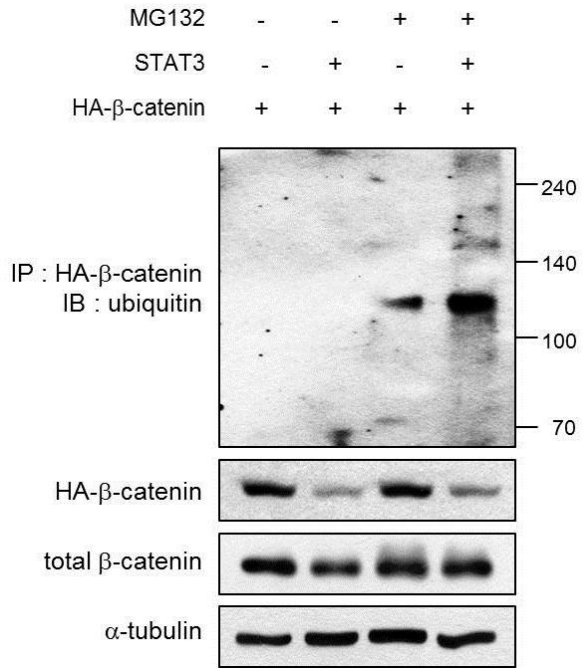

MG132 - - + +
STAT3 - + - +
HA-β-catenin + + + +
IP : HA-β-catenin
IB : ubiquitin
240
140
100
70
HA-β-catenin
total β-catenin
α-tubulin

Figure 5. STAT3 enhances proteasomal degradation of β-catenin.

(**A**) HEK293T cells were transfected with STAT3wt and β-catenin. After 24 h later, cells were harvested immunoprecipitation was performed with anti-HA-antibody and western blotting was done with HA-tag, SIAH-1 antibody. (**B**) HEK293T cells were transfected with STAT3wt and β-catenin, and then MG132 10 μM was treated 10 h before harvest. After 24 h later of transfection, cells were harvested immunoprecipitation was done as explained above.

DISCUSSION

STAT3 is responsible for various cellular processes such as survival, proliferation, and differentiation. Canoncial Wnt signaling co-activator β-catenin is also involved in diverse cellular procedures like as those of STAT3. Nevertheless, little is known about whether these molecules relate to each other, or if so, can regulate each other. In this report, I found that STAT3 controls active β-catenin protein levels through regulation of SIAH-1 that leads to ubiquitin-dependent proteasomal degradation of active β-catenin in HEK293T cells.

STAT3 activation suppressed β-catenin-TCF-4-dependent transcriptional activity, which could be restored by inhibition of STAT3 activity, when STAT3 was inactivated (Figure 1B). To determine whether STAT3-dependent the inhibition of β-catenin-dependent reporter activity occurs through downregulation of protein levels of β-catenin, I evaluated protein levels of β-catenin using STAT3 siRNA in HEK293T cells. When endogenous β-catenin was increased by treatment with GSK3

inhibitor, dephosphorylated β-catenin, in other words, active β-catenin not committed to degradation pathway, was elevated by knockdown of STAT3 (Figure 2A). AG490 and STAT3 inhibitor treatment also enhanced the levels of HA-β-catenin, which is same as active β-catenin since serine 33 phosphorylation site of β-catenin is mutated so that cannot be degraded by E3 ubiquitin protein ligase β-Trcp-dependent degradation pathway. Protein kinases CK1 and GSK3 sequentially phosphorylate β-catenin, and these 'designated β-catenin' is degraded by β-Trcp involved ubiquitin-dependent proteasomal degradation [19]. These results suggest that STAT3 activation leads to downregulation of active β-catenin through proteasomal degradation.

It has been reported that wild-type and nonphosphorylatable mutant β-catenin can be degraded through E3 ubiquitin protein ligase SIAH-1, and SIAH-1 induction is sufficient to degrade β-catenin [7]. SIAH-1 showed similar expression pattern of STAT3 (Figure 3A and 3B), and the activation of STAT3 also decreased one of the β-catenin target genes, c-Myc (Figure 3B). To investigate how STAT3 regulates SIAH-1, immunoprecipitation assay between STAT3 and SIAH-1 was performed. The results

showed that STAT3 interacts with endogenous SIAH-1, whereas tyrosine 705 mutated STAT3 resulted in decrease interaction between STAT3 and SIAH-1 (Figure 4A). These findings suggest that STAT3 activation promotes degradation of active β-catenin through stabilization of SIAH-1. These notion are clearly supported that STAT3 enhances the interaction between SIAH-1 and active β-catenin, which leads to polyubiquitination of active β-catenin (Figure 5).

In this reports, I found that STAT3 stabilizes SIAH-1 protein levels and enhances the interaction between SIAH-1 and active β-catenin, resulting in ubiquitin-dependent proteasomal degradation of β-catenin in HEK293T cells. During developmental processes, both STAT3 and β-catenin play important roles, involving cell survival, maintenance of pluripotency, and specific cellular differentiation. It has been revealed that Wnt/β-catenin pathway acts to prevent ES cell differentiation through convergence on the LIF/JAK-STAT3 pathway [20]. However, genetic and molecular evidence show that the ability and sensitivity of embryonic stem cells to differentiate into the three germ layers is inhibited by increased

doses of β-catenin by specific Apc mutations [21]. Results performed in this study suggest that activated STAT3 can downregulate protein levels of active β-catenin by SIAH-1-dependent manner during differentiation process. Therefore, I suggest that STAT3 activation is involved in the degradation of active β-catenin via SIAH-1 in HEK293T cells.

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국문초록

STAT3 and β-Catenin 의 연관성에 관한 연구

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STAT3 는 다양한 외부신호에 의해 activation 되어 proliferation, survival, oncogenesis, pluripotency 와 differentiation 에 관여하는 다양한 유전자들의 발현을 조절한다. 정상 발달 과정에 있어서 중요한 또 다른 핵심 molecule 인 β-catenin 은 Wnt signaling 의

co-activator 로 작용하여 STAT3 와 유사하게 전사 인자로 직접 작용해 cellular proliferation, stemness maintenance 등에 관여하는 다양한 유전자들을 조절하는 것으로 알려져 있다. 이러한 기능적인 측면에서 유사한 두 molecule 이 서로 어떠한 연관성을 가지고 있는지는 거의 밝혀진 바가 없다. 특히, 정상 발달 과정에 있어서, 분화가 진행될 때 JAK-STAT3 signaling 은 Wnt/β-catenin signaling 이 모두 중요하다고 알려져 있다. 하지만 이 두 signaling 이 서로 연관성을 가지고 있는지 그렇다면 어떻게 조절될 수 있는지에 대해서는 아직 연구가 미흡한 상황이다. 따라서, 본 연구에서 HEK293T cells 에서 STAT3wt, STAT3-Y705F, HA-β-catenin 등을 이용하여 STAT3 와 β-catenin 의 관계를 알아보고자 하였다. STAT3 의 활성증가는 β-catenin 의존적인 전사활성도 감소시키고, active β-catenin 의 단백질도 감소시키는 것을 발견할 수 있었다. 또한 β-catenin 의 타겟 유전자인 c-Myc 또한 감소시키는 것을 확인 할 수 있었다. 이는 STAT3 가 E3 ubiquitin ligase 인 SIAH-1 과 결합하여 active β-catenin 을 분해시킬 수 있다는 것을 알 수 있었다. 이상의 연구 결과는 STAT3 가 active β-catenin 의

감소에 관여할 수 있으며, 이는 β-catenin 을 분해시킬 수 있는 SIAH-1 의 안정화를 통해서 이루어 질 수 있음을 의미한다.

주요어 : STAT3, β-catenin, SIAH-1

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