The biological function and clinical significance of STAT1 in esophageal squamous cell carcinoma

by

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Abstract

Although esophageal cancer makes up only 1% of all the cancers in western countries, it is a common death cancer in other areas, especially China. Esophageal squamous cell carcinoma (ESCC) is the most common type of esophageal cancer in Chaoshan, an area with high incidence rate of this disease in China. However, the pathogenesis of ESCC is incompletely understood until now. Signal transducer and activator of transcription 1 (STAT1) is considered as a tumor suppressor in many types of cancer, such as breast cancer and melanoma. However, the biological and clinical significance of STAT1 in ESCC has never been clearly demonstrated. In this thesis, I hypothesized that STAT1 plays a tumor suppressor role in ESCC via distinct mechanisms, including activavtion of downstream genes and interaction with some other factors. I found that constitutive activated STAT1 induced cell apoptosis, cell growth inhibition and cell cycle arrest in ESCC cell lines, whereas, knockdown of STAT1 led to the opposite results. The *in-vivo* study revealed that STAT1 is downregulated in ESCC compared to case-matched normal esophageal epithelial tissues. Importantly, the loss of STAT1 correlated with a worse clinical outcome in a large cohort of ESCC patients. In addition, STAT1 can regulate several apoptosis and cell cycle target genes, such as BCL-xL, p21, cyclin D1, which was also confirmed by using immunohistochemistry in ESCC patient samples.

Then we found that ERK was an effective negative regulator of STAT1 signaling in ESCC, by promoting its proteasomal degradation and decreasing IFN-γ production. The correlation and clinical significance of STAT1 and ERK were confirmed by using immunohistochemistry in a large cohort of ESCC patients. Finally, we examined the cross-regulation of STAT1α and STAT1β, two isoforms of STAT1 in ESCC. STAT1β was found to enhance the tumor suppressor function of STAT1 by interacting with STAT1α to protect it from protein degradation. The clinical significance of STAT1β was also explored in a large cohort of ESCC patient samples, which confirmed the hypothesis that STAT1β plays a tumor suppressor role in ESCC.

All together, this study provides novel insights into the biological function and clinical significance of STAT1 in ESCC, which favors to understand the importance of STAT1 signaling in cancer. These findings have provided new therapeutic strategies for ESCC by targeting STAT1. We may predict the survival of ESCC patients based on the STAT1 expression. In addition, the anti-tumor drugs, aiming at activating STAT1, could be designed to treat the ESCC patients.

Preface

This thesis is an original work by Ying Zhang. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board. Project name: The clinical and biological significance of Sox2 in breast cancer, Project ID: RES0018569, Human ethical application number: Pro00044942, Date: May 8, 2014.

The research project, of which this thesis is a part, received research ethics approval from the Shantou University Medical College. Project name: The clinical and biological significance of STAT1 in esophageal squamous cell carcinoma, Human ethical application number: SUMC-2015-33, Date: September 11, 2011.

This thesis represents collaborative work, led by Dr. Raymond Lai at the University of Alberta and Dr Min Su at Shantou University Medical College.

Chapter 2 of this thesis has been published as:

Zhang Y, Molavi O, Su M, Lai R. The clinical and biological significance of STAT1 in esophageal squamous cell carcinoma. BMC Cancer. 2014 Oct 29; 14: 791. I contributed to this work as outlined on **page 121** on this thesis.

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Zhang Y, Zhang Y, Yun H, Lai R, Su M. Correlation of STAT1 with apoptosis and cell-cycle markers in esophageal squamous cell carcinoma. PLoS One. 2014 Dec 1; 9(12):e113928. I was the first author of this manuscript, and contributed to the work as outlined on **page 171** of this thesis.

Chapter 4 of this thesis represents work that will be included in a manuscript to be shortly submitted;

Zhang Y, Yun H, Lai R, Su M. ERK promotes proteasomal degradation of STAT1 and high ERK expression correlates with a worse clinical outcome in esophageal squamous cell carcinoma. I was the first author of this work, and contributed as outlined on **page 199** of this thesis.

Chapter 5 of this thesis represents work that will be included in a manuscript to be shortly submitted;

Zhang Y, Yun HL, Zhang HF, Lai R, Su M. STAT1β enhances the tumor suppress effects of STAT1α in esophageal squamous cell carcinoma. I was the first author of this work, and contributed as outlined on **page 251** of this thesis.

Dedicated to My parents

Baozhong Zhang and Hui Liang

To my husband Zhaoyong Liu and my lovely kid Kaize Liu, Kaiqi Liu

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List of Abbreviations

5-FU – 5-fluorouracil

ATP – adenosine triphosphate

AML – acute myelocytic leukemia

BCL2 – B cell lymphoma 2

BCL–xL – B cell lymphoma-extra large

BRCA1 – breast cancer 1

BARD1 – BRCA1-associated RING domain

Ca – cancer

CAMKII – calmodulin kinase II

CDK – cyclin dependent kinase

CECA – cervical carcinoma

CHRY– 3-methyl-1, 8-dihydroxy-9-anthrone

Co-IP/Co-IPP – co-immunoprecipitation

COX2 – cyclooxygenase-2

CPB – CREB binding protein

CRC – colorectal cancer

CXCL10 – C-X-C motif chemokine 10

DAB – 3, 3'-diaminobenzidine

DBD – DNA binding domain

DMEM – Dulbecco's modified Eagle's media

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

EAC – esophageal adenocarcinomas

EC – esophageal cancer

ECL – enhanced chemiluminescence

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

ELISA – enzyme-linked immunosorbent assay

EPC – epidermoid carcinoma

EPCC – epithelial cell carcinoma

ERK – extracellular signal-related kinase

ES – Ewing's sarcoma

ESCC – esophageal squamous cell carcinoma

EV – empty vector

FADD – Fas associated death domain protein

FBS – fetal bovine serum

FGF – fibroblast growth factor

FITC – fluorescein isothiocyanate

GAPDH – glyceraldehyde-3-phosphate dehydrogenase

GAS – gamma activated sequence

GLBL– glioblastoma

GM-CSF – granulocyte macrophage colony-stimulating factor

GNAS – guanine nucleotide binding protein

GWAS – genome-wide association study

HAT– histone acetyltransferas

H&E – hematoxylin and eosin

HDAC1 – histone deacetylase 1

HEP – hepatocyte

HGF – hepatocyte growth factor

HIF – hypoxia inducible factor

HPV – Human Papillomavirus

HMLH1 – human mutL homolog 1

HNC – head and neck cancer

HNSSC – head and neck squamous cell carcinoma

IAP – inhibitor of apoptosis

ICAM1 – intercellular adhesion molecule 1

IHC – immunohistochemistry

IFN – interferon

IGF – insulin-like growth factor

iNOS – inducible nitric oxide synthase

IL – interleukin

IP – immunoprecipitation

IRF – IFN regulatory protein

ISGylation – interferon-stimulated gene conjugation

ISRE – interferon-sensitive response element

JAK – Janus kinase

JH – JAK homology domain

JNK – c-Jun N-terminal kinase

KMT2D – lysine (K)-specific methyltransferase 2D

LEUK – leukemia

LPS – lipopolysaccharide

LYMP – lymphoma

mAB – monoclonal antibody

MAPK – mitogen activated protein kinase

MAPKK – MAPK kinase

MAPKKK – MAPKK kinase

MBL – medulloblastoma

MCL1 – myeloid cell leukemia sequence 1

MCM5 – maintenance complex component 5

MDM2 – mouse double minute 2

MEK – mitogen-activated protein kinase

MEK1/2 – MAP kinase/ERK kinase 1/2

MHC – major histocompatibility complex

MM – multiple myeloma

MMP – matrix metalloproteinase

MT1G – metallothionein-1G

MTS – 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-

sulfophenyl)-2H-tetrazolium

N – normal

NBL – neuroblastoma

NF-ƙB – nuclear factor kappa B

N-terminal – amino terminal

N/A – not available

OPN – osteopontin

OSM – oncostain M

OVCA – ovarian cancer

PY – phosphorylated tyrosine

PARP1 – poly (ADP ribose) polymerase 1

PBS – phosphate buffered saline

PCA – pancreatic cancer

PCNA – proliferating cell nuclear antigen

PCR – polymerase chain reaction

PCT– pheocromocytoma

PDGF – platelet-derived growth factor

PGP 9.5 – protein gene product 9.5

PH – pleckstrin homology

PKC-δ – protein kinase C-δ

PI – propidium iodide

PKR – RNA protein kinase

PRMT–protein arginine methylation transferase

PTP – protein tyrosine phosphatase

PTPN6 – tyrosine phosphatase non-receptor type 6

RB1 – retinoblastoma 1

RNA – ribonucleic acid

ROS – reactive oxygen species

RT-PCR – reverse transcriptase -polymerase chain reaction

Runx2 – runt-related transcription factor 2

SAR – sarcoma

SCC – squamous cell carcinoma

SDS-PAGE – sodium docedyl sulfate-polyacrylamide gel electrophoresis

SH2 – src homology 2

SKP2 – s-phase kinase-associated protein 2

siRNA – short interfering RNA

SLIM – STAT interacting LIM

SMUF – smad ubiquitination regulation factor

SOCS – suppressor of cytokine signaling

SUMOylation – small ubiquitin-like modifier conjugation

STAT – signal transducer and activator of transcription

STAT1C – constitutively active STAT1

TAP – antigen processing

Th – T helper

TNF– tumor necrosis factor

TRADD – tumor necrosis factor receptor type 1-associated DEATH domain

TRAF1 – TNFR-associated factor 1

TRAILR –TRAIL receptor

TYK2 – tyrosine kinase 2

Tyr – tyrosine

Ub – ubiquitin

uPA – urokinase-type plasminogen activator

UPP – ubiquitination proteasome degradation

UV – ultraviolet

VEGF – vascular endothelial growth factor

WHO – World health organization

CHAPTER 1

Introduction

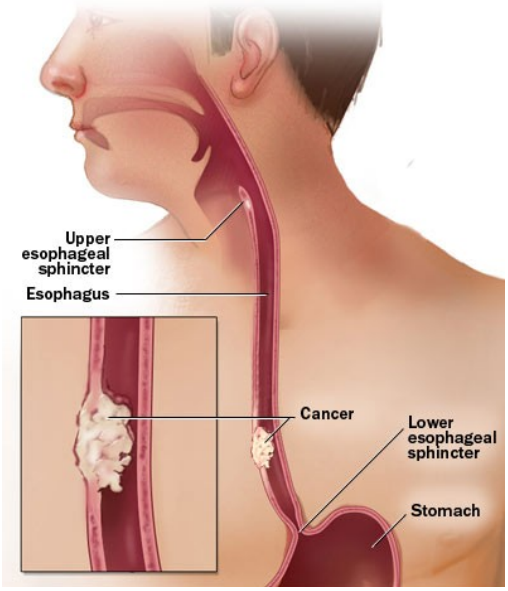
1. Introduction

1.1 Esophageal cancer-overview

Esophageal cancer (EC) involves a type of cancer generated from the esophagus (**Figure 1.1**). The age-standardized five-year survival rate of EC is around 16.9%, which means that most patients die of this type of cancer in the first two years after diagnosis **[1]**. There are two major types of EC; the first type, esophageal squamous cell carcinoma (ESCC) arises from stratified squamous epithelial cells. The other type of EC, adenocarcinomas (EAC) EC, affects columnar glandular cells that replace the squamous epithelium **[1]**. In addition, some rare types of cancer, such as sarcomas and small cell carcinomas, represent less than 1%-2% of all ECs **[2]**. These other carcinomas, including melanomas and lymphomas, may also develop in the esophagus on rare occasions **[2]**. ESCC comprises 90-95% of all cases of EC in Central Asia, especially China. In other countries, especially Western countries, EAC constitutes the dominant type of EC **[3]**.

Many risk factors, including genetic, environmental, and lifestyle, contribute to the development of ESCC. Studies show that for genetic risk factors, a variety of genes and/or microRNAs mediate aberrant cell growth, metabolism, and deoxyribonucleic acid (DNA) repair in ESCC **[4]**. For example, some well-known cell cycle and apoptosis regulation genes

A



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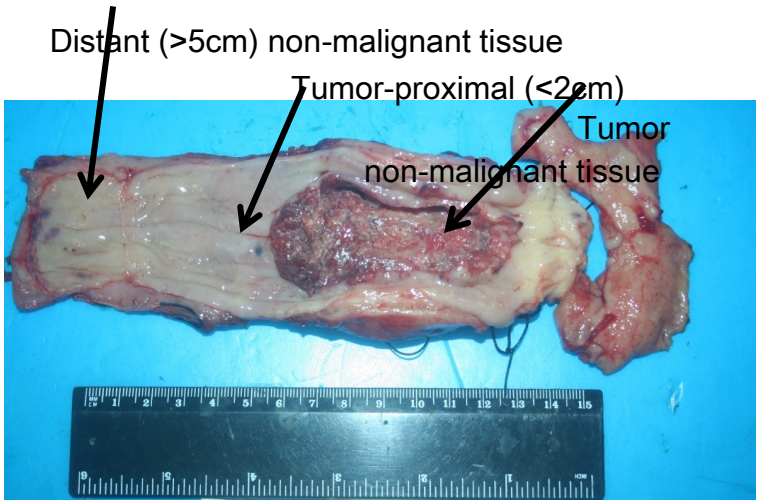

Figure 1.1 Schematic illustrations of esophageal cancer and sample collection. A. EC can occur anywhere in the esophagus, but is usually located in the middle portion of esophageal. B. For experiment preparation, we collected the EC samples in three different locations in one EC case, including distant and tumor proximal non-malignant tissues as well as the EC tissue.

as well as histone modifier genes were mutated in a cohort of Chinese ESCC patients **[5]**. The first set of genes included p53, cyclin D1, and Retinoblastoma (RB1), while the histone modifier genes incorporated lysine (K)-specific methyltransferase 2D (KMT2D), KMT2C, and KDM6A. Environmental risk factors, such as human papillomavirus (HPV) infection as well as N-nitroso compounds, can cause inflammation, toxicity, local irritation, and mutation in esophageal tissues **[6]**. Furthermore, lifestyle-related factors for EC include the consumption of hot foods and beverages as well as smoking and alcohol, especially in the case of ESCC **[1]**. The data generated from a genome-wide gene-environment interaction analysis in a large cohort of ESCC patients revealed the presence of six ESCC susceptibility loci **[7]**. Four of these areas, located at chromosomes 4q23, 16q12.1, 22q12, and 3q27, had a significant effect on ESCC; however, the other two areas, 2q22 and 13q33 were associated with ESCC only in the case of alcohol consumption. These results demonstrated that alcohol is a key factor that interacts with ESCC genetic contribution **[7]**.

Among all countries, China has the highest incidence of EC worldwide. However, the incidence of EC is regionally distributed throughout the nation (**Figure 1.2**). Specifically, the Chaoshan region represents a well-known high-risk area for EC. In this region, the annual average age-standardized incidence rate of EC is 74.5 /100,000 people, which is

Figure 1.2 Two high incidence areas of esophageal cancer in China. China has highest incidence of EC worldwide and unique geographic distribution pattern of EC. There are two areas with high incidence rate of EC, Linzhou and Chaoshan. Linzhou located in the middle and Chaoshan region located at the southeast of China. Nanao island, belonged to Chaoshan area, is well-known for top age-standardized incidence rate of EC, which is almost $100/10^5$ populations. The blue lines show that there is a human immigration from center China to Chaoshan area.

10 times the average global rate of EC per capita **[8]**. These figures suggest the possibility of potential genetic and/or environmental factors predisposing this particular population to EC. In a recently published review, the authors clearly demonstrated that several specific risk factors contribute to ESCC in the Chaoshan region, including the consumption of hot tea and smoking **[9]**. Additionally, studies have shown that the occurrence of injury and inflammation through exposure to pathogens, in combination with inappropriate gene activation or silencing, represent major factors for promoting ESCC in the Chaoshan area of China **[9]**.

In previously published papers, researchers isolated the presence of many cell-signaling pathways involved in the development of ESCC. Specifically, the Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) signaling pathway constitutes one of most common pathways in ESCC.

1.2 JAK-STAT signaling pathway

JAK-STAT signaling pathway is an essential component of the cytokine receptor system. This pathway consists of three main components: receptors, JAKs, and STATs. As shown in **Figure 1.3**, upon stimulation from interferon (IFN), growth factors, hormone or other chemical messengers, the isolated receptors on the cell membrane become assembled to activate JAK family members, resulting in the

phosphorylation of JAKs. JAKs can phosphorylate the receptors on their tyrosine residues and provide docking sites for the STATs. Subsequently, STATs can bind to the receptors and phosphorylate on their tyrosine residue site. The phosphorylated STAT molecules form dimers and migrate into the nucleus, where they bind to the DNA consensus sequence and induce target gene transcription. The STAT dimers activate gene expression by binding to the different IFN responsive elements, such as the gamma-activated sequence (GAS) or the interferon-sensitive response element (ISRE) **[10].**

As shown in **Table 1.1**, JAK-STAT signaling can be activated by many stimuli, such as IFNs, Interleukin (IL)-6, IL-10 and IL-21 cytokine families, growth factors and hormones **[11]**. In response to the stimuli, STATs accumulate in the cell nucleus over a short period of time and then return to the cytoplasm for the next term of signaling. This process involves the negative regulation of JAK-STAT signaling, which can occur on multiple levels **[12, 13]**. For example, the suppressor of cytokine signaling (SOCS) family members can inhibit JAK activation by competing with STATs for phosphorylation binding sites on the cytokine receptors. Moreover, protein tyrosine phosphatases (PTPs) negatively regulate the signaling by dephosphorylating STATs both in the nucleus and cytoplasm. The protein inhibitor of activated STAT (PIAS) family members can inhibit the DNA binding and transcriptional activity of STATs by interacting with

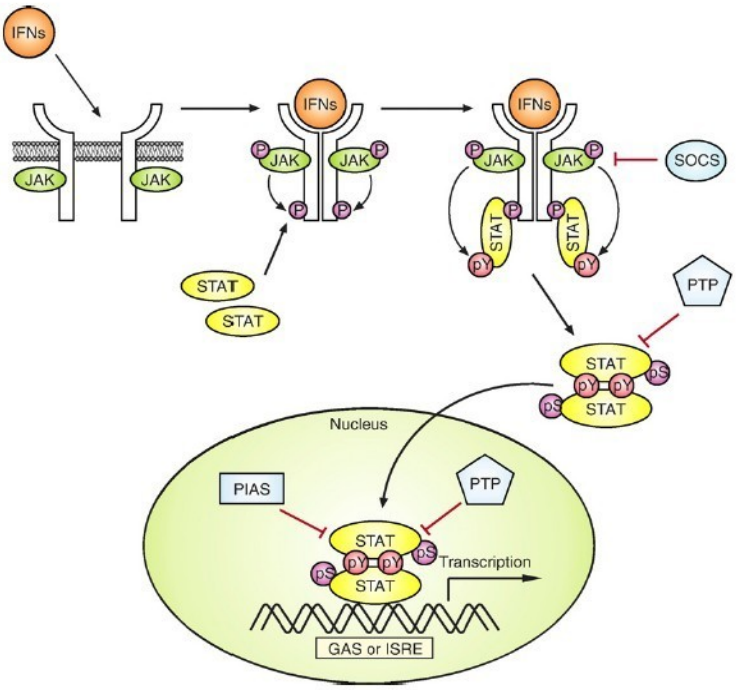

Figure 1.3 JAK-STAT signal pathway and its negative regulators. In response to IFN stimulation, IFN receptors form dimers to phosphorylate JAKs, which is followed by tyrosine phosphorylation of receptors. Then, STAT dimers translocate from cytoplasm to the nucleus to bind to the IFN responsive element, GAS or ISRE. Various protein families can negatively regulate the JAK-STAT pathway. SOCS proteins directly bind to tyrosine phosphorylated of JAKs to inhibit its activation. PTPs can negatively regulate STATs in both the nucleus and cytoplasm, as well as PIASs directly inhibit the transcriptional activity and DNA-binding of STATs.

Table 1.1 Stimuli of JAKs and STATs

		Cytokines	Growth factors	Hormones	Others
JAKs					
	JAK1	IFNs, IL2, IL10, IL6 family	EGF, VEGF, FGF, HGF, IGF1, PDGF	Growth hormones	OSM
	JAK2	IFN-α/β, IL3, IL5, IL6	EGF, VEGF, FGF, HGF, IGF1, PDGF	Growth hormones	GM-CSF
	JAK3	IL2, IL4, IL7, IL9, IL15, IL21	EGF, VEGF	Growth hormones	
	TYK2	IFN-α/β, IL6, IL10, IL12, IL23	VEGF		Cardiotrophin 1, cardiotrophin-like cytokine
STATs					
	STAT1	IFNs, IL2, IL10, IL6 family	EGF, VEGF, FGF, HGF, IGF1	Growth hormones	OSM
	STAT2	IFN-α/β, IL3, IL5, IL6			
	STAT3	IFNs, IL2, IL10, IL6 family, IL21, IL27	EGF PDGF	Growth hormones	F-CSF, leptin
	STAT4	IL12, IL23			
	STAT5	IL3, IL5, IL2, IL7, IL9, IL15, IL21		Growth hormones	GM-CSF
	STAT6	IL4, IL13			

Abbreviations: EGF: Epidermal growth factor; FGF: fibroblast growth factors; GM-CSF: Granulocyte macrophage colony-stimulating factor; HGF: Hepatocyte growth factor; IGF1: Insulin-like growth factor; OSM: Oncostatin M; PDGF: Platelet-derived growth factor; VEGF: vascular endothelial growth factors;.

STATs in response to cytokine stimulation **[14, 15]**.

1.2.1 JAKs

JAKs are an intracellular tyrosine kinase family that comprises four family members: JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2) **[16]**. All of these members appear to undergo ubiquitous expression, with the exception JAK3, which is normally expressed in lymphoid cells. As shown in **Figure 1.4**, JAKs range in size from 120-140 kDa, and these molecules are structurally unique in possessing the JAK homology domain (JH) 1-7. JH1, a C-terminal kinase domain, is important for the enzymatic activity of JAKs. JH2, a pseudo kinase domain, lacks enzymatic activity; however, this domain performs a critical regulatory function. The JH3-JH4 domains share a homology with the Src homology 2 (SH2) domains **[17]**. The N-terminal domain, JH4-JH7, known as the FERM domain (F: 4.1 protein, E: Erin, R: Radixin and M: Moesin), is essential for the mediation of cytokine receptors **[17]**.

As shown in **Table 1.1**, JAKs can be activated upon the multimerization of ligand-mediated receptors. For example, JAK1 and JAK2 are involved in IFN-γ stimulation, whereas JAK1 and TYK2 are stimulated by IFN-α and IFN-β. In response to cytokines, the receptors bind to ligands to change the conformation and cause JAKs to phosphorylate each other. The phosphorylated JAKs can subsequently activate both the receptors and

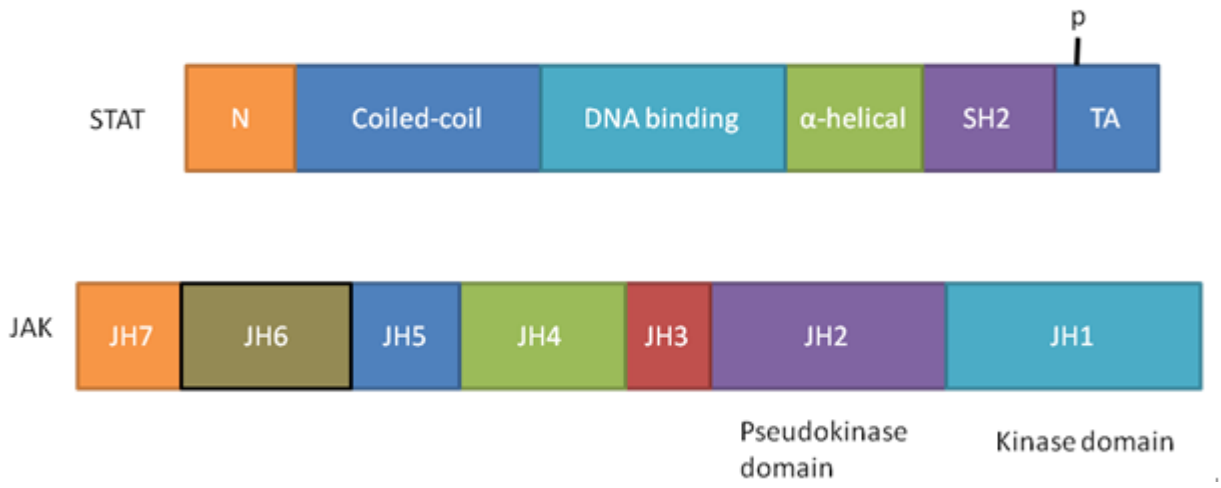

Figure 1.4 Structures of JAKs and STATs. STATs are composed of six functional domains, including N-terminal domain, coiled-coil domain, DNA binding domain, α-helical domain, src homology 2 (SH2) domain and transcription activation domain (TAD). Tyrosine phosphorylation is usually located in the TAD. JAKs have tandem kinase and kinase-like domains, which is very unique structure. The C-terminal domain of JAKs is JH1, a kinase domain. The JH2 is the kinase-like domain. The functions of the JH3-JH7 domains have not been well understood. P, phosphorylation.

the major substrates, such as STATs **[17]**.

1.2.2 STATs

The STAT family comprises seven transcription factors: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 **[16]**. Most of these proteins vary in length from approximately 750 to 800 amino acids; however, STAT2 and STAT6 consist of approximately 850 amino acids. As shown in **Figure 1.4**, STATs usually have six functional domains, including an N-terminal domain, a DNA-binding domain (DBD), a SH2 domain, a coiled-coil domain, a linking domain, and a transcriptional activation (TA) domain. The SH2 domain mediates the dimerization of STATs, while the N-terminal region fulfills a function in the protein binding of STATs. In the TA domain, the phosphorylation of tyrosine enables STAT dimerization, and the serine phosphorylation site has been identified in some STATs, such as STAT1 and STAT3.

As shown in **Table 1.1,** STATs can undergo activation by various cytokines, growth factors, and hormones. JAKs stimulate the tyrosine and serine phosphorylation in the transactivation domain, which allows STATs to dimerize and translocate into the nucleus. Subsequently, STATs bind to the target gene promoters to induce gene expression **[17]**. STAT proteins fulfill important roles in mediating cell biological functions, such as cell proliferation, apoptosis, and development. Among all STAT family

members, STAT1 and STAT3 have received the most research attention.

1.3 STAT1

1.3.1 STAT1 structure

The first STAT family member, STAT1, provides an essential part of IFN-signaling **[18]**. STAT1 is a 91 kDa protein composed of 750 amino acids. However, as in the case of STAT3 and STAT4, STAT1 has two isoforms: the longer STAT1α (91 kDa) and the shorter STAT1β (84 kDa). Spliced from STAT1, STAT1β comprises a truncated version of STAT1. This molecule lacks 38 amino acids due to the deletion of the last 118 nucleotides **[19]**. Thus, compared to STAT1α, STAT1β lacks most of the transactivation domain as well as a serine 727 phosphorylation site in the C-terminus. Intriguingly, the expression level ratio of STAT1α and STAT1β can change in response to specific physiological stimulation. For example, the expression of STAT1β can undergo dramatic upregulation in response to *Mycobacterium avium intracellularae* and *Leishmania mexicana* infections in macrophage cells **[20, 21]**.

As shown in **Figure 1.5**, STAT1 is composed of six functionally conserved domains, including an N-terminal domain, a coiled-coil domain, a DBD, a α-helical domain, a SH2 domain and C-terminal TA domain **[10]**. These domains have distinct functions; first, the N-terminal of STAT1 dimerizes

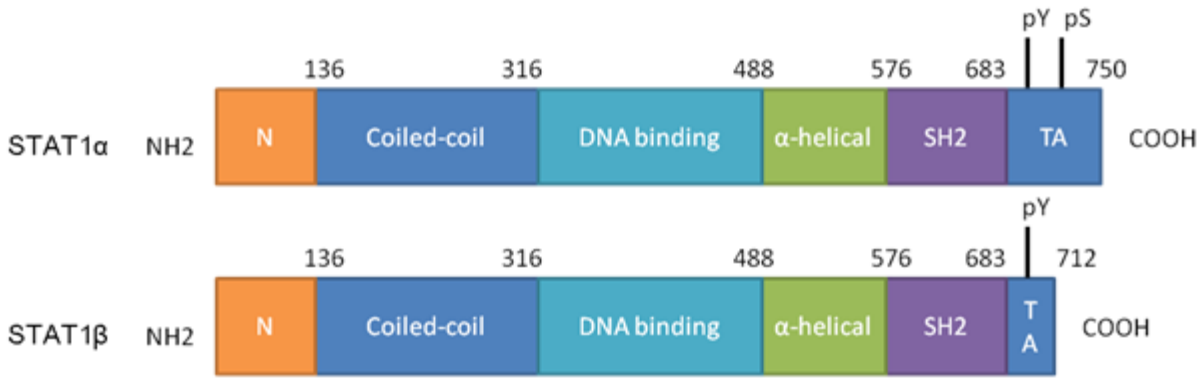

Figure 1.5 Structure of STAT1. STAT1 is composing of six functional domains: N-terminal domain (N), coiled-coil domain, DNA-binding domain, α-helical link domain, src homology (SH) 2 domain, and C-terminal transcriptional activation (TA) domain. STAT1α is full length of STAT1 and STAT1β lacks 38 amino acids and most of TA domain. pY, tyrosine phosphorylation; pS, serine phosphorylation.

unphosphorylated STAT1, resulting in antiparallel dimers **[22].** In addition, this domain also facilitates the interaction between STAT1 and other proteins, such as the CREB binding protein (CPB)/300 **[23]**. The coiled-coil domain not only participates in STAT1 protein interaction, such as that involving IFN regulatory protein 9 (IRF9/p48) **[24]**, but it also regulates the nucleus-cytoplasmic shuttling of STAT1 **[25]**. The DBD plays a role in the IFN-induced nuclear translocation of phosphorylated parallel STAT1 dimers **[26]**. While research has not yet established the function of the α-helical domain, studies have recently reported that this domain participates in STAT1 DNA binding and transcriptional activation upon IFN-γ stimulation. In addition, the α-helical domain assists in the nucleocytoplasmic shuttling of phosphorylated STAT1 **[27]**. The SH2 domain, the most conserved domain in STAT1, plays an essential role in STAT1 binding to the tyrosine phosphorylated receptor and dimerization **[28]**. The TA domain contains two important phosphorylation sites for STAT1: tyrosine 701 phosphorylation and serine 727 phosphorylation. While tyrosine 701 phosphorylation facilitates STAT1 dimerization, DNA binding and transcription activity, serine 727 phosphorylation maximizes the transcription of STAT1 **[29]**. In addition, various proteins, such as CBP/p300, breast cancer 1 (BRCA1), and minichromosome maintenance complex component 5 (MCM5), can interact with STAT1 by binding to this doamin **[30-32]**.

1.3.2 STAT1 signaling regulation

The activation and inactivation of the STAT1 signaling pathway are regulated by several mechanisms. These functions mainly depend on protein modification, such as phosphorylation and methylation, as well as the interaction between STAT1 and other regulatory proteins, including its downstream genes and STAT3.

1.3.2.1 STAT1 activation

STAT1 has been reported as a mediator of several cell functions in response to stimulation by cytokines, growth factors, and hormones, such as IFNs, IL6, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and growth hormone **[10]**. In particular, the essential role of STAT1 in IFN signaling has been demonstrated in multiple review papers **[10-18]**. In short, STAT1 usually undergoes localization as an inactive form in cell cytoplasm. Once IFNs activate STAT1, the phosphorylated JAKs provide docking sites for the recruiting molecules that recognize the phosphotyrosine-binding domain. The STAT1 SH2 domains recognize the tyrosine receptor and subsequently recruit STAT1 to the receptor complex, where it undergoes phosphorylation on a tyrosine residue. The activated STAT1 forms homodimers or heterodimers, which translocate into the nucleus and bind to the promoters of target genes to induce specific gene expression. Various

cytokines stimulate the formation of different dimers. For example, IFN-γ induces STAT1 homodimers, which bind to the IFN-γ responsive element, GAS. However, IFN-α/β mainly stimulates the heterodimerization of STAT1 and STAT2; subsequently, the heterodimers bind to the IFN-α/β responsive element, ISRE **[10] (Figure 1.6)**.

There are two main forms of active STAT1: tyrosine 701 phosphorylation STAT1 and serine 727 phosphorylation STAT1. Although each form regulates distinct downstream signaling pathways and undergoes stimulation from different cytokines and pathological conditions, both forms possess similar functions, including the promotion of cell apoptosis and the inhibition of cell proliferation **[10]**. As previously discussed, tyrosine 701 phosphorylation, stimulated by IFN-γ, performs several STAT1 functions, such as dimerization, nuclear translocation, and DNA binding **[10, 18]**. Serine 727 phosphorylation STAT1 is mediated by several kinases, such as calmodulin kinase II (CaMKII), double-stranded RNA-activated protein kinase (PKR), protein kinase C-δ (PKC-δ), and mitogen-activated protein kinases (MAPKs), in response to various stresses, such as IFN-γ, reactive oxygen species (ROS), lipopolysaccharides (LPS), and ultraviolet (UV) exposure **[33-41] (Table 1.2)**. The potential functions of serine phosphorylation of STAT1 involve enhancing the antiviral and anti-proliferation functions of IFN-γ as well as maximizing the transcription activity of STAT1 **[29, 42]**.

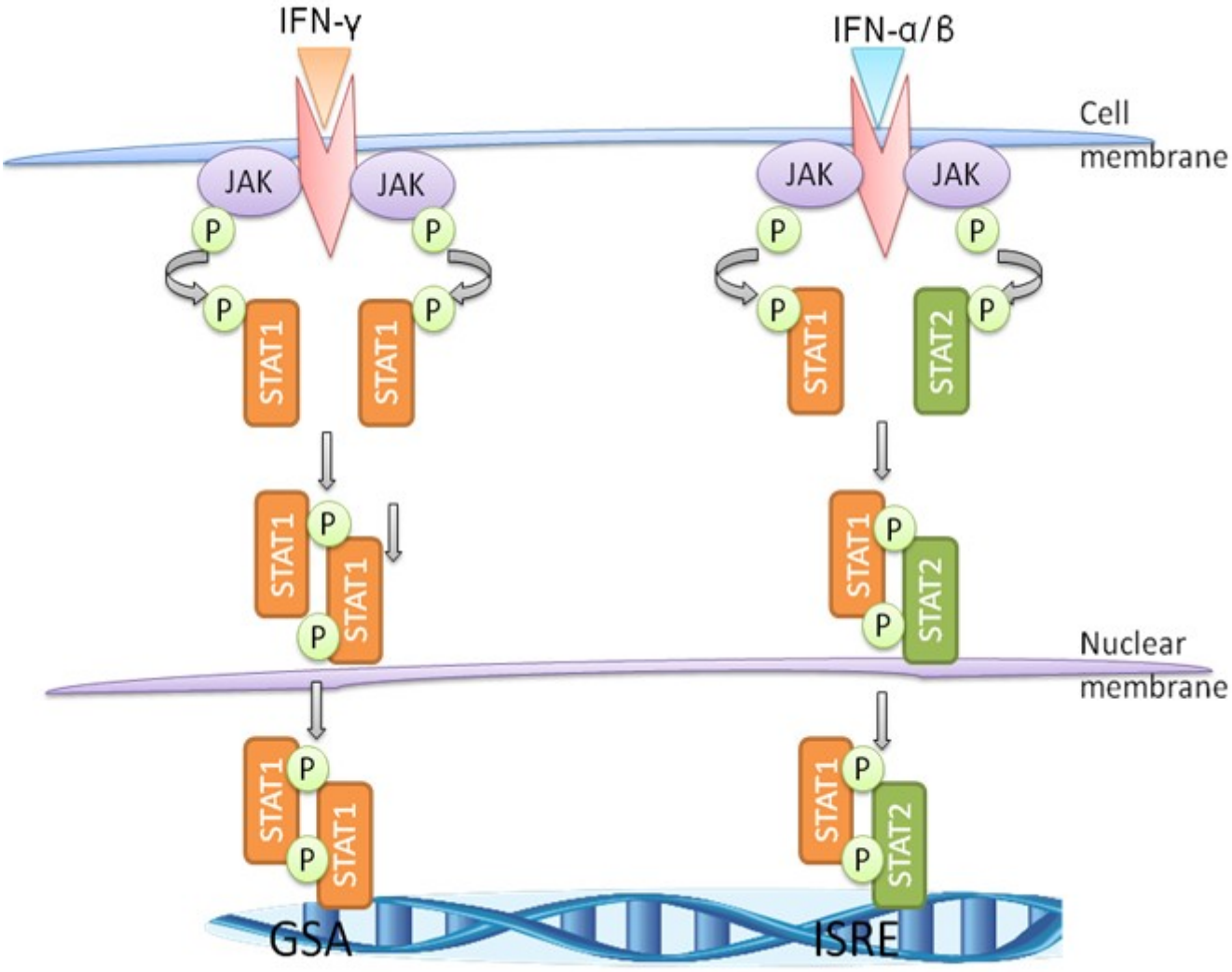

Figure 1.6 IFN-STAT1 pathways. Once IFN activates STAT1, the phosphorylated JAKs provide docking sites to STAT1 to recognize the tyrosine phosphorylation binding domain. Then STAT1 phosphorylated on a conserved tyrosine residue. The activated STAT1 forms homodimers or heterodimers, which translocate into the nucleus and bind to the promoters of target genes to induce gene expression. STAT1 homodimers bind to the GAS and STAT1-STAT2 heterodimers bind to the ISRE.

Table 1.2 STAT1 phosphorylation

Activation	Site	Stimulus	Kinase and modify proteins	Ref
Tyrosine	701	IFN-γ	JAK1 and JAK2	10
phosphorylation	701	IFN-α/β	JAK1 and TYK2	10
Serine phosphorylation	727	TNF	p38	36
	727	ROS	p38	37
	727	IFN-γ	p38	38
	727	LPS	p38	36
	727	UV	p38	36
	727	IFN-γ	PKCδ	41
	727	IFN-γ	CAMKII	39
	727	IFN-α	PKCδ	40
	727	IFN-γ	ERK	33
	727	IFN-α/β	ERK2	34
	727	ischemia/reperfusion	Fas/FasL	35

Modified from [STAT1 as a key modulator of cell death. Hun Sik Kim and Myung-Shik Lee. Cell signaling.19. Copyright © 2006 Elsevier Inc.] with permission (license number 3692071473332)

An initial report demonstrated that serine phosphorylation of STAT1 depended entirely on IFN-γ-induced tyrosine phosphorylation. The evidence for this theory arose from research using a STAT1-deficient cell line **[43]**. Meanwhile, other studies found that the phosphorylation of STAT1 on serine residue occurred independently of tyrosine phosphorylation under certain stress conditions, such as UV and LPS treatment **[44-46]**. These distinct findings may link to the observations that the phosphorylation of STAT1 was mediated by different kinases. Furthermore, the full transcriptional activity of STAT1 required serine 727 phosphorylation. This phenomenon may result from the positive influence of serine phosphorylation on STAT1 tyrosine phosphorylation through the activation of receptors and JAKs **[29]**.

The literature has also proposed other STAT1 activation mechanisms, including acetylation, methylation, and IFN-stimulated gene conjugation (ISGylation) as well as small ubiquitin-like modifier conjugation (SUMOylation) **[47-51]**. STAT1 can undergo modification via gene acetylation on its lysine 410 and/or 413 residues, which block the cell anti-apoptosis function by binding with the NF-κB p65 complex. This process was demonstrated in studies investigating human melanoma cell lines **[52]**. Intriguingly, the acetylation of STAT1 depends on the balance between STAT1-associated histone acetyltransferases (HATs) and histone deacetylases (HDACs), such as CBP/p300. This acetylation

process was also essential for the tyrosine 701 dephosphorylation of STAT1, suggesting that the balance between STAT1 acetylation and phosphorylation can modulate its signaling **[53]**.

Studies showed that STAT1 underwent methylation on arginine 31 residues by means of the protein arginine methyl-transferase 1 (PRMT1). As a result, STAT1 increased its DNA binding ability to enhance the IFN-γ response **[54]**. However, recent studies concluded that this form of activation results from PIAS1 methylation rather than from STAT1 **[55]**. Research initially reported that STAT1 ISGylation constituted a positive regulator of JAK-STAT1 signaling, as induced by IFN **[56]**. However, some reports consider this change as a non-functional STAT1 modification in response to vesicular stomatitis and virus infection, such as *lymphocytic choriomeningitis* **[57]**. Studies have reported that STAT1 can be SUMOylated by PIAS family members; however, these findings failed to demonstrate the significance of this process. Some authors found that STAT1 SUMOylation from PIAS1 could negatively regulate the IFN/STAT1 pathway **[58]**, while other studies reported that STAT1 SUMOylation by PIASx-α lacked the ability to affect STAT1 activation **[59].**

1.3.2.2 STAT1 negative regulation

To avoid the inappropriately sustained activation of the STAT1 signaling

pathway, a variety of negative regulators exist at several levels, including the PTP family, the PIAS family and the SOCS family members (**Figure 1.3**).

1) STAT1 dephosphorylation.

Since STAT1 activation mainly depends on tyrosine 701 phosphorylation, dephosphorylation of STAT1 by PTPs plays a crucial role in downregulating STAT1 signaling **[60-64]**. STAT1 dephosphorylation represents a key step in stimulating the transport of STAT1 from the nucleus to the cytoplasm. PTPs comprise a group of enzymes that work with protein tyrosine kinases to remove phosphate from tyrosine phosphorylation residues on proteins. In addition, PTPs fulfill an equally important role as protein kinases in signal transduction **[60]**. PTPs, such as TC45 and SHP2, can function in both the cytoplasm and nucleus **[61, 62]**.

Several studies have reported that one type of T cell PTP, TC45, dephosphorylates STAT1 **[62]**. Initially, this function was demonstrated in the mice embryonic fibroblast (MEF) cells. Researchers found that the IFN-induced dephosphorylation of tyrosine-phosphorylated STAT1 demonstrated defectiveness in TC45-null MEF cells **[63]**. In most cases, TC45 works in the nucleus; however, these cells can function in the cytoplasm at a low level of expression **[62]**.

SHP2, a SH2 domain containing non-transmembrane PTP, constitutes another important phosphatase that targets STAT1 tyrosine site dephosphorylation **[61]**. Studies have shown IFN-y promotes both the tyrosine and serine phosphorylation of STAT1, causing this gene to become enhanced and prolonged in SHP2-deficient MEF cells. On the other hand, enhanced SHP2 expression inhibits the phosphorylation and transcription activity of STAT1 **[64]**. However, PTPs lack the ability to affect the protein expression of STAT1.

2) Inhibition of STAT1 binding to DNA.

PIAS functions as an E3 SUMO protein ligase. This molecule can downregulate STAT1 by inhibiting its binding to DNA. The PIAS family is composed of four members: PIAS1, PIASx, PIAS3 and PIASy. PIAS1 can specifically inhibit STAT1 transcription activity by blocking STAT1 from binding to DNA in the nucleus **[65]**. The inhibitory activity of PIAS1 depends on the PIAS1-STAT1 interaction and tyrosine 701 phosphorylation **[65]**. Research has shown that another PIAS family member, PIASy, represses the activation of STAT-mediated genes without affecting its DNA-binding ability **[66]**. These findings suggest that PIAS family proteins can inhibit STAT1 functioning through distinct mechanisms.

3) Inhibition of STAT1 binding to JAKs.

STAT1 signaling activation comprises a transient process that causes the levels of phosphorylated STAT1 proteins to diminish within hours of activation, mainly due to inhibition from SOCS proteins. The SOCS family is composed of eight members, including SOCS1-7 and CISH, a cytokine-inducible SH2 protein involved in inhibiting the JAK-STAT signaling pathway **[67]**. Similar to PTPs and PIAS, the SOCS proteins reduce the activation of STATs without affecting their protein levels. SOCS1 is a relatively small protein molecule that contains an SH2 domain, which can directly bind to JAKs and act as a competitive inhibitor of STAT1 **[68-70]**. Under normal conditions, SOCS1 remains in the cell cytoplasm at a low level with potential induction from various cytokines and growth factors. Upon cytokine stimulation, SOCS1 is dramatically induced by JAK/STAT1 signaling and forms a negative-feedback loop, which subsequently inhibits STAT1 activation **[71, 72]**. Some research shows that SOCS1 functions as a mediator to prevent over-activation of the JAK-STAT1 signaling pathway. These *in-vivo* studies demonstrate that STAT1 activation prolongs and intensifies the IFN-γ proinflammatory effect in SOCS1-deficient mice **[73-75]**.

4) Degradation of STAT1

Furthermore, STAT1 can also undergo downregulation by many types of protein through the ubiquitin proteasome pathway (UPP). This theory has

received support from evidence of proteasome inhibitors that prolong the activation and increase the total expression of STAT1 **[76]**. Several ubiquitin-ligases (E3 ligases) can mediate the ubiquitination and degradation of STAT1. For example, both STAT-interacting LIM (SLIM) and SMAD ubiquitination-regulatory factor 1 (Smurf1) apparently promote STAT1 ubiquitination and degradation **[77, 78]**. These processes occurred independently of STAT1 phosphorylation in mouse macrophage cells and human embryonic kidney 293 (HEK293) cells **[77, 78]**. However, other F-box E3 ligases, such as βTRCP, were reported to promote STAT1 proteasome degradation, which was mainly dependent on the serine 727 phosphorylation of STAT1 in MEF cell lines **[79]**. Some viruses resist eradication from the immune system by targeting STAT1 for degradation through UPP **[80]**. Thus, the degradation of STAT1 through UPP can affect both its activation and protein expression.

5) Increased expression of STAT1β

Studies have reported that STAT1α and STAT1β have significantly different biological properties **[81-84]**. The STAT1α isoform has traditionally been considered as the physiologically active form of STAT1, which regulates cell biology activity, such as stimulating the immune system, inhibiting cell growth, and inducing cell death. However, the β isoform is considered as a physiological inhibitor of STAT1 **[81-83]**.

Although research demonstrated that STAT1β bound to the promoter regions of regulatory genes, such as low molecular mass polypeptide 2 (LMP2), interferon regulatory factor 1 (IRF1), and antigen peptide transporter 1(TAP1) to induce cell apoptosis, scholars still consider this isoform as the transcription-inactive form of STAT1 **[81]**. Previous studies have shown that overexpressed STAT1β inhibited STAT1 activation, including tyrosine 701 phosphorylation, DNA-binding activity, and transcriptional activity in human B cells, resulting in the cells receiving protection from fludarabine-induced apoptosis **[81]**. Consistent with these observations, subsequent studies revealed that *Mycobacterium avium intracellularae* and *Leishmania mexicana* can overcome an IFN-γ-triggered host cell defense by inducing STAT1β expression to inhibit STAT1α function **[20, 21]**. However, a recent paper has challenged the role of STAT1β as a domain-negative STAT1 **[84]**. This study showed that STAT1β performed transcriptional activity in response to IFN-γ. STAT1β transfection showed prolonged IFN-γ-induced phosphorylation and promoter binding in the absence of STAT1α. The mechanism involves SOCS1 reduction, which is caused by STAT1β transfection **[84]**. However, studies have neglected to demonstrate the function of STAT1β and its effect on STAT1.

1.3.3 STAT1 function in normal cells

STAT1 performs various important biological functions in normal cells, such as cell death promotion, cell growth inhibition, immune system stimulation, and cell differentiation regulation (**Figure 1.7**).

1.3.3.1 Apoptosis and cell growth arrest

Many studies have identified STAT1 as a key regulator of cell death **[10]** First, STAT1 mediates the pro-apoptosis and anti-proliferation of IFN signaling, which is supported by the inability of IFN-γ to inhibit the growth of STAT1-deficient cells **[85-87]**. Furthermore, the constitutively active form of STAT1 can induce cell apoptosis **[88]**. There are two distinct mechanisms for STAT1-induced cell death: transcription-dependent and transcription-independent.

1.3.3.1.1 Transcription-dependent mechanisms

In transcription-dependent mechanisms, STAT1 induces the transcription of various pro-apoptosis and pro-survival genes, such as caspases, inducible nitric oxide synthase (iNOS), B-cell lymphoma-2 (BCL-2) family members, as well as death receptor and ligands **[10, 89]**. STAT1 also regulates cell cycle genes, including inducing the cyclin-dependent kinase (CDK) inhibitors, such as $p21^{WAF1/CIP1}$ and $p27^{KIP1}$, and repressing cell cycle regulators, such as cyclin A, cyclin D1, and c-Myc **[10, 90]**.

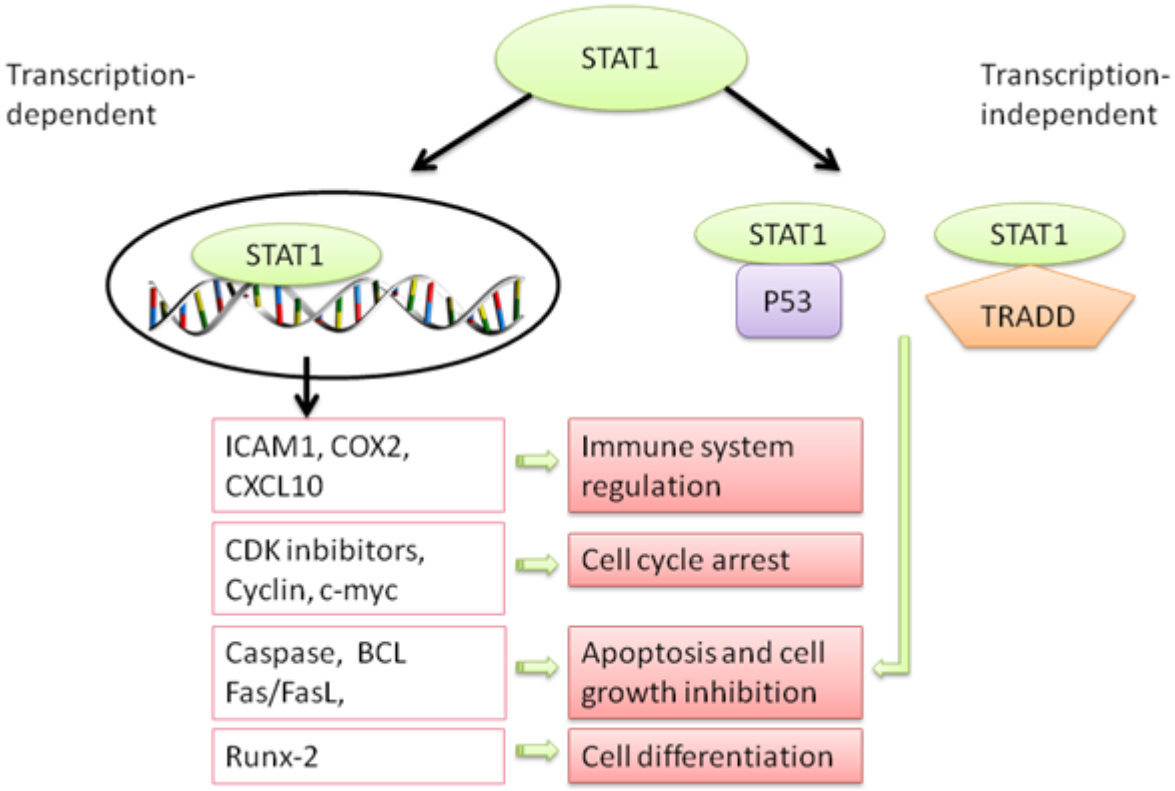

Figure 1.7 Schematic of STAT1 function in normal cells. STAT1 regulate various cellular biological processes in normal cells, such as cell apoptosis, cell cycle arrest, cell differentiation as well as the immune system regulation. STAT1 can promote cell death through both transcription-dependent and transcription-independent mechanisms. STAT1 regulated lots of pro-apoptosis genes, such as BCL family members, caspases, death receptors and ligands, NO and cell-cycle regulated genes, such as p27 and p21. STAT1 also promote apoptosis via working with cell death-modulating proteins, such as, p53 and TRADD.

1) Caspases

Researchers have found that the constitutive activation of caspase 1, 2, and 3 was STAT1-dependent in human fibrosarcoma cells **[87]**. Interestingly, this pro-apoptosis function of STAT1 did not require phosphorylation or dimerization. Moreover, studies show that with external stimuli, such as IFNs, IL1, IL21, tumor necrosis factor-alpha (TNF-α), and EGF, STAT1 promotes cell death by regulating caspase expression in various cell types, such as fibroblast cells and microglia cells **[87, 91-95]**. For example, STAT1-deficient lymphocytes failed to promote IFN-γ-induced apoptosis due to defective caspase-8 expression **[94]**. One study provided direct evidence that STAT1 promotes cell apoptosis by regulating caspase expression; these authors showed that a constitutively active form of STAT1 induced cell death by upregulating the expression of caspases 2, 3, and 7 in human fibrosarcoma cells **[88]**.

2) iNOS

Furthermore, STAT1 promotes cell death when its activation induces iNOS expression, which subsequently produces nitric oxide (NO). As a highly reactive free radical, NO modulates several cell biological functions, such as cell proliferation and death, migration, and angiogenesis **[96]**. The mechanism underlying NO-induced cell death participates in activating p53, a well-known tumor suppressor **[97, 98]**. Studies reported

that lipopolysaccharide (LPS)-induced STAT1 activation regulates the expression of iNOS in intestinal epithelial cells and macrophages, **[99,100]**. STAT1 activation and subsequent IRF-1 transcription activation induced iNOS in murine macrophages, and pancreatic islets cells **[101, 102]**.

3) Death receptor and ligands

STAT1 activation induces cell death by upregulating Fas/FasL and TRAIL/TRAIL-R, the cell death receptors and ligands **[103-106]**. For instance, STAT1 fulfills a critical role in the induction of Fas receptor and Fas ligand expression by invoking ischemia in cardiomyocytes, with a significant contribution from STAT1 Ser727 phosphorylation **[86, 103]**. Similar findings were also demonstrated in the fibrosarcoma cell line U3A and microglial cells **[86,104, 105]**. IFN-γ upregulates the expression of Fas/FasL in STAT1-transfected cells rather than in STAT1-deficient cells. This finding supports the concept that STAT1 induces cell death by stimulating Fas/FasL **[86]**. In addition, STAT1 can also induce apoptosis by upregulating TRAIL/TRAIL-R. A previous study revealed that IFN-γ induces the expression of TRAIL and subsequently mediates human hematoma cell apoptosis by activating STAT1 **[106]**.

4) BCL2 family

The apoptosis regulator BCL2 comprises a family of evolutionarily related proteins, including the pro-apoptotic subgroup, Bax and BAD; and the pro-survival subgroup, BCL-xL and BCL2 **[107]**. Activated STAT1 negatively regulates the promoters of pro-survival genes, such as BCL2 and BCL-xL **[89,108]**. Upon TNF-α and/or IFN-γ treatment, the upregulation of STAT1 and Bim expression were observed in human pancreatic β-cells **[109]**. Moreover, other findings reported that a decrease in the STAT1 gene expression and/or activation upregulated BCL2, thus diminishing cell apoptosis **[110]**. These results suggest that STAT1-induced apoptosis may depend upon BCL2 family members.

5) Cell cycle regulated genes

The cell growth inhibition effect mediated by STAT1 mainly participates in regulating cell-cycle regulatory genes, including $p21^{WAF1/CIP1}$, $p27^{KIP1}$, cyclin D1, cyclin A, and c-myc **[111-114]**. IFN-γ dramatically reduces cyclin D1 and CDK4 protein expression as well as the interaction between STAT1 and cyclin D1, suggesting that STAT1 can regulate the cell-cycle G1 phase **[112]**. Upon the presence of IFN-γ in U-937 cells, the increased expression level of the cell-cycle inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ provides additional support for the fact that cell proliferation inhibition may depend on STAT1 transcriptional activity **[113]**. In addition, STAT1 regulated c-myc, a repressor of $p21^{WAF1/CIP1}$, in wild-type MEF cells. The

studies have shown that IFN-γ can suppress c-myc expression, which is STAT1-dependent **[114]**.

1.3.3.1.2 Transcription-independent mechanisms

The transcription-independent mechanisms by which STAT1 induces cell death involve several apoptosis-modulating proteins, including the tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD) and p53.

1) TRADD

TRADD is a death domain protein that interacts with the TNF-α receptor 1 (TNFR1) and mediates TNF-α-induced responses, such as programmed cell death and NF-kB activation **[115]**. Studies have reported that STAT1 may interact with TRADD to inhibit the activation of NF-kB **[116]**. Researchers used HeLa cells as a model to detect STAT1 involvement in TNFR1-TRADD signaling. These authors found that STAT1 directly interacted with TRADD and a Fas-associated death domain protein (FADD) in a TNF-α dependent process. Furthermore, findings showed that TNF-α enhanced NF-kB activation by promoting IkB degradation in fibroblast cells, while the overexpression of STAT1 blocked TNF-α-induced NF-kB activation by interacting with TRADD **[116]**. These results support the theory that STAT1 interacts with TRADD to suppress

NF-kB activation, thus promoting cell death.

2) P53

P53, a well-known transcription factor and cell cycle regulator, interacts with STAT1 to enhance its pro-apoptosis function in cells **[117]**. For instance, Mouse double minute 2 (MDM2), a negative regulator of p53, was upregulated in STAT1-deficient cells **[118]**. Furthermore, STAT1 increased the transcription activity of p53 to regulate downstream genes, such as Noxa, Fas, and Bax **[118]**. The importance of p53-STAT1 interaction was also supported by *in-vivo* data demonstrating that STAT1-p53-deficient mice developed a more rapid and broader spectrum of tumors in comparison to p53-deficient mice **[119]**.

1.3.3.2 Immune system

Previous studies have shown that IFN-γ plays a critical role in mediating immunity against intracellular bacteria, viruses, and parasites **[120-126]**. However, the immune protective function of IFN-γ largely depends on STAT1 activation. This notion has received support from the high susceptibility of STAT1-deficient mice to pathogens, such as *Listeria monocytogenes*, *Candida albicans,* and *Plasmodium berghei* **[125-130]**. Similarly, STAT1-mutated human cells were predisposed to bacterial and viral infections, including *mycobacterium* **[131-133]**. Thus, *in-vitro* and

in-vivo research has demonstrated that the loss of STAT1 expression and/or activity is associated with the susceptibility to infectious diseases. In addition, studies have shown the involvement of STAT1 in immune system regulation through its interaction with LMP2, IRF1, and antigen processing (TAP) 1 **[134-136]**. Research has also reported the role of STAT1 in the maintenance of immunological self-tolerance, suggesting that STAT1 functions as a mediator in broad levels of immune system homeostasis **[137]**.

STAT1 also acts as a key inflammation factor both indirectly and directly. Indirectly, IFN-γ induces a number of proinflammation genes through STAT1 activation, such as intercellular adhesion molecule 1 (ICAM1), C-X-C motif chemokine 10 (CXCL10), and cyclooxygenase-2 (COX2), which favors the recruitment of immune cells and activation macrophages **[138,139]**. For instance, ICAM1 helps to increase the adhesion of leukocytes to endothelial cells and cellular extravagantes; in addition, CXCL10 demonstrates potent chemotactic activity for T cells **[140,141]**. STAT1 also mediates oxidant stress, which provides a key link between inflammation and cellular transformation **[142]**. In a direct way, STAT1 drives the development and differentiation of T helper 1 (Th1) lymphocytes **[143]**. These cells constitute host immunity effectors against intracellular bacteria and protozoa **[144]**. Taken together, these findings clearly show that STAT1 is a key mediator of the immune system.

1.3.3.3 Cell differentiation

A few studies demonstrate the function of STAT1 in cell differentiation. Specifically, STAT1 induces the expression of runt-related transcription factor 2 (Runx2), a transcriptional factor associated with the differentiation of osteoblast cells **[145,146]**. In human cells, STAT1 functions as an important mediator in bryostatin-1-induced chronic lymphocytic leukemia cell differentiation **[147]**. Moreover, the serine 727 phosphorylation of STAT1 comprises an essential process for the differentiation of acute promyelocytic leukemia cells, which is induced by retinoic acid **[148]**.

1.3.4 STAT1 function in cancer

1.3.4.1 Tumor suppressor effects of STAT1

Results generated from most STAT1 studies support the concept of STAT1 as a tumor suppressor and thus its potential role in fighting cancer **[149-166]**. First, the loss of activation and/or expression of STAT1 can occur in malignant cells derived from various histological types of tumors, such as head and neck cancer, breast cancer, melanoma, gastric cancer, and lymphoma **[149-155].** Second, the literature shows that cancer patients with high STAT1 expression always have a better clinical outcome than those with low or negative expression of STAT1. This finding occurs in many cancers, such as gastrointestinal cancers,

melanoma, breast cancer, and hepatocellular carcinoma **[156-160]**. In particular, Wilms' tumors patients with serine 727 phosphorylation had a positive prognosis **[161]**. These important observations correlated strongly with the current theory that STAT1 represents an important tumor suppressor.

More direct evidence supporting the tumor suppressor functions of STAT1 originates from studies using STAT1C, a constitutively active STAT1 mutant plasmid **[162,163]**. STAT1C converts cysteine into Arg-656 and Asn-658 residues with the C-terminal loop of the STAT1 SH2 domain, mimicking the homodimerization of two STAT1 molecules that exist in the normal STAT1 activation process **[163].** In these studies, STAT1C increased the transcription and expression of many genes, such as myeloid cell leukemia sequence 1 (MCL-1) and Noxa, which are key mediators that inhibit cellular proliferation in multiple myeloma **[163]**. Moreover, enhanced STAT1 has been consistently reported to inhibit cell growth in different neoplastic types by regulating the transcription of several pro-apoptotic and cell-cycle genes, such as caspases, BCL-2, BCL-xL, p27, and $p21^{WAF1}$ **[162]**. However, the most convincing evidence for the role of STAT1 as a tumor suppressor occurs in STAT1 knockout mice, which demonstrated increased susceptibility to experimentally-induced tumors and spontaneously developed mammary adenocarcinomas and ovarian teratomas **[164-166]**.

1.3.4.1.1 Mechanisms underlying loss of STAT1 in cancer

Certain types of tumors demonstrate insensitivity to IFN treatment, thus having the ability to promote cell proliferation, escape immune detection, and metastasize. While the genetic mechanisms behind these phenomena lack a clear explanation, the loss activation or expression of STAT1 in tumors may comprise the key factor regulating a lack of sensitivity to IFN treatment. There are at least three mechanisms contributing to this feature: 1) protein degradation; 2) promoter methylation; and 3) aberrant activation of the STAT1 negative regulator.

1) Protein degradation

Proteasome degradation comprises a key mechanism that regulates the cancer-related proteins, such as p53, BRCA1-BARD1 and p27 **[167-169]**. These proteins represent ubiquitination targets in tumor cells. However, the aberrations in proteasome degradation systems can promote cancer development by enhancing the effect of oncoproteins and/or reducing the level of tumor suppressor proteins.

Research has reported that osteopontin (OPN), a secreted and highly phosphorylated sialoprotein that mediates bone cell-matrix interactions, can target STAT1 to promote its ubiquitination and degradation in human breast cancer and murine mammary epithelial tumor cells **[170,171]**.

These findings suggest that the loss of STAT1 in certain types of cancer may partly result from protein degradation.

2) Promoter methylation

Promoter methylation is an important mechanism for gene inactivation. This type of methylation constitutes a common feature of tumor suppressor genes silencing in cancer cells **[172-174]**. For example, the methylation of metallothionein-1G (MT1G) and neuromedin U was found in ESCC; protein gene product 9.5 (PGP9.5) showed the highest frequency of cancer-specific methylation in head and neck cancer **[172, 173]**. Previous studies have revealed that the promoter methylation comprises one of the primary mechanisms of STAT1 downregulation in human head and neck squamous cell carcinoma (HNSCC) **[174]**. Using bisulfite sequencing and methylation-specific polymerase chain reaction in a cohort of HNSCC patients, Xi S et al. found that STAT1 promoter methylation decreased STAT1 expression in tumors. These results suggest that STAT1 silencing via promoter methylation may contribute to HNSCC tumorigenic properties **[174]**.

3) Aberrant activation of the STAT1 negative regulator

As previously discussed, multiple STAT1 negative regulators are essential for STAT1 nucleus-cytoplasm shuttling in order to avoid

continuous activation. In this regard, the aberrant activation of these regulators, such as PIAS1, SOCS1 and PTPs, may contribute to STAT1 inactivation in cancer cells. For example, the expression of PIAS1 increased in prostate cancer and breast cancer. The knockdown of PIAS1 can diminish cell proliferation and tumor growth in cancer cells [**175, 176]**. In addition to PIAS1, the abnormal expression of SOCS1 has also been detected in various human cancers **[177]**. For example, the expression of SOCS1 appeared stronger in breast cancer tissues than in case-matched normal breast tissues **[178]**. Similar findings were reported for melanoma, suggesting that SOCS1 has a tumor-promoting function. In conjunction with this proposed theory, researchers found an inverse correlation between the expression of SOCS1 and p-STAT1 **[179]**.

Many PTPs, such as SHP1 and SHP2, are aberrantly activated in cancer cells **[180]**. The tyrosine phosphatase non-receptor type 6 (PTPN6) was found to be overexpressed in human epithelial ovarian cancer and SHP-1 protein is over-expressed in some non-lymphocytic cell lines, such as prostate cancer, ovariancancer and breast cancer cell lines **[181, 182]**. SHP2 underwent significant upregulation in head and neck cancer. The process of silencing SHP2 by siRNA has proven to directly upregulate p-STAT1, whereas the overexpression of SHP2 resulted in the loss of p-STAT1 in head and neck cancer cells **[183]**.

1.3.4.1.2 Mechanisms that mediate the tumor suppressor function of STAT1

In canonical mechanisms, the tumor suppressor function of STAT1 is mainly depends on its phosphorylation in tyrosine 701 residue **[184]**. The gene expression induced by activated STAT1 can regulate various aspects of tumor suppression, such as cell growth arrest, apoptosis and angiogenesis inhibition (**Table 1.3**).

1) Apoptosis and cell cycle arrest

As discussed previously, STAT1 regulates cell death via transcription-dependent and transcription-independent mechanisms. These processes involve including inducing the transcription of genes, such as caspases and BCL2 as well as interacting with other proteins, such as p53 and TRADD. Similar mechanisms by which STAT1 promotes cell death are also detected in various cancer cell types.

STAT1 can transcriptionally increase the expression of various pro-apoptosis genes. For instance, IFN-γ induces caspases 1 and 8 in a STAT1-dependent manner for T-cell leukemia, epithelial cell carcinoma, neuroblastoa and Ewing tumor cells **[185,186]**. In addition, STAT1 can also regulate some pro-survival and pro-apoptosis genes, including NO, BCL-2 and BCL-xL in hepatoma, T lyphocyte, pancreatic carcinoma

Table 1.3 STAT1-regulated molecules in cancers

Function	Target molecules	Cancer types	Reference
Apoptosis	Caspases	T-cell leukemia, Epithelial cell carcinoma, Ewing tumor, Neuroblastoa	185,186
	NO, BCL2	Hepatoma, T lyphocyte, Pancreatic carcinoma, Melanoma	187-190
	Fas, TRAIL,	Multiple myeloma , Fibrosarcoma, Colorectal cancer, Melanoma	86,191,192
Cell cycle arrest	$p21^{WAF1/CIP1}$, CDKs,$p27^{KIP1}$	Ovarian cancer , Lymphoma,	113,189,197
Angiogenesis inhibition	IP10/CXCL10 MMP2, MMP9	Many types of cancer Fibrosarcoma	199
Immunosurveillance	LMP2, LMP7, TAP1, TAP2	Renal cell carcinoma, Head and neck cancer	202,203
	MHCI,MHCII	Melanoma	201, 204
	CIITA	Multiple myeloma , Melanoma, Lymphoma , Neuroblastoma	205-207
Metastasis	uPA	Breast cancer	209,210

and melanoma cell lines **[187-190]**. STAT1 also stimulates the expression of surface receptors and their ligands, such as Fas/FasL, to promote cell death in hematopoietic and colon carcinoma **[86]**. Moreover, TRAIL and its receptor activation, induced by STAT1, can occur in myeloma and melanoma cells, fibrosarcoma, myeloma, and colorectal cancer **[191,192].**

In addition to transcription-dependent mechanisms, STAT1 promotes apoptosis in cancer cells through transcription-independent mechanisms. Current studies suggest that p53 and STAT1 can cooperatively to induce apoptosis in cancer cells **[193-195]**. In particular, STAT1 favors p53 activation to promote cell death induced by many chemotherapeutic drugs, such as fludarabine, doxorubicin, and cisplatin **[196]**.

In cancer cells, STAT1 regulates the transcription and expression of multiple cell cycle regulatory genes, including $p21^{WAF1/CIP1}$, $p27^{Kip1}$ and cyclin D1 **[113, 189, 197]**. For example, activated STAT1 was reported to negatively regulate cell cycles by inducing $p21^{WAF1/CIP1}$ and $p27^{Kip1}$, a CDK inhibitor, thus triggering G0/G1 arrest in leukemic monocyte lymphoma cells and ovarian cancer cells **[113, 197]**.

2) Immunosurveillance, angiogenesis and metastasis

One of the fundamental ways in which tumor cells metastasize involves

their ability to escape surveillance from the body's immune system. Furthermore, progressive tumor cell growth and metastasis depend upon angiogenesis. In many types of cancer, STAT1 is considered as a negative regulator of tumor angiogenesis **[151, 198-200]**. STAT1 suppressed the biological function of VEGF in human umbilical vein endothelial cells by inhibiting various genes, including angiopoietin-2 (ANGTP2), COX2 and VEGFR2, which are required for the VEGF response **[199]**. Researchers also showed that the enforced expression of STAT1 in fibrosarcoma cells can repress their tumorigenicity and metastasis in nude mice by downregulating of pro-angiogenic molecules, such as matrix metalloproteinase (MMP) 2 and 9 as well as fibroblast growth factor (FGF-β) **[151]**. Since these genes are essential for angiogenesis and neovascularization, STAT1's downregulation of such genes supports its role in negatively regulating cancer metastasis.

Previous findings suggest that major histocompatibility complexes (MHC) I and II play important roles in immunoevasion of melanomas **[201]**. IFN-γ/STAT1 signaling activation promotes the processing and presentation of tumor antigens in association with MHC Class I or Class II molecules. On one hand, IFN-γ/STAT1 can directly mediate the expression of MHC class I antigens in cancer cells, such as LMP2 and LMP7, as well as TAP 1 and 2 **[202, 203]**. In particular, literature showed that the induction of MHC Class I molecules correlated with STAT1

phosphorylation in melanoma cells **[204]**. Other studies demonstrated that the expression of the MHC Class II transactivator CIITA, which is silenced or downregulated in cancer cells, experienced mediation by STAT1 in response to IFN-γ for many cancer types, such as multiple myeloma, neuroblastoma, and melanoma **[205-207]**.

Additionally, IFN/STAT1 signaling experienced downregulation in the T-lymphocytes derived from patients with metastatic melanoma **[208]**. This finding suggested that STAT1 represents a key mediator of cancer cell metastasis**.** Further studies revealed that STAT1 fulfilled an anti-metastatic role in breast cancer by inhibiting the expression of urokinase-type plasminogen activator (uPA) gene, a serine protease. **[209]**. This gene has been implicated in cell proliferation and invasion as well as metastasis **[210, 211]**. In conjunction, these findings propose the activation of STAT1 as a novel, dominant mechanism of immunosurveillance, inhibiting angiogenesis and metastasis in cancer cells.

1.3.4.2 The oncogenic potential of STAT1

Despite these promising studies, the role of STAT1 as a tumor suppressor still contains controversy. Aberrant STAT1 activation has been detected in some types of human cancer, such as breast cancer, lung cancer, head and neck cancer, and lymphoma **[212-215]**. The phosphorylation of

STAT1 at serine 727 sites promotes tumor cell growth; this finding further supports the critical role of STAT1 in the pathogenesis of Wilms' tumor and leukemia **[216, 217]**. Moreover, STAT1 might influence the resistance of tumor cells to apoptosis, thus promoting further tumor development. This theory gains support from the observation that overexpressed STAT1 is associated with the resistance of tumor cells to chemotherapeutic agents and radiation therapy **[218]**. On the other hand, the silencing of STAT1 can enhance cell apoptosis induced by chemotherapeutic agents and radiation in breast cancer, chronic lymphocytic leukemia, ovarian cancer, and myeloma **[219-222]**.

The majority of papers have evaluated the clinical significance of STAT1 and/or p-STAT1 as improved prognostic markers. However, some studies reported contradictory observations showing that that patients with high expressions of STAT1 and/or p-STAT1 in cancer tissues experience worse clinical outcomes in comparison to patients with low levels of STAT1 and/or p-STAT1, such as breast cancer, sarcoma and Wilm's tumor **[216, 223-226]**. For example, an analysis of 123 human soft tissue sarcoma specimens revealed that patients with highly unphosphorylated STAT1 expressions experienced a lower survival rate than those with low unphosphorylated STAT1. In contrast, patients with high levels of p-STAT1 had a better outcome than patients with low p-STAT1 expression **[223]**. These findings revealed that phosphorylated

status of STAT1 determines its function as a tumor suppressor or promoter. In addition, the co-expression of mucin 1 (MUC1) and STAT1 were significantly associated with worse overall survival than the single expression of MUC1 in 327 breast cancer patients **[224]**. Other studies found that a high p-STAT1 expression was associated with poor overall survival and an advanced clinical stage in premenopausal breast cancer patients **[225]**. In addition, patients with active STAT1 had a worse clinical outcome for glioblastoma multiforme than patients with inactive STAT1 **[226]**.

The *in-vivo* studies further support the role of STAT1 in promoting carcinogenesis. STAT1-/- mice were partially protected from leukemia due to their low MHC Class I expression levels **[227]**. In combination, all of these findings clearly support that STAT1 acts as a tumor promoter.

1.3.4.2.1 Mechanisms that mediate the tumor promoter function of STAT1

The previous findings demonstrated that STAT1 can also function as a critical mediator of oncogenic signaling and can participate in the development and progression of human tumors. However, the mechanisms behind these processes lack a clear understanding.

Several explanations account for the tumor promoter role of STAT1;

however, none of these theories have mutual exclusivity. First, the aberrant STAT1 activation found in cancer cells may result from the loss of STAT1 negative regulators, such as SOCS1 and PIAS1. For example, SOCS1 underwent silencing in hepatocellular carcinoma, gastric cancer, and multiple myeloma **[228-231]**. This biochemical aberrance may contribute to STAT1 activation in cancer cells. Second, the transcription activation of STAT1 induces various genes involved in controlling cell proliferation and survival. The active form of STAT1 induces the high expression of many anti-apoptotic genes, such as inhibitors of apoptosis 1 (IAP-1), IAP-2, and TNFR-associated factor 1 (TRAF1), which protects Hodgkin's lymphoma cells from apoptosis **[232]**.

In addition to other factors, chronic inflammation comprises an important contributor to tumorigenesis and metastasis. Long periods of inflammation create favorable microenvironments for tumor cell transformation and growth. Thus, the third possible explanation for why STAT1 promotes tumor cell growth involves its ability to upregulate numerous pro-inflammatory cytokines and chemokines, such as TNF-α, COX2, and iNOS, all of which contribute to tumorigenesis **[233, 234]**. These chemokines are considered as important targets to prevent cancer cells for anti-inflammatory agents **[235,236]**. Such chemokines are considered as important targets for preventing cancer cells through their function as anti-inflammatory agents **[236]**. Some *in-vivo* studies have

supported this concept. By using a mouse skin multistage carcinogenesis model, researchers found that 3-methyl-1, 8-dihydroxy-9-anthrone (CHRY) promoted skin tumors by inducing COX2 and iNOS synthesis. These processes are STAT1-dependent **[237]**, thus indicating the role of STAT1 in promoting tumor growth and development.

To summarize the previous findings, STAT1 can act as either a tumor suppressor or promoter in different types of cancer; in fact, contraindications have been reported in some types of cancer, such as breast cancer and leukemia. These discrepancies occurred from differences in the genetics and environments of the patient cohorts rather than from the specific type of cancer. Overall, these findings suggest the hypothesis that the activation of STAT1 may promote a “switch” from a tumor survival to a tumor death phenotype.

1.3.4.3 STAT1 and EC

A paucity of current research investigates STAT1 expression and function in ESCC. In previously published studies, Watanabe et al. found that IFN-γ-induced apoptosis of ESCC may depend on STAT1 activation. These authors suggested that STAT1 activation represents a useful therapeutic approach for treating EC **[238]**. Researchers also reported the dysfunctional nature of the EGF-STAT1 signaling pathway in a considerable amount of ESCC patients, demonstrating that the loss of

this signaling correlated with a worse clinical outcome **[239]**. Another study revealed that STAT1 activation is essential for inhibiting ESCC cell growth **[240]**. These observations correlated strongly with the current concept that STAT1 works as a tumor suppressor in ESCC.

These few studies indicate that the literature has not clearly demonstrated the biological function and clinical significance of STAT1 in ESCC.

1.4 STAT3

Another important transcription factor in the STAT family, STAT3, can undergo activation by IL-6 family cytokines, growth factors and IFNs **[241]**. STAT3 performs both pro-apoptosis and pro-survival functions in normal cells and regulates genes that are involved in cell proliferation, development, and cell death **[241]**. This substance fulfills critical roles in a wide variety of physiological processes and developments. For example, STAT3 mediates inflammation factors in the immune system to control the bodily response to bacteria and fungal infection or injury **[242]**. In the nervous system, STAT3 undergoes activation by a ciliary neurotropic factor, which develops sensory neurons necessary for the normal development and maintenance of the brain **[243]**. Studies using conditional STAT3 knockout mice have also shown that STAT3 is essential for the function and development of T-cells, the thymus, and

mammary glands **[244, 245]**.

1.4.1 STAT3 functions in cancer

The majority of studies reveal that STAT3 functions as an oncogene (**Figure 1.8**), which indicates that it fulfills an opposite role to that of STAT1 **[246]**. Direct evidence supporting STAT3 as an oncogene occurs in its constitutive activity of promoting cell proliferation and malignant transformation in a number of tumor cells **[247-249]**. Research has found that STAT3 regulates various target genes, including the pro-survival genes, such as MCL1, BCL-2, BCL-xL, and survivin, as well as cell cycle arrest genes, such as cyclin D1 and c-Myc **[250, 251].** The aberrant activation of STAT3 occurs in various types of cancer, such as melanoma, head and neck carcinoma, breast cancer, and EC **[252-254].** Furthermore, clinical observations also support the role of STAT3 as a tumor promoter. Patients with phosphorylated STAT3 had a significantly worse survival rate than those without phosphorylated STAT3 in cervical cancer, ESCC, HNSCC, B-cell lymphoma and thymic epithelial cancer **[255-261]**.

Although the oncogenic effects of STAT3 have been shown in several papers, a small number of studies demonstrated that STAT3 functions as a tumor suppressor **[262-266]**. In comparison to mice with STAT3 expression, STAT3-deficient mice formed more chronic carbon

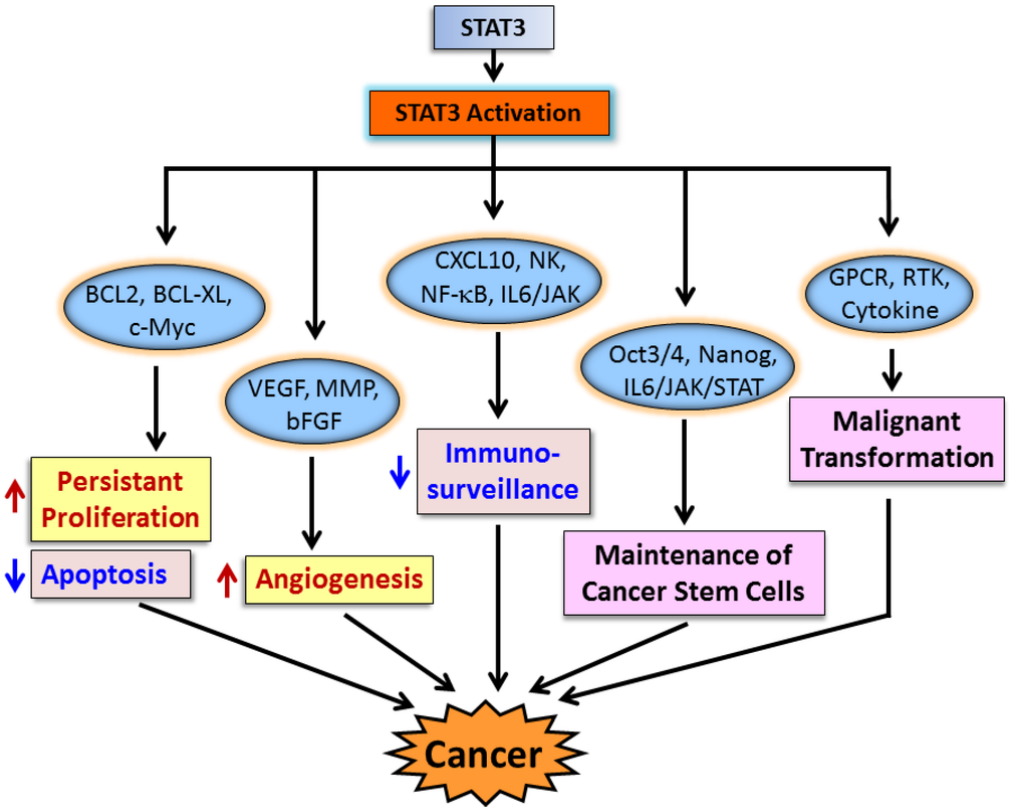

Figure 1.8 Schematic mechanisms of STAT3 contributing to carcinogenesis. Aberrantly active STAT3 signaling promotes carcinogenesis through regulating its downstream genes, such as BCL2, c-Myc. Those genes control various cellular biological processes, including cell proliferation and apoptosis, cell angiogenesis, immunesurveillance, maintenance of cancer stem cells and malignant transformation.

tetrachloride-induced tumors **[265]**. Recently, researchers found that the knockdown STAT3 significantly inhibited thyroid cancer cell growth **[266]**. Moreover, high expression levels of p-STAT3 or nuclear STAT3 was found to be associated with a longer survival rate in head and neck cancers as well as breast cancer **[267-269]**.

Taken together, the literature indicates that STAT3 can act as a tumor suppressor as well as an oncoprotein. According to a recently published review, one explanation of these contradictory findings involves the ratio of STAT3α and STAT3β in distinct cancer types **[270]**. STAT3β, a truncated isoform of STAT3, is considered as a dominant negative mutation of STAT3 and inhibits the transcription activity of full-length STAT3 **[271].** Specifically, STAT3β inhibits tumor growth and promotes cell apoptosis by abolishing the transcription activation of some STAT3 downstream genes, such as BCL-xL, p21 and MCL1 **[272-274]**. Thus, STAT3β functions as a tumor suppressor in melanoma, breast cancer and lung cancer **[275-279]**.

1.4.2 STAT1 and STAT3 cross regulation

The cross-regulation of STAT1 and STAT3 has been demonstrated in a number of papers **[280-282]**, suggesting that STAT1activation is not only experiences regulation by proteins, as discussed above, but also by other STATs, especially STAT3.

The expression and/or activation balance of STAT1 and STAT3 constitute important mechanisms in controlling their responses to cytokines and growth factors. For instance, a study found that STAT3 directly blocked the DNA binding of STAT1 in human myeloid cells **[282]**. Certain cytokines, such as IL6, IL10, and IFN-γ, can activate both STAT1 and STAT3 in normal cells **[283-286]**. In particular, IL10, which is an anti-inflammation cytokine, predominantly activates STAT3 under normal conditions. However, in the cells pre-cultured with IFNs, IL10 mainly mediates the phosphorylation of STAT1 rather than that of STAT3 and fulfills a pro-inflammatory role in the cell **[283, 284]**. Other research using STAT-deficient cells indicates the significance of the balanced expression and/or activation of STAT1 and STAT3. For instance, IL-6 prolongs STAT1 activation and induces the expression of multiple IFN-γ-dependent genes in STAT3-deficient MEF cells **[285]**. In comparison to wild-type MEF cells, STAT1-deficient MEF cells induced a much stronger STAT3 activation in response to IFN-γ **[286]**. In conclusion, the network of cytokine crosstalk can activate both STAT1 and STAT3 to perform opposing functions, subsequently causing these molecules to block each other.

STAT1 and STAT3 fulfill different roles in cancer cell proliferation, apoptosis, inflammation, and tumorigenesis. Specifically, STAT1 induces anti-proliferative and pro-apoptotic genes that directly hamper tumor growth, while STAT3 promotes cell survival and proliferation **[287, 288]**.

Some research has speculated that any interference with the activation of STAT1 or STAT3 may cause these molecules to activate or repress each other **[289-292]**. One study found that STAT1 promotes the cell apoptosis effect of STAT3 inhibition in HNSCC, as the knockdown of STAT1 by siRNA may obstruct the anticancer effect of the STAT3 inhibitor **[290]**. However, other researchers showed that the therapeutic mechanism of a STAT3 inhibitor is independent of STAT1 activation in HNSCC **[291]**. Interestingly, STAT3 significantly enhanced STAT1 gene expression by binding to the STAT1 promoter and synergizing with EGFR in breast cancer **[292]**. These results suggest that the reciprocal regulation between STAT1 and STAT3 might exist in tumor cells; in addition, the cross-regulation of STAT1 and STAT3 can play a role in tumor cell biology

1.4.2.1 Mechanisms of STAT1: STAT3 cross-regulation

The mechanisms by which STAT1 and STAT3 conflict with each other require further investigation **(Figure 1.9)**. One speculation suggests that STAT1 and STAT3 may compete for the same receptor docking sites, target gene promoters, and other cofactors. In fact, STAT1 and STAT3 compete for docking sites, such as IFN gamma receptors (IFNGR), which comprise essential parts for the activation of STATs via tyrosine

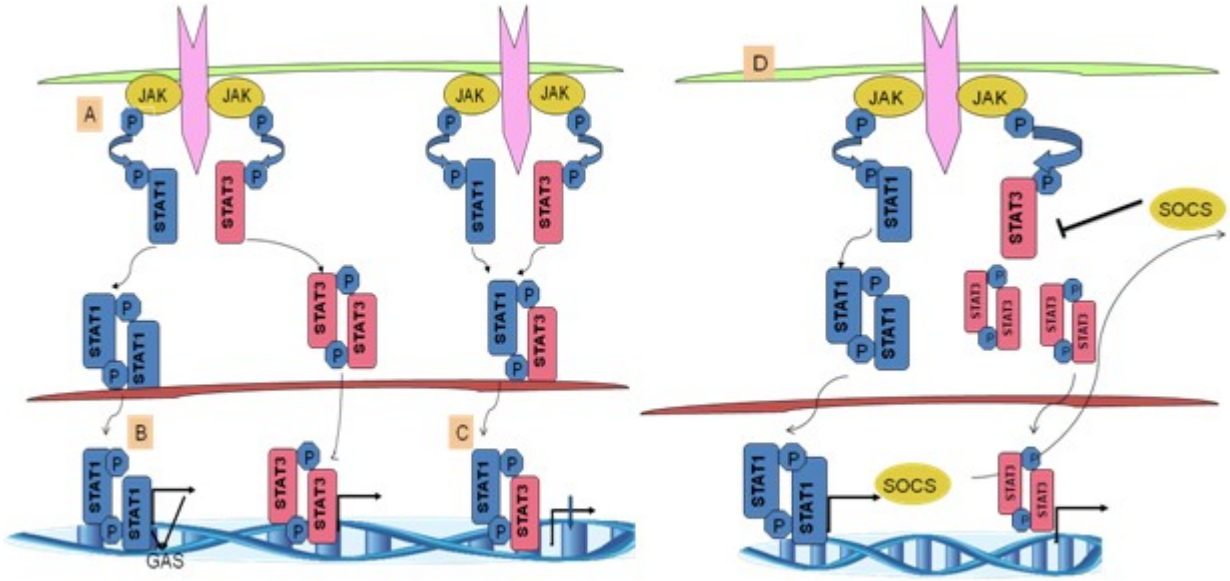

Figure 1.9 Mechanisms by which STAT1 can opposite with STAT3. A. STAT1 and STAT3 compete for the same receptor-docking sites, for example, JAKs. B. STAT1 and STAT3 compete for same DNA binding elements, and/or binding cofactors. C. In response to cytokines, STAT1 and STAT3 form STAT1/STAT3 heterodimers, which may be less active than STAT1 and STAT3 homodimers in gene induction or possess alternative functions. D. Although not experimentally proven, it is conceivable that a given STAT1 can transcription activate negative regulator SOCS, which can in turn inhibit STAT3 activation.

phosphorylation. This theory has received support from research showing that STAT1 suppresses STAT3 tyrosine phosphorylation in response to IFNs and other cytokines **[293,294]**, Furthermore, STAT1 and STAT3 can suppress each other's functions by competing for the same DNA binding sequence. The DNA binding sequences for STAT1 and STAT3 resemble one another so closely that the amino acid sequences for these factors are 72% identical **[294]**. However, there is limited research to demonstrate the mechanisms with which STATs compete for target gene promoters.

One possible explanation suggests the reason that STAT1 inhibits STAT3 without suppressing its tyrosine phosphorylation. This theory proposes that heterodimers of STAT1 and STAT3 may sequester the functions of these factors. Upon cytokine stimulation, STAT1 and STAT3 can form three different dimers: a STAT1:STAT1 homodimer, a STAT3:STAT3 homodimer, and a STAT1:STAT3 heterodimer **[163].** STAT1 upregulation leads to the sequestration of STAT3 into STAT1:STAT3 heterodimers, which are considered as transcription-inactive or less transcription-active. This finding correlates with the suppression of the STAT3:STAT3 homodimer formation and the STAT3 function. Thus, this theory explains the way in which STAT1 inhibits the functionality of STAT3 without suppressing its tyrosine phosphorylation.

Other explanations have proposed that STAT1 can suppress STAT3 by regulating SOCS family members to restrain the functions of other STATs. The activation of STAT1 can induce SOCS expression in response to IFN-γ **[295]**. As a negative regulator of the IFN-dependent pathway, SOCS1 can suppress other signaling pathways, such as IL-4, which is the key cytokine for activating STAT6 **[296-299]**. Thus, STAT1 can potentially cross-inhibit STAT3 activation via the induction of SOCS. However, studies have yet to provide firm evidence that supports this hypothesis.

1.5 The MAPK/ERK signaling

The MAPK-extracellular signal regulated kinase (ERK) pathway, also known as the Ras-Raf-MEK-ERK pathway, comprises a line of cascades that form the essential part of the intracellular signaling network **[300,301].** As shown in **Figure 1.10,** signal transmission via MAPK cascades is usually induced by activation of a small G protein, such as Ras, and subsequently followed by the activation of several sets of cytoplasmic protein kinase. The signaling contains complex networks of interacting proteins, including three core protein cascades: a MAPK kinase-kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK. Generally, the MAPK family consists of several subgroups: ERK, p38, and the N-terminal kinase (JNK) **[302]**. The ERK pathway experiences activation by growth factor-stimulated cell surface receptors, whereas the JNK and

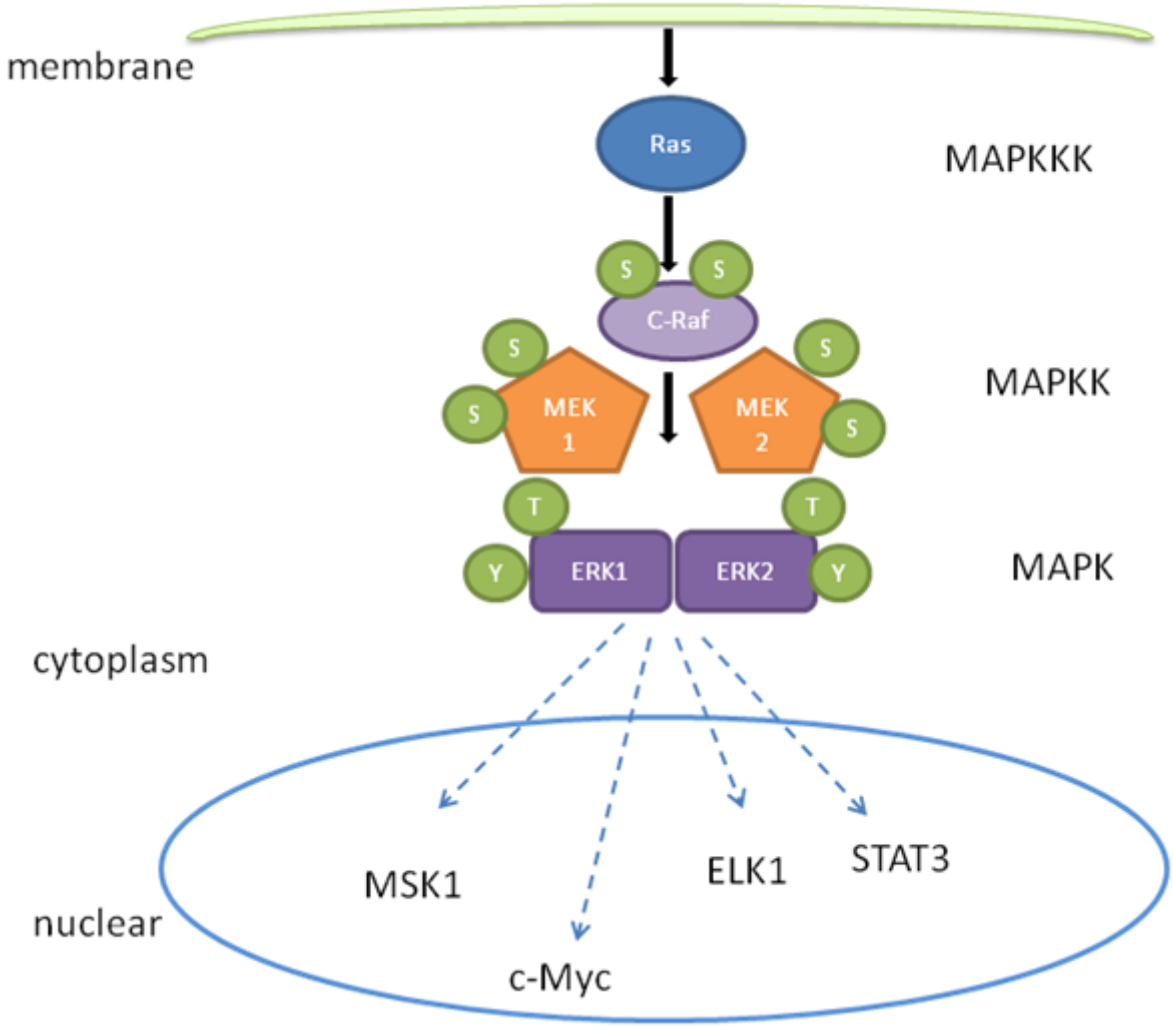

Figure 1.10 Overview of Ras-Raf-MEK-ERK pathway. The Raf/MEK/ERK pathway is regulated by cytokine receptors. The signals transmission via MAPK cascades is often initiated by activation of a small G protein, such as Ras, which sequentially activate several cytoplasmic protein kinases. The signaling is mainly comprised of three protein kinases: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK.

p38 pathways are activated by growth factors and stress **[303]**.

The MAPK-ERK pathway regulates various cellular processes, such as cell growth, cell differentiation, and cell death **[304]**. In the case of cancer cells, this pathway undergoes aberrant activation by the upstream induction of EGFR and the Ras superfamily of small guanosine triphosphatases (GTPases) **[305]**. In addition, the activated MAPK-ERK pathway also promotes cell metastasis **[306].** Many MAPK/ERK inhibitors have been developed and approved by United States Food and Drug Administration (USFDA) to treat breast cancer, colon cancer and non-small cell lung cancers **[307-310]**.

1.5.1 ERK

ERK, which comprises a key component of the MAPK-ERK pathway, contains two isozymes, ERK1 and ERK2, whose molecular weights are 44-and 42-kDa respectively. ERK1 and ERK2 are 83% identical, with the greatest difference occurring outside of the kinase core **[311]**. The full activation of ERK requires the phosphorylation of both threonine $^{202/185}$ and tyrosine $^{204/187}$, which results in the phosphorylation of various substrates upon stimulation. These substrates can be categorized into several groups, including transcription factors, protein kinases, protein phosphatases, receptors, and other types of proteins **[312].** An activated ERK cascade controls cell proliferation, differentiation, survival, and

apoptosis as well as regulating the cell cycle. These cascades also regulate cell meiosis, mitosis, and postmitotic processes in differentiated cells by phosphorylating several molecules, including transcription factors, cytoskeleton elements, apoptosis regulators, translation regulators, and a variety of other signaling-related molecules **[313]**.

The function of ERK in cell proliferation and differentiation has been well documented in various papers **[314-317]**. In general, ERK fulfills the role of an intracellular checkpoint for cellular mitogenesis. The activation of ERK can promote the cell cycle to progress from the G1 phase to the S phase, thus enhancing cell proliferation. Functional studies using ERK-deficient mice suggest that ERK also participates in aspects of embryogenesis, such as the process of thymocyte development. Moreover, recent studies have revealed that ERK1 and ERK2 perform different functions. One investigation found that ERK1-deficient mice upregulate ERK2 expression **[314]**. The failure of ERK2-deficient embryos to form a mesoderm reveals that ERK2, rather than ERK1, fulfills an essential role in embryo development, as ERK2 can serve a compensatory function in this regard **[315]**. By using MEF and NIH 3T3 cells as models, researchers have found that ERK2 has a positive effect on controlling cell proliferation, whereas ERK1 obstructs ERK2 function and subsequently affects the overall signaling output of the cell **[316]**. The separate knockdowns of ERK1 and ERK2 have distinct effects on cell

proliferation and biological phenotypes **[317]**. These findings suggest that the two isozymes function differently in the physiological and developmental processes of cells.

An aberrant ERK cascade activation occurs in a high proportion of human cancers, including human renal cell carcinoma and prostate cancer **[318-320]**. The inappropriate activation of upstream molecules, such as EGFR, Raf, and Ras, constitute major forces controlling the constitutive activation of the ERK pathway in tumor cells **[321-326]**. Research identifies this pathway as a promising chemotherapy target. Specifically, the inhibition of the ERK pathway represents an efficient way of blocking proliferation, metastasis, and angiogenesis in cancer cells **[327]**. Since ERK fulfills an essential role in responding to hepatocyte growth factor (HGF) **[328]**, the obstruction of this pathway downregulates the hypoxia-inducible factor 1a (HIF-1a) **[329-331]**, a master regulator of angiogenesis, as well as MMP-3/-9/-14 and CD44, important regulators of cell invasion **[332]**.

1.6 Protein degradation-overview

Protein levels are determined by not only the rate of synthesis but also by the rate of degradation. There are two major pathways involved in protein degradation: the ubiquitin (Ub)-proteasome pathway (UPP) and lysosomal proteolysis. In eukaryotic cells, the majority of intracellular

proteins, nearly 70-90 %, undergo ubiquitin labeling and experience degradation by UPP **[333]**.

1.6.1 UPP

Ubiquitin is a small protein containing 76 amino acids and having a molecular mass of approximately 9 kDa **[334]**. This protein attaches to target proteins on lysine residue as a label; subsequently long ubiquitins join the protein to form a long polyubiquitin chain for further degradation **[335]**. This ubiquitin modification constitutes a multistep adenosine triphosphate (ATP)-dependent process involved with three classes of critical enzymes **[335]**. First, ubiquitin-activating enzymes, known as E1 enzymes, are responsible for activing ubiquitin. Subsequently, the activated ubiquitin is then transfered to ubiquitin-conjugating enzymes (E2). The final transfer of ubiquitin to the target protein is mediated by E3, also known as ubiquitin ligase. In some cases, E2 and E3 work together to recognize and conjugate ubiquitin to the substrate protein. Finally, the marked protein is recognized by the 26S proteasome, a proteasome complex that includes 20S protein subunits and two 19S regulatory cap subunits (**Figure 1.11**).

1.6.2 UPP function in diseases

The function of the UPP involves removing damaged and redundant

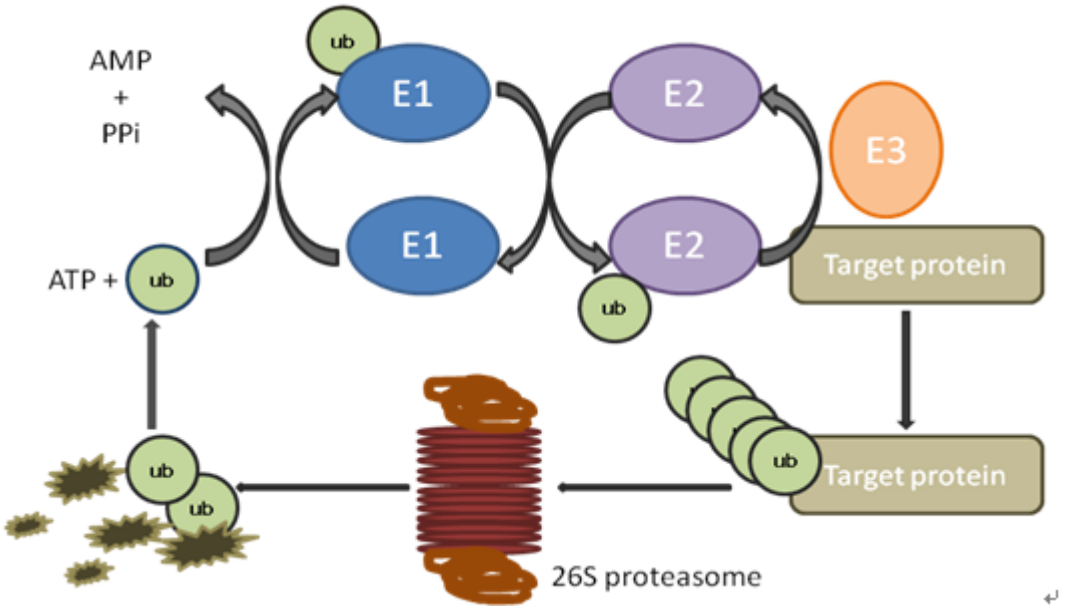

Figure 1.11 Protein degradation by the ubiquitin–proteasome pathway. A chain of 4 or more ubiquitin molecules is attached by the action of a series of ubiquitin ligases (E1, E2, E3) to 1 or more lysine residues on the target protein to be degraded. The ubiquitin–protein complex is transported to the proteasome, where the ubiquitin chain is removed, allowing the target protein to be unfolded by an ATP-dependent process and translocated to the interior of the proteasome, where it is degraded by 3 threonine proteases to yield peptide fragments.

proteins from the cells. This process occurs in the regulation of several cellular processes, including cell proliferation, cell death, cell division, DNA repair, and cell differentiation. Aberrations of this pathway occur in the pathogenesis of various human diseases, especially chronic inflammatory diseases and neurodegenerative disorders, such as Alzheimer disease **[336]**.

The diseases associated with the UPP can be categorized into two subgroups based on distinct mechanisms. The first group of diseases results from mutations in UPP enzymes or target substrates. The mutations may affect protein stabilization and lead to the loss of protein functions. For example, the mutation of E6-AP, an E3 enzyme, occurs in Angelman syndrome **[337]**. Furthermore, the renal sodium channel in Liddle syndrome, a substrate of the UPP, reportedly mutates in the α/β subunit **[335]**. The second category of diseases results from the degradation of target proteins. For instance, some E3 enzymes, such as MDM2 and s-phase kinase-associated protein 2 (SKP2), are overexpressed in various malignant conditions **[335]**.

1.6.3 UPP function in cancer

Cancer-related regulators, including cell surface receptors, cell cycle regulators, and oncogenic transcription regulators are regulated by UPP**.** In breast cancer, mutations in *BRCA1-BRAD1,* a heterodimeric RING

finger complex, occurred in the early stages of breast tumors **[338]**. P27, a well-known tumor suppressor and cell-cycle inhibitor, experienced a low expression level in various types of cancer, such as prostate cancer, lung cancer, gliomas, and lymphomas **[168]**. The downregulation of p27 in cancer results from abnormally enhanced degradation by UPP **[168]**. An overexpression of SKP2, the E3 enzyme,promotes the responsible for recognition and ubiquitination of p27 in colorectal cancer, breast cancer, and prostate cancer **[339]**.

UPP mediates a wide array of cellular processes and provides a broad platform for the treatment of various diseases and the creation of anti-tumor drugs. Moreover, proteasome inhibitors have emerged as a novel chemotherapeutic agent for treating cancers. In recently published papers, researchers have shown that proteasome inhibitors demonstrate more sensitivity to malignant cells than to normal cells. These *in-vivo* studies have also revealed the anti-tumor function of proteasome inhibitors **[340]**. These findings suggest the possible existence of a therapeutic potential for proteasome inhibitors. The USFDA has already approved the proteasome inhibitor Bortezomib as an anticancer drug for the treatment of resistant multiple myeloma. This drug achieves its anti-tumor function by inhibiting the UPP **[340,341]**. In conclusion, UPP comprises a key mediator that regulates cellular functions ranging from cell cycle regulation to malignant transformation.

1.7 Thesis overview and objective

1.7.1 Rationale

Although ESCC represents one of the most common types of cancer in China, its biological mechanisms remain unclear. The Chaoshan region is a well-known high-risk region for this disease, suggesting the existence of potential genetic and/or environmental factors predisposing this particular population to EC. STAT1, a tumor suppressor, has been aberrantly expressed in numerous types of cancer, such as breast cancer, multiple myeloma, head and neck cancer, and leukemia. The literature has reported that certain cytokines, such as IFN-γ and EGRF, can induce cell apoptosis through STAT1 activation in ESCC. Nevertheless, the biological function and clinical significance of STAT1 in ESCC still require further exploration.

1.7.2 Hypothesis

Based on previous findings, the current study hypothesizes that STAT1 is a tumor suppressor in ESCC via multiple mechanisms, including interaction with its downstream genes and STAT3. Meanwhile, the loss of STAT1 contributes to ESCC carcinogenesis, which is mediated by ERK.

1.7.3 Objectives

To test this hypothesis, this research proposes three specific aims regarding the biological function and clinical significance of STAT1 in ESCC:

The first objective aims to validate the tumor suppressor role of STAT1 in ESCC as well as possible mechanisms underlying these processes. This topic is covered in **Chapters 2 and 3**, where ESCC cell lines are used for detecting the STAT1 function with a constitutively active form of STAT1: STAT1C. The biological function of STAT1 will undergo detection with multiple assays. Furthermore, Western blot assays and immunohistochemistry will be used to detect STAT1 expression in patient samples collected from the Chaoshan region. Finally, this part of the project will assess the mechanisms that contribute to tumor suppressor function of STAT1. In **Chapter 3**, *in vitro* findings will be confirmed using a cohort of ESCC patient samples. The correlations of STAT1 with the cell cycle and apoptosis regulatory genes are evaluated.

The second objective, as described in **Chapter 4**, involves validating the mechanisms by which STAT1 demonstrates lower levels in ESCC as compared to case-matched normal esophageal tissues. Based on the previously published paper, I hypothesize that activated ERK, which is a classical MAP kinase, promotes STAT1 protein ubiquitination and degradation in ESCC.

In **Chapter 5,** the biological function of STAT1β, a short STAT1 isoform, is reported in cancer cell lines for the first time. By using ESCC as a study model, the interaction of STAT1α and STAT1β is examined. Moreover, the clinical parameters and prognostic factors of STAT1β in ESCC are determined by using immunohistochemistry in a cohort of patient samples.

In conclusion, this thesis evaluates the biological function and clinical significance of STAT1 by using ESCC cell lines and patient samples. Further knowledge about the mechanisms influencing STAT1 activation enhances the understanding of the role that STAT1 fulfills in ESCC. The proposed studies are highly relevant in furthering the understanding of STAT1 in the pathogenesis of ESCC, which may lead to the development of new therapeutic strategies.

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CHAPTER 2

Clinical and Biological Significance of STAT1 in Esophageal Squamous Cell Carcinoma

This chapter has been modified from a previous publication:

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As first author, I designed and performed all the experiments described here, except for the following: Molavi O performed some IHC staining shown in **Figure 2.1**; I also wrote the manuscript.

2.1 Abstract

Loss of STAT1 (Signal Transducer and Activator of Transcription-1) has been implicated in the pathobiology of a number of cancer types. Nonetheless, the biological and clinical significance of STAT1 in esophageal squamous cell carcinomas (ESCC) has not been comprehensively studied. Using immunohistochemistry, we found that the expression of STAT1 was heterogeneous in ESCC, with 64 (49.0%) strongly positive cases, 59 (45.0%) weakly positive cases and 8 (6.1%) negative cases. STAT1 expression inversely correlated with the depth of tumor invasion and tumor size (p=0.047 and p=0.029, respectively, Chi square). Furthermore, patients with STAT1-strong/weak tumors had a significantly longer survival compared to those with STAT1-negative tumors (33.6 months versus 13.1 months, p=0.019). In patients carrying tumors of aggressive cytology (n=50), those with STAT1-strong tumors survived significantly longer than those with STAT1-weak/negative tumors (34.6 months versus 20.5 months, p=0.011). *In-vitro* experiments using enforced gene transfection of *STAT1C* into two STAT1-weak/negative ESCC cell lines and siRNA knockdown of STAT1 in two STAT1-strong ESCC cell lines revealed that STAT1 is pro-apoptotic and inhibitory to cell-cycle progression and colony formation. Lastly, we found evidence that STAT1 signaling in ESCC cells down-regulated the expression and/or

activity of NF-κB and STAT3, both of which are known to have oncogenic potential. To conclude, our findings suggest that STAT1 is a tumor suppressor in ESCC. Loss of STAT1, which is frequent in ESCC, contributes to the pathogenesis of these tumors.

2.2 Introduction

Members of the STAT protein family are known to regulate various cellular processes involved in oncogenesis, including cell cycle progression, apoptosis, angiogenesis, invasion, metastasis, and evasion of the immune system **[1]**. STAT1, as the first discovered member of the STAT family, serves as the principal mediator of both type I and type II interferon activation **[2]**. Recent studies have revealed that the expression of STAT1 is frequently lost in various types of human cancer such as breast cancer, head and neck cancer, multiple myeloma and leukemia **[3]**. Furthermore, it has been reported that STAT1 can inhibit the growth of benign and neoplastic cells by regulating the transcription and expression of a host of pro-apoptotic and anti-proliferative genes, such as caspases, BCL-xL and $p21^{waf1}$ **[4]**. Overall, these observations suggest that STAT1 carries tumor suppressor functions.

Esophageal cancer is one of the leading causes of cancer-related deaths worldwide **[5]**. This type of cancer is known to be highly frequent in specific geographic regions in China, such as the Chaoshan area.

Specifically, the annual average age-standardized incidence rate of esophageal cancer in Chaoshan is 74.5 /100,000 people **[6]**, as compared to 7.0/100,000 people worldwide. This finding suggests that various genetic and/or environmental factors may predispose the population in Chaoshan to esophageal cancer. Interestingly, esophageal cancer found in Chaoshan predominantly carries the histology of squamous cell carcinoma, in contrast with that in the Western world which predominantly carries the histology of adenocarcinoma **[7]**. The pathogenesis of ESCC is incompletely understood. The overall survival of these patients remains to be relatively poor, with the overall 5-year survival rate being approximately 15% **[8]**. In one previous study, it was found that γ-interferon can induce significant apoptosis in ESCC cell lines and this process correlates with STAT1 activation **[9]**. In parallel with this observation, it was reported that the EGF-STAT1 signaling pathway, which is active in normal esophageal epithelial cells, is lost in a considerable fraction of esophageal cancer; furthermore, loss of EGF-STAT1 signaling was found to correlate with a worse clinical outcome **[10]**. Nevertheless, the clinical and biological significance of STAT1 in ESCC has never been directly or comprehensively examined.

In the present study, we tested our hypothesis that STAT1 is a tumor suppressor in ESCC. First, using immunohistochemistry and Western blots, we comprehensively evaluated the expression of STAT1 in a large

cohort of ESCC harvested from patients from Chaoshan. Second, we evaluated the clinical and prognostic significance of STAT1 in ESCC. Third, we used an *in-vitro* model to assess the biological functions of STAT1 in ESCC cells.

2.3 Materials and methods

2.3.1 ESCC tumor samples and cell lines

We randomly collected 131 consecutive ESCC tumors at the Shantou Tumor Hospital between 2005 and 2012. All patients underwent potentially curative surgery without preoperative chemotherapy or radiotherapy. In this cohort, 98 were men and 33 were women; the age was 36-78 years, with a median of 57 years. Follow-up data was available for 74 patients; most (58, 78.4%) died during the follow-up period (median, 31.4 months). The study was approved by the ethical review committees of the Medical College of Shantou University. All participants involved in our study were given written informed consents.

Four ESCC cell lines (EC1, EC109, KYESE150 and KYSE510) and 4 human esophageal immortalized epithelial cell lines (SHEE, NE2, NE3, and NE6) were included in this study. The ESCC cell lines were gifts from Shantou University Medical College and esophageal immortalized epithelial cell lines were gifts from University of Hong Kong. All of them

were cultured in DMEM supplemented with 10% fetal bovine serum at 37˚C under 5% CO_2.

2.3.2 Antibodies and Western blotting

Western blot analysis was performed using standard techniques as previously described **[11]**.Briefly, speaking, preparation of cell lysates for Western blots was done as follows: cells were washed twice with cold phosphate-buffered saline (PBS, pH=7.0), and scraped in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50mM Tris pH 8.0) supplemented with 40.0 µg/mL leupeptin, 1 µM pepstatin, 0.1 mM phenylmethylsulfonyl-fluoride and sodium orthovandate. Cell lysates were incubated on ice for 30 minutes and centrifuged for 15 minutes at 15000g at 4℃. Proteins in the supernatant were then extracted and quantified using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Subsequently, cell lysates were then loaded with 4x loading dye (Tris-HCl pH 7.4, 1%SDS, glycerol, dithiothreitol, and bromophenol blue), electrophoresed on 8% or 10% SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). After the membranes were blocked with 5% milk in Tris buffered saline (TBS) with Tween, they were incubated with primary antibodies. After washings with TBS supplemented with 0.05% Tween-20 for 30 minutes between steps, secondary antibody conjugated with the

horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA, USA) was added to the membrane. The following antibodies were employed: anti-STAT1 (1:1000) and anti-p-STAT1(Tyr-701)(1:1000), anti-FLAG (1:1000), anti-caspase 3 (1:1000), anti-survivin (1:1000), anti-BCL-2 (1:1000), anti-p21 (1:1000) and anti-cyclin D1(1:1000), all of which were purchased from Cell Signaling (Danvers, MA, USA). Anti-STAT3 (1:1000), anti-p-STAT3 (Tyr-705) (1:1000), anti-BCL-xL (1:1000) and anti-β-actin (1:1000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Densitometric analysis was performed using the ImageJ analysis system (Bethesda, WA, USA); the values for the STAT1 bands were normalized to those of the β-actin bands.

2.3.3 Immunohistochemistry

Formalin fixed, paraffin embedded ESCC tumors were used for this study. All cases were retrieved from the file at the Department of Pathology, Shantou University Medical College. The diagnosis of these cases was based on the criteria established by the World Health Organization classification scheme. Immunohistochemistry to detect STAT1 expression was performed using a method similar to that described previously **[12]**. Briefly, formalin-fixed, paraffin embedded tissue sections of 4 μm thickness were deparaffinized in xylene and hydrated in graded ethanol (100% to 50%). Antigen retrieval was performed using citrate buffer (pH

6.0) microwaved in a pressure cooker for 20 minutes and left to cool for 20 minutes. After antigen retrieval, tissue sections were incubated with 10% hydrogen peroxide (H_2O_2) and methanol for 10 minutes to block endogenous peroxidase activity, followed by washing in running tap water for 5 minutes. Subsequently, the sections were incubated for 20 minutes in antibody diluent (Dako, Mississauga, ON, Canada), followed by incubation overnight at 4°C with a rabbit polyclonal antibody reactive with anti-STAT1 (1:75 dilution, Cell signaling). The antibodies were diluted in antibody diluent (Dako) Immunostaining was visualized with a labeled streptavidin-biotin method (20 minutes in biotinylated link and 20 minutes in streptavidin horse radish peroxidase, both from Dako) using (3, 3-diaminobenzidine/ H_2O_2) DAB as a chromogen (Dako). Hematoxylin was used as a counter stain. Following staining, sections were dehydrated in graded ethanol (50%-100%), followed by xylene incubation. Coverslips were applied using permount solution (Fisher Scientific).

Using the same antibody we employed for our Western blot studies, we performed immunohistochemistry and the staining results were independently evaluated by two pathologists who were blinded to the clinical data and concordance between two pathologies was guaranteed. For each case, the percentages of cells showing negative, weak or strong cytoplasmic STAT1 staining was recorded. Using our scoring system (the sum of % of cells strongly positive for STAT1 x 3 and % of cells weakly

positive for STAT1 x 1), we determined that a cut-off of 80 points allowed us to achieve the lowest p-values in our statistical analysis. Thus, tumors with a score of <80 point were classified as STAT1-weak whereas those with a score of ≥80 points were classified as STAT1-strong.

2.3.4 Co-immunoprecipitation

A total of 2 µg of anti-STAT3 monoclonal antibody (Santa Cruz Biotechnology) was added to 500 µg of protein lysate isolated in cell lytic M (Sigma Aldrich, St Louis, MD, USA) and the samples were rotated overnight at 4 °C. Subsequently, 30 µl of protein G Plus/A beads (Emdmillipore, Billerica, MA, USA) was added to the samples and rocked overnight at 4°C. The beads were then washed 3 times with cold phosphate-buffered saline followed by the final wash using cold cell lysis buffer. Western blot analysis was then performed using standard techniques as previously described **[11]**.

2.3.5 Plasmids and cell transfection

FLAG-tagged *STAT1C* cloned into the backbone of pcDNA3.1 was a gift from Dr. Ouchi (University of New York) **[13]**. For each experiment, 1×10^6 ESCC cells were transiently transfected with 10 µg of *STAT1C* vector or the pcDNA3.1 empty vector (Invitrogen, Burlington, Ontario, CA) in 6-well plates using the lipofectamine 2000 reagent (Invitrogen) as per

manufacturer's suggested protocol.

2.3.6 Short interfering RNA and gene transfection

5×10^6 ESCC cells in 2 ml of culture medium were transfected with 100 pmol of SMARTpool-designed siRNA against STAT1 obtained from Dharmacon (Lafoyetle, CO, USA). Cells transfected with scrambled siRNA (Dharmacon) were used as the negative controls. Gene transfection was performed by using lipofectamine RNAiMax (Invitrogen) as per manufacturer's suggested protocol.

2.3.7 Cell-cycle analysis by flow cytometry and assessment of cell growth

Flow cytometry analyses were performed at the University of Alberta flow cytometry core facility as previously described **[12]**. Cells transfected with scrambled siRNA or STAT1 siRNA, as well as empty vector and *STAT1C* were fixed with ice-cold 70% ethanol 24 hours after gene transfection. These cells were then subjected to RNase treatment and propidium iodide staining. DNA content was determined using a FACSCalibur flow cytometer (BD Biosciences). Data acquisition was gated to exclude cell doublets, and the cell-cycle phase distribution was determined using the CellQuest program (20 000 events were counted). To assess cell growth, ESCC cells were plated at a density of 20,000/ml of culture medium. Cell

count, done daily for 4 days, was performed using trypan blue staining (Sigma-Aldrich) according to the manufacturer's protocol.

2.3.8 Colony formation assay

After *STAT1C* transfection, 500 cells/well were plated in six-well plates and incubated 10 days at 37°C. The cells were fixed with 4% buffered formalin for 15 min and then stained with 1% crystal violet (Sigma Aldrich) for 30 min. The plates were gently washed with PBS and dried before microscopic evaluation. Cell clusters with >30 cells were considered as a colony.

2.3.9 Quantitative RT-PCR

Using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), total cellular RNA was extracted from cells following the manufacture's protocol. Reverse transcription was performed using 1μg of total RNA and superscript reverse transcriptase obtained from Invitrogen. Quantitative PCR was performed using SYBR green (Invitrogen), and the primer sets for STAT1 and GAPDH were purchased from Invitrogen. For both primer sets, the PCR conditions were as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Samples were processed on an ABI 9700 HT system (Applied Biosystems Inc., Foster City, CA). Results were examined using the SDS 2.2 software, and

the relative expression levels of STAT1 were calculated by normalizing with those of GAPDH.

2.3.10 Cell invasion assay

The invasion assays were done using basement membrane (Cell biolabs, NY, USA).The ESCC cell treat with *STAT1C* or empty vector were prepared before the experiment. Then, 5×10^5 cells in 300 μL serum-free DMEM supplemented were seeded into the upper part of each chamber, whereas the lower compartments were filled with 500μl of 10% fetal bovine serum media. Following incubation for 48 hours at 37°C, the insert was incubated in the cell detachment solution. The invasiveness was determined by fluorescence measurement, and the extent of invasion was expressed as an average number of cells per microscopic field.

2.3.11 Luciferase activity assay

The NF-κB and STAT3 transcriptional activity analyses were performed as previously described **[12]**. The transcription activity is measured by their luciferase reporter. Luciferase activity was measured with the Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega) as described. After 48 hours of transfection of the reporter plasmid as well as Renilla luciferase plasmid as an internal control, together with STAT1C or control plasmid were harvested and cell

extracts were prepared using a lysis buffer. Data were normalized for transfection efficiency by division of firefly luciferase activity with that of Renilla luciferase.

2.3.12 Subcellular fractionation and DNA binding assays

Nuclear and cytoplasmic protein of ESCC cells were extracted using the NE-PER protein extraction Kit (Thermo Scientific) according to the manufacturer's instructions. For Western blot analysis, α-tubulin and histone deacetylase 1 (HDAC1) were using as the cytoplasmic and nuclear loading control, respectively.

Oligonucleotide pull-down assays were performed with an annealed nucleotide comprising the STAT3 consensus site: 5'-GAT CCT TCT GGG AAT TCC TAG ATC with a biotin label. Nuclear extracts (50-100 g) were incubated for 1 hour at 4°C with 1g oligonucleotide in binding buffer. 50μL sepharose–streptavidin (Sigma) was added for 2 hours at 4°C. After 3 washes in PBS buffer, the complexes were resuspended in SDS sample buffer and processed for Western blotting as described above using anti-STAT3 antibody (Cell Signaling).

2.3.13 Statistical Analysis

Statistical analysis was performed with the SPSS15.0 software. The association between expression of STAT1 and survival was analyzed

using the Kaplan-Meier's analysis. The correlation between STAT1 and other clinical parameters was evaluated using Chi-square or Student's t-test. A value of $p<0.05$ was considered as statistically significant.

2.4 Results

2.4.1 Expression of STAT1 in esophageal squamous cell carcinoma (ESCC)

To survey the expression of STAT1 expression in our cohort of ESCC, we performed immunohistochemistry (IHC) applied to paraffin-embedded tissues. STAT1 immunoreactivity, assessed based on the presence of cytoplasmic staining, was detectable in the vast majority of cases (123 of 131, 93.8%). The staining intensity was categorized as strong (n=64, 49.0%) or weak (n=59, 45.0%) (**Figure 2.1A a-c**). Of these 123 STAT1-positive tumors, nuclear staining was detectable in 58 (47.2%) cases(**Figure 2.1A d**). The remaining 8 (6.1%) cases had no detectable cytoplasmic or nuclear STAT1 expression. Benign esophageal epithelial cells had relatively strong STAT1 immunostaining in both their nuclei and cytoplasm (**Figure 2.1A e and 2.1A f**).

We then validated the IHC findings using Western blots. Of the 131 cases studied by IHC, fresh tumor tissues were available in 57 cases. STAT1 at 91 kD was detectable in all tumors examined, although the intensity was

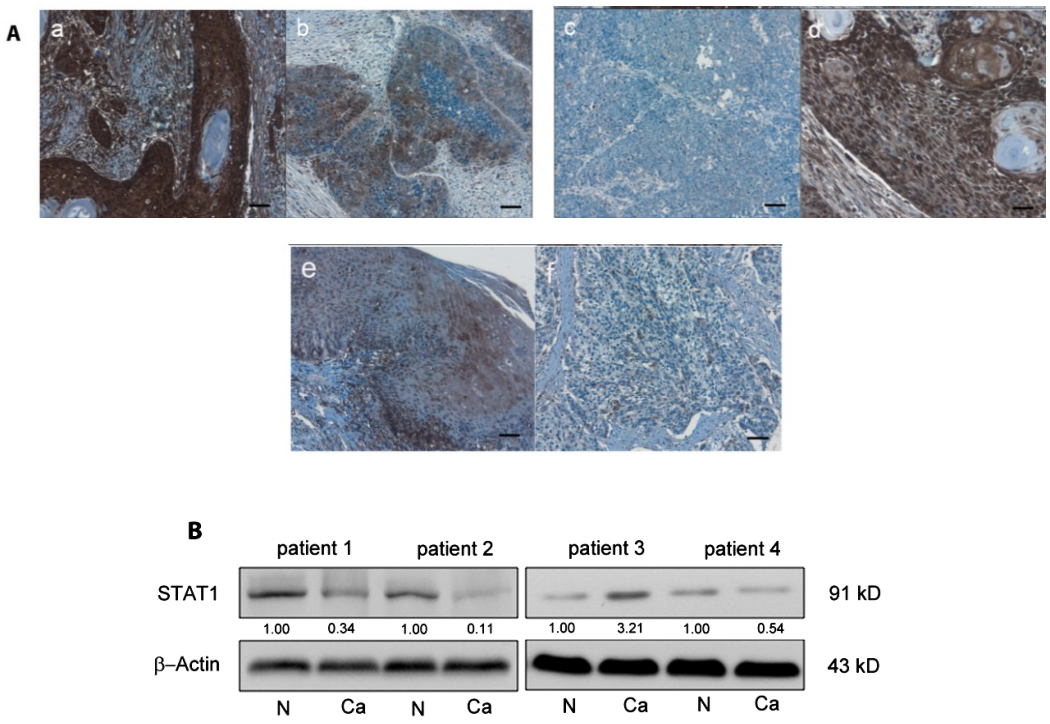


Figure 2.1 Heterogeneous STAT1 expressions in ESCC. (A) Variable levels of STAT1 were detectable in most ESCC tumors examined by immunohistochemistry. Based on the cytoplasmic staining intensity, tumors were categorized into STAT1-strong (a), weak (b) and negative (c) (scale bar, 20 μm). Nuclear staining of STAT1 was detected in some ESCC cases (d) (scale bar, 50 μm). The normal epithelium (e) from a STAT1-weak tumor (f) was also illustrated (scale bar, 20 μm). B. By Western blots, STAT1 expression in ESCC tumors was examined. Compared to the benign esophageal tissue harvested at the surgical margins in the same specimens (labeled as N) cancerous tissues (labeled as Ca) often expressed a lower level of STAT1. Thus, tumors from patient #1, 2 and 4 were categorized as STAT1-low. A small subset of tumors (e.g. that from patient #3) were categorized as STAT1-high.

Table 2.1 STAT1 expression in ESCC: significant correlation between IHC and Western blot data

STAT1 expression level by IHC[#]	STAT1 expression level by Western blot			
	High	**Low**	**Total**	
Strong	19	11	30	
Weak	4	23	27	
negative	0	0	0	
Total	23	34	57	P=0.0003*

*p<0.05

[#]all cases in this cohort were positive for STAT1.

variable, as illustrated in **Figure 2.1B**. Densitometry analysis was performed to generate a value for the STAT1 band derived from each of the 57 cases. Based on these values, the 57 cases were categorized as STAT1-high (n=23, 40.4%) or STAT1-low (n=34, 59.6%). As shown in **Table 2.1**, data generated from the IHC and Western blot studies significantly correlate with each other (p=0.0003, Fisher exact test). Specifically, 19 (33.3%) cases showing strong IHC for STAT1 were STAT1-high by Western blots; 23 (40.4%) cases showing weak/negative IHC for STAT1 were STAT1-low by western blots.

2.4.2 The clinical significance of STAT1 expression in ESCC

We then assessed if STAT1 expression detectable by IHC correlated with various and clinical and pathologic parameters, including gender, location and size of the tumor, lymph node metastasis, histologic grade, depth of tumor invasion and the overall clinical stage. As summarized in **Table 2.2**, we found that STAT1 expression inversely correlated with the depth of tumor invasion and tumor size (p=0.047 and p=0.029, respectively, Chi square). Cases with strong STAT1 expression also showed a trend toward a higher degree of histologic differentiation (p=0.074, Chi square). Compared to the poorly differentiated tumors (n=12), well- or intermediate-differentiated tumors (n=129) significantly correlated with strong STAT1 immunostaining (p=0.032, Fisher square). Nuclear

expression of STAT1 did not show significant correlation with any of these clinicopathologic parameters.

Clinical follow-up data was available for 74 of the 131 patients included in this study. The survival data was analyzed using Kaplan-Meier's. Based on the intensity and percentage of immunostained cells described above, approximately half of these tumors (34 of 74, 45.9%) were assessed strongly positive for STAT1 by IHC, 32 (43.2%) were assessed weak positive and 8 (10.9%) were assessed negative. As shown in **Figure 2.2**, the overall survival of patients with STAT1-strong tumors (n=34) was found to be similar to that of patients with STAT1-weak/negative tumors (n=40) (35.9 months versus 31.1 months, $p>0.05$). In contrast, patients with STAT1-strong/weak (positive) tumors (n=66) had a significantly longer survival compared to those with STAT1-negative tumors (n=8) (33.6 months versus 13.1 months, $p=0.019$). Furthermore, of the 74 patients for whom follow-up data was available, 50 carried poorly or intermediate-differentiated tumors. In this sub-group, patients with STAT1-strong tumors (n=23) survived significantly longer than those with STAT1-weak/negative (n=27) tumors (34.6 months versus 20.5 months, $p=0.011$). Nuclear STAT1 expression again did not significantly correlate with the overall survival in this sub-group.

2.4.3 Roles of STAT1 in ESCC cell lines

Table 2.2 Correlations between STAT1 expression and various clinicopathologic parameters in 131 ESCC patients

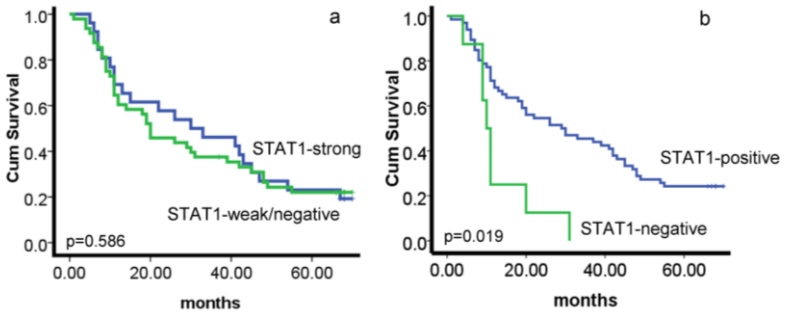
Parameter		Case NO.	STAT1 Expression by IHC		Result
			Negative/Weak	Strong	
Age	**≤57**	66	31	35	p=0.337
	>58	65	36	29	
Gender	**Male**	98	49	49	p=0.653
	Female	33	18	15	
	Upper	13	8	5	p=0.572
Tumor site	**Middle**	104	51	53	
	lower	14	8	6	
Differentiation	**Poor**	12	10	2	p=0.074
	Intermediate	75	37	38	
	Well	44	20	22	
Tumor size	**>5cm**	82	48	34	p=0.029*
	<5cm	49	19	30	
Depth of invasion	**T1-T2**	103	48	55	p=0.047*
	T3-T4	28	19	9	
Lymph metastasis	**Yes**	68	32	36	p=0.286
	No	63	35	27	
Clinical Stage[#]	**1**	6	4	2	p=0.257
	2	55	30	23	
	3	64	32	32	
	4	6	1	5	

*p<0.05

[#]**Clinical stage** is based on the TNM classification for esophageal cancer

Abbreviation: T1: Tumor invades lamina propria, muscularis mucosae, or submucosa; T2: Tumor invades muscularis propria; T3: Tumor invades adventitia; T4: Tumor invades adjacent structures.

Whole follow-up patients (n=74)



Poorly and intermediate-differentiated patients (n=50)

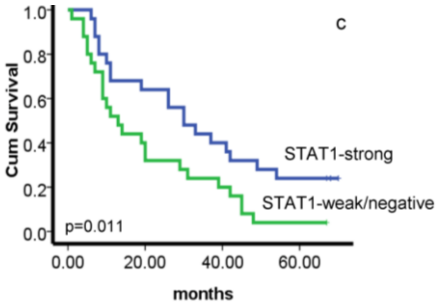


Figure 2.2 Survival analysis of STAT1 in ESCC patients. By Kaplan-Meier analysis, we found no significant correlation between overall survival and the expression level of STAT1, when the two groups were defined as STAT1-strong and STAT1-weak/negative (a). In contrast, we found a significant correlation between overall survival and the expression level of STAT1 protein levels when the two groups were defined as STAT1-positive or STAT1-negative (b). With the subset of patients carrying poorly or intermediate-differentiated tumors, those with STAT1-strong tumors survived significantly longer than those with STAT1-weak/negative tumors (c).

2.4.3.1 STAT1 expression in ESCC cell lines

In light of the clinical significance of STAT1 in ESCC, we examined its roles in ESCC using an *in-vitro* model. The expression of STAT1 in a cohort of human ESCC cell lines (EC1, EC109, KYSE150 and KYSE510) as well as a cohort of human immortalized esophageal epithelial cell lines (SHEE, NE2, NE3 and NE6) was examined using Western blots. MCF7, an estrogen receptor-positive breast cancer cell line, served as the positive control for STAT1. As shown in **Figure 2.3**, we were able to detect STAT1 in 6 of these 8 cell lines; EC109 and SHEE were STAT1-negative. In the 6 STAT1-positive cell lines, EC1 and KYSE150 expressed STAT1 relatively weakly, whereas KYSE510, NE2, NE3 and NE6 expressed STAT1 relatively strongly. The expression of the phosphorylated/activated form of STAT1 (p-STAT1) in these cell lines was also assessed in these 8 cell lines. Except for EC1, all STAT1-positive cell lines expressed p-STAT1, although all of the immortalized cell lines (including NE2, NE3 and NE6) expressed p-STAT1 relatively weakly.

2.4.3.2 The biological impact of STAT1 in ESCC cell lines

Using the ESCC cell lines, we then performed specific *in-vitro* studies. First, we examined the biological impact of enforced expression of the constitutively active form of STAT1 (i.e. *STAT1C*) in ESCC. To this end, EC1 and EC109, both of which were STAT1-weak/negative cell lines,

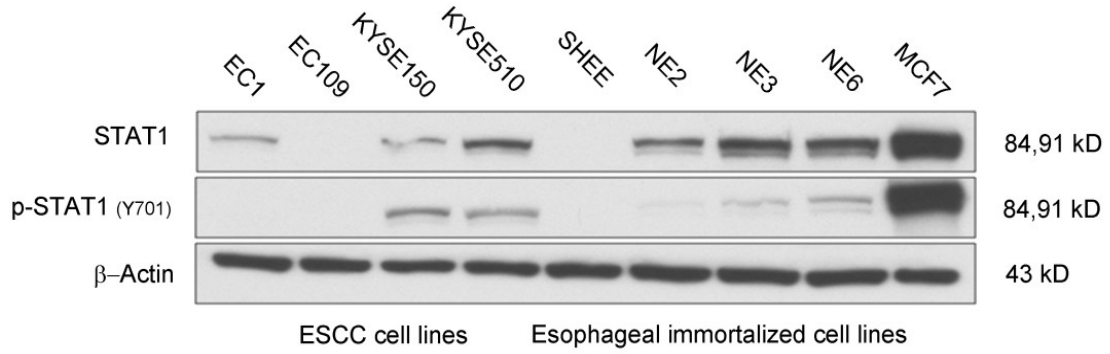


Figure 2.3 Expression of STAT1 and phospho-STAT1 in ESCC (n=4) and esophageal immortalized cell lines (n=4). ESCC cell lines included EC1, EC109, KYESE150 and KYSE510 and human esophageal immortalized cell lines included SHEE, NE2, NE3 and NE6. MCF7, a breast cancer cell line, served as a positive control. The expression of STAT1 was heterogeneous among these cell lines, and the expression of phospho-STAT1 was generally in parallel with the expression of STAT1.

were employed and they were subjected to gene transfection of *STAT1C*.

As shown in **Figure 2.4A**, the expression of STAT1C was confirmed by the high intensity of the total STAT1 band and the strong expression of FLAG, which was tagged to the *STAT1C* construct. These changes correlated with a significant decrease in the number of viable cells, as assessed using the trypan blue exclusion assay (**Figure 2.4B**). As shown in **Figure 2. 4C and D**, STAT1C transfection in EC1 and EC109 cells led to a significant decrease in colony formation and cell invasion, as compared to cells transfected with the empty vector ($p<0.001$ and $p<0.05$ in both cell lines). As shown in **Figure 2.5A**, the occurrence of apoptosis was supported by the expression of cleaved caspase 3 in both cell lines. Correlating with these changes, there was a marked reduction in the expression levels of several anti-apoptotic proteins including BCL-2, BCL-xL and survivin. Furthermore, we also observed changes in two proteins known to regulate G_1 cell-cycle progression including $p21^{Waf1}$ and cyclin D1. Specifically, transfection of *STAT1C* into EC1 and EC109 substantially upregulated $p21^{Waf1}$, a negative regulator of G_1 cell-cycle progression **[14]**. Cyclin D1, a promoter of G_1 cell-cycle progression **[15]**, was downregulated. Based on the results of the time-course experiment (**Figure 2.5B**), the decrease in the cyclin D1 protein level began as early as 6 hours after *STAT1C* gene transfection, indicating that the decrease in cyclin D1 was not due to the apoptotic activity. As shown in **Figure 2.6**,

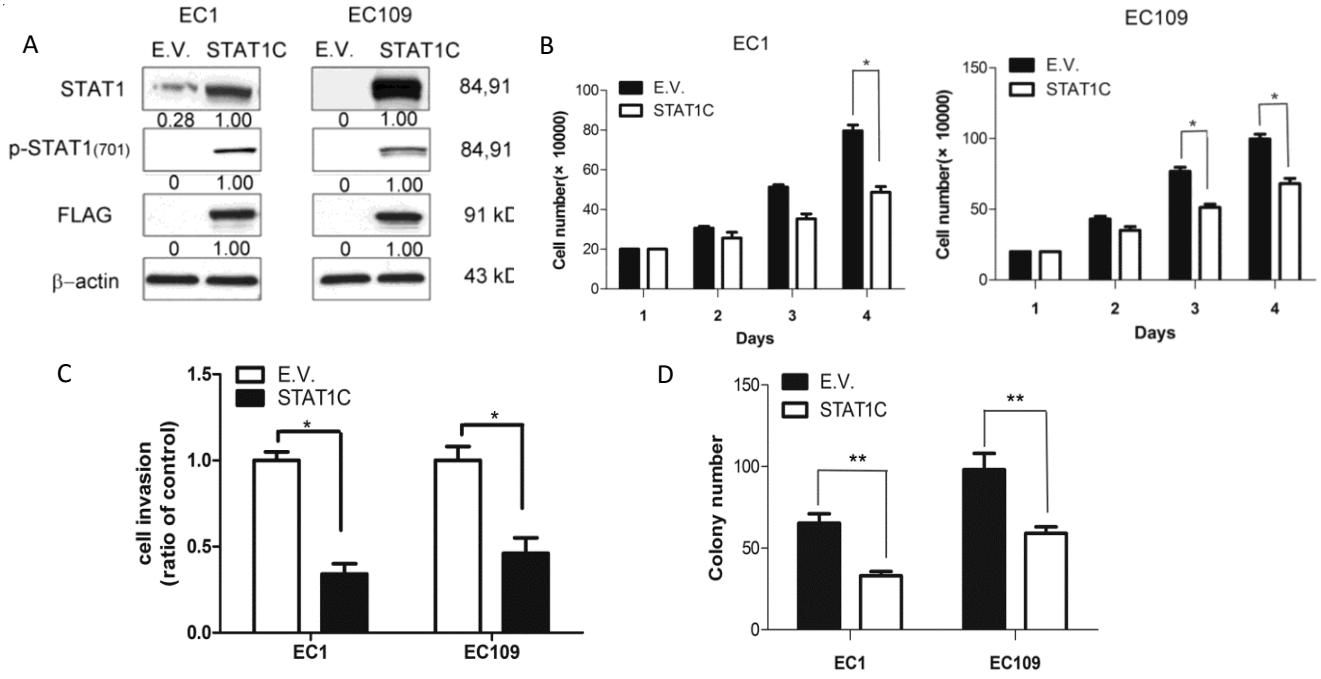


Figure 2.4 STAT1C decreases the cell growth, colony formation ability and cell invasion in ESCC. A. Using Western blot analysis, the gene transfection of *STAT1C* in EC1 and EC109 cells was shown to be effective, since the levels of STAT1, phospho-STAT1 and FLAG were dramatically increased 2 days after STAT1C transfection. B. Cell growth, as assessed by trypan blue cell counting, was found to be significantly decreased after *STAT1C* transfection in EC1 and EC109 cells (*p<0.05). C. Colony number was significantly lower in EC1 and EC109 cells transfected with *STAT1C*, as compared to an empty vector (**p<0.001). D. Transwell invasion assay showed that the transfection of STAT1C significantly inhibited cell invasion both ESCC cell lines. Results shown are representative of three independent experiments. (E.V.: empty vector)

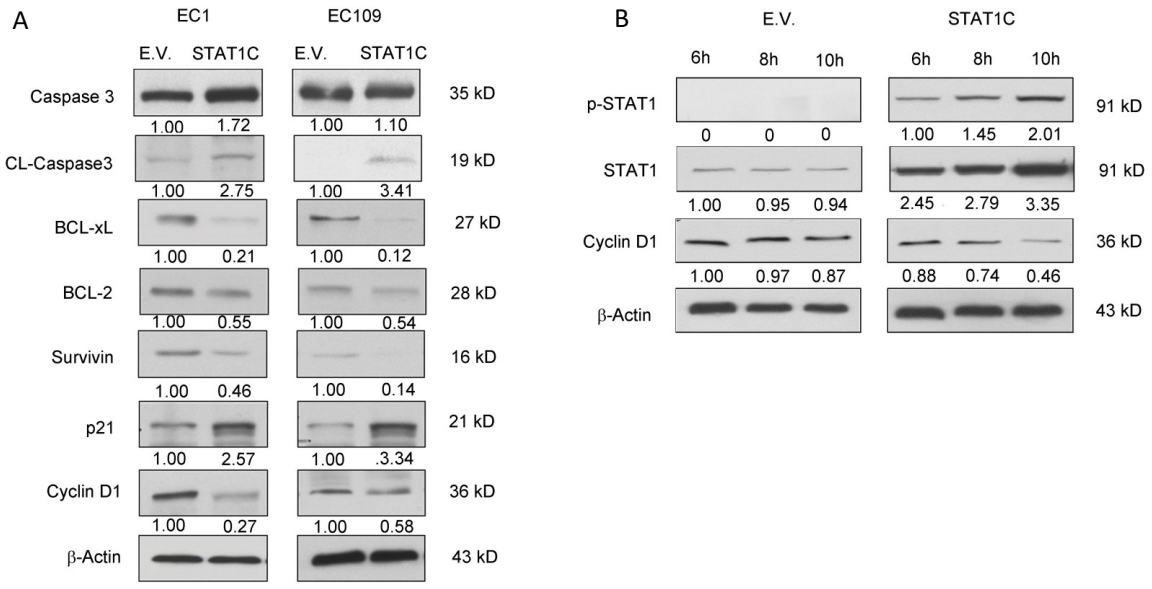


Figure 2.5 STAT1C regulates apoptosis and cell cycle markers in ESCC. A. By western blots, gene transfection of *STAT1C* into ESCC cell lines induced cleavages of caspase 3, downregulated several pro-apoptotic proteins (including BCL-2, BCL-xL, survivin), and promoted G1 cell-cycle arrest by decreasing cyclin D1 and increasing p21. Cell lysates were collected 2 days after the gene transfection of *STAT1C* in EC1 and EC109. B. Time course experiments were performed, and the decrease in cyclin D1 expression was detectable as early as 6 hours after STAT1C transfection in EC1 cells. Results shown are representative of three independent experiments. (E.V.: empty vector)

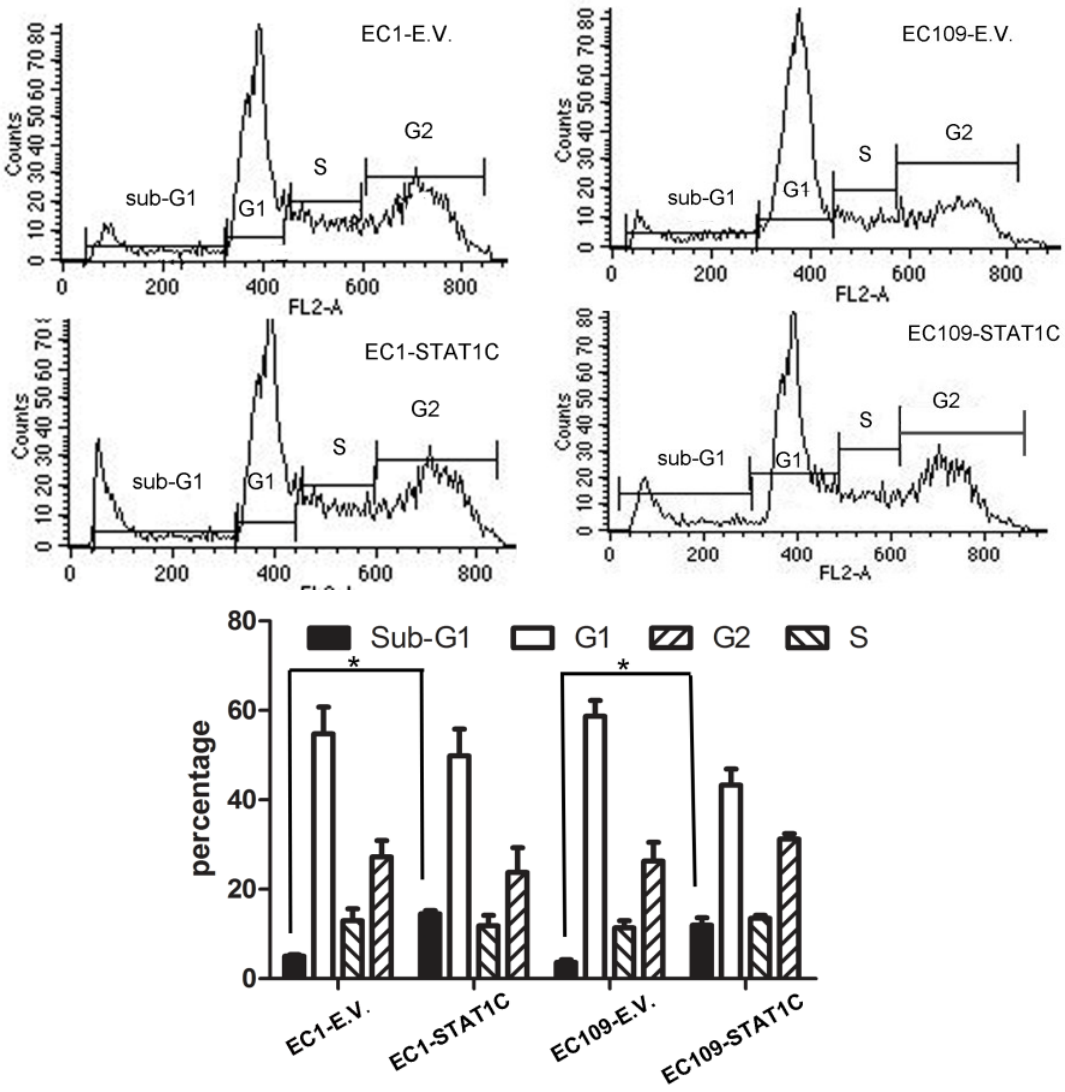


Figure 2.6 STAT1C increases sub-G1 cell-cycle fraction in ESCC cells. Cell cycle analysis using flow cytometry revealed that STAT1C induced a significant increase in the sub-G1 fraction in both cell lines, EC1 and EC109. All experiments were performed in triplicate, and results from a representative run are shown. (*p<0.05) (E.V.: empty vector)

cell cycle analysis showed a significant increase in the sub-G_1 fractions in EC1 and EC109 cells transfected with *STAT1C*.

2.4.3.3 The biological impact of siRNA knockdown of STAT1 in ESCC cell lines

The biological effects of siRNA knockdown of STAT1 in ESCC cells were evaluated. KYSE150 and KYSE510 cells, which showed the highest level of STAT1 expression among the eight examined cell lines, were treated with STAT1 siRNA. As shown in **Figure 2.7A**, STAT1 siRNA induced a dramatic reduction in the STAT1 expression level in both cell lines. With this experimental system, we found that siRNA knockdown of STAT1 significantly increased the number of viable cells, which was assessed by using the trypan blue exclusion assay ($p<0.05$ for both cell lines) (**Figure 2.7B**). Furthermore, using colony formation assay, we found that siRNA knockdown of STAT1 of KYSE150 and KYSE510 induced a significant decrease in colony formation ($p<0.0001$, $p<0.0001$, respectively) (**Figure 2.8A**). As shown in **Figure 2.8B**, Western blot studies showed changes in the expression of $p21^{waf1}$, cyclin D1, BCL-2 and BCL-xL in a pattern opposite to that seen in EC1 and EC109 cells transfected with *STAT1C*. As shown in **Figure 2.9**, cell cycle analysis showed a significant decrease in the sub-G_1 fractions in KYSE150 and KYSE510 cells transfected with siRNA against STAT1, compared with the control.

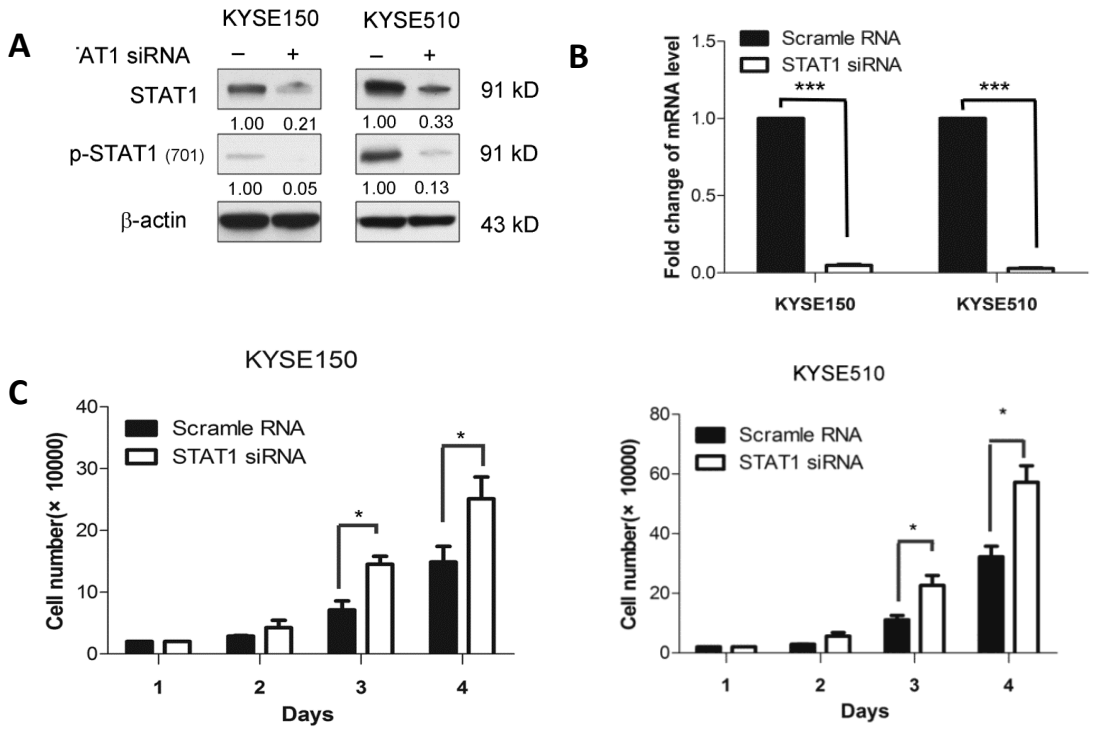


Figure 2.7 siRNA knockdown of STAT1 increases the cell growth in ESCC. A. By Western blot analysis, the protein level of STAT1 and phospho-STAT1 were dramatically decreased in KYSE150 and KYSE510 treated with siRNA against STAT1. Cell lysates were collected 2 days after the siRNA transfection. B. The decrease in *STAT1* expression after siRNA treatment was further supported by quantitative RT-PCR (***p<0.0001). C. siRNA knockdown of *STAT1* induced a significant decrease in cell growth, assessed by trypan blue assay. The cell numbers were assessed on day 4 after siRNA transfection. Triplicate experiments were performed and the results of a representative experiment are illustrated (* p<0.05)

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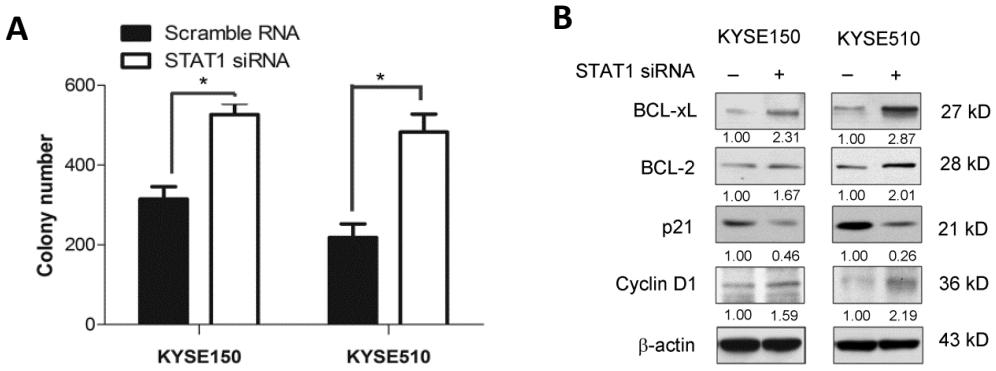

Figure 2.8 siRNA knockdown of STAT1 increases colony formation and regulates its downstream genes. A. Transfection of *STAT1* siRNA into KYSE150 and KYSE510 cells led to a significant reduction in the number of colonies formed, as compared to cells transfected with scrambled siRNA. Triplicate experiments were performed and the results of a representative experiment are shown (*$p<0.05$). B. By Western blots, transfection of *STAT1* siRNA resulted in an appreciable increase in BCL-xL, BCL-2, cyclin D1 and a corresponding decrease in $p21^{waf1}$. Cells treated with scrambled siRNA served as the negative controls.

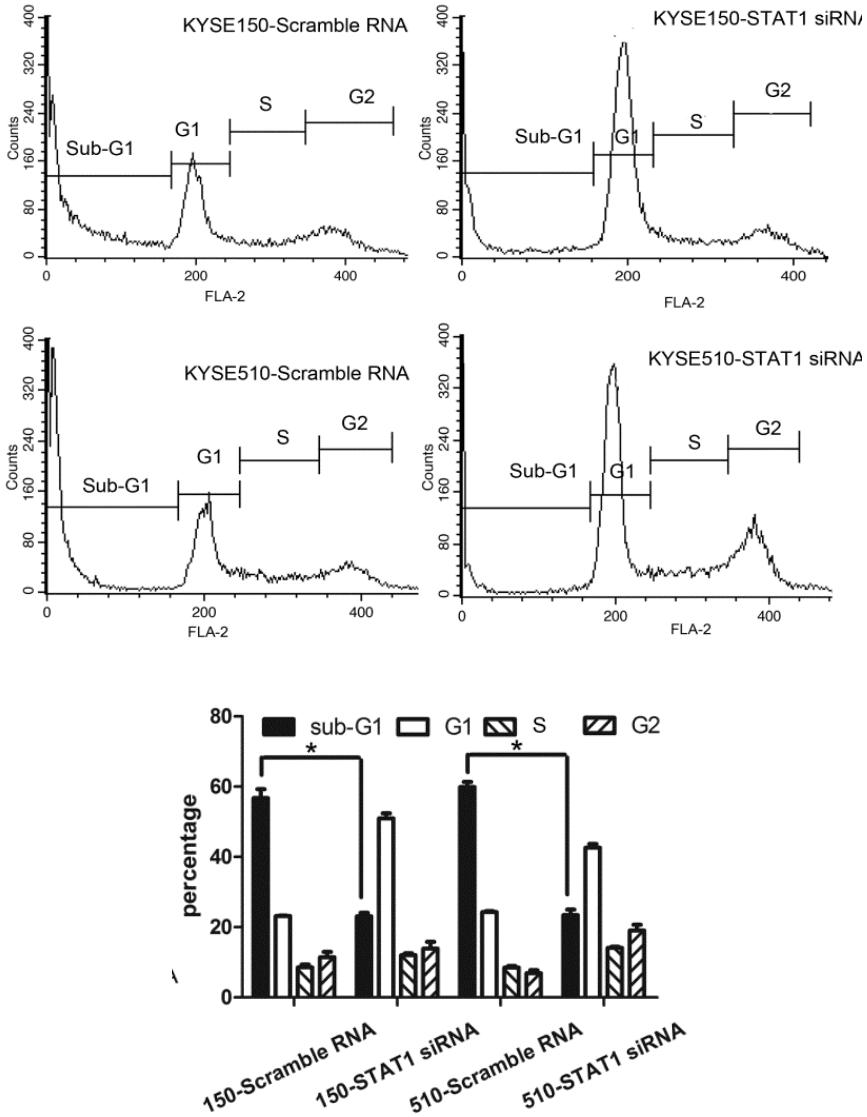

Figure 2.9 STAT1 siRNA decreases sub-G1 cell cycle fraction in ESCC. Cell cycle analysis using flow cytometry revealed that STAT1 siRNA induced a significant decrease in the sub-G1 fraction in both cell lines, KYSE150 and KYSE510. Results shown are representative of three independent experiments. (*$p<0.05$)

2.4.4 STAT1 inhibits NF-κB

Previous studies have shown that STAT1 can block NF-κB by downregulating TNF-α **[16]**. In view of the importance of NF-κB in the biology of ESCC **[17, 18]**, we hypothesized that the biological effects of *STAT1C* in ESCC may be mediated by down-regulating the NF-κB signaling. In keeping with this concept, we found that transfection of *STAT1C* into EC1 and EC109 cells resulted in a substantial decrease in the phosphorylation of NF-κB p65, a marker of NF-κB activation **[19]** (**Figure 2.10A**). By subcellular fractionation, we also found that *STAT1C* transfection induced a dramatic decrease in the nuclear localization of NF-κB p65 or phospho-NF-κB p65 in both cell lines (**Figure 2.10B**). Lastly, we assessed the transcriptional activity of NF-κB using a commercially available luciferase reporter construct. As shown in **Figure 2.10C**, there was a significant down-regulation of NF-κB transcriptional activity after *STAT1C* transfection in both ESCC cells.

2.4.5 *STAT1C* transfection downregulates STAT3 expression and activation

Since STAT1 and STAT3 are known to counteract each other during their regulations of various cellular processes **[20, 21]**, we asked if the modulation of STAT1 may have an impact on the expression and/or activation of STAT3. As shown in **Figure 2.11A**, we found that the

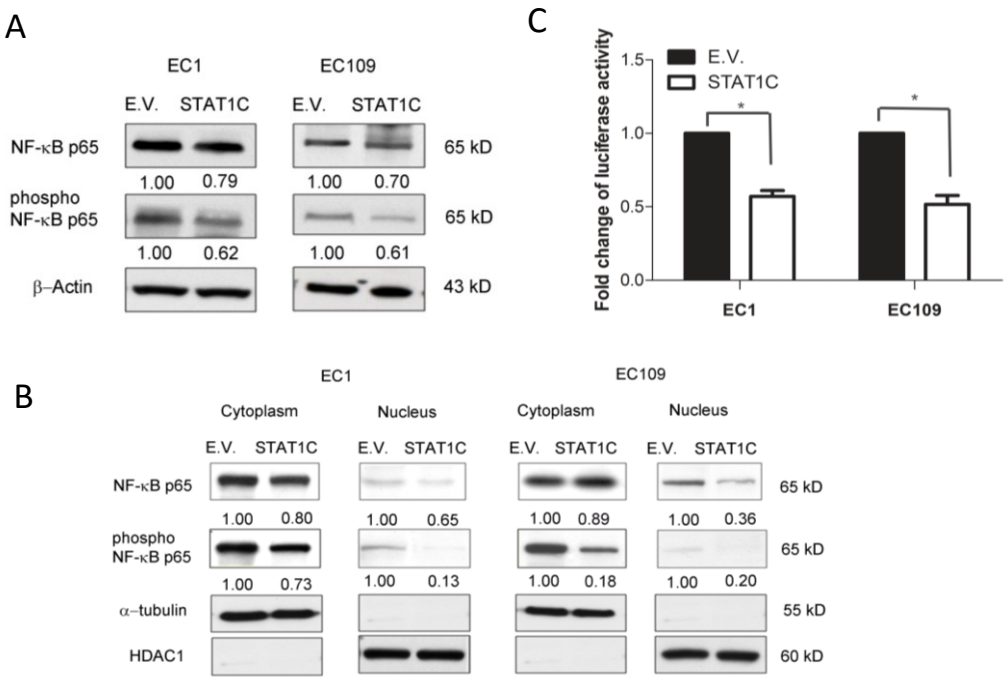

Figure 2.10 STAT1C inhibits NF-κB signaling. A.Western blot results showed a detectable down-regulation of total p65 and phospho-p65 after *STAT1C* transfection in EC1 and EC109 cells.B. In the same experiment, nuclear/cytoplasmic fractionation studies showed that *STAT1C* induced a substantial decrease in nuclear p65 and phospho-p65. C. Using a NF-κB/luciferase reporter, we found that *STAT1C* gene transfection induce a significant down-regulation of the NF-κB transcription activity in ESCC cells transfected with *STAT1C* cells were harvested 48 hours after the gene transfection. (*p<0.05) (E.V.: empty vector)

expression levels for STAT3 and p-STAT3 were decreased 48 hours after *STAT1C* transfection into EC109 and EC1 cells. Correlating with these results, siRNA knockdown of STAT1 substantially increased the expression level of STAT3 and p-STAT3 in KYSE150 and KYSE510 (**Figure 2.11A and B**). Moreover, by using biotin pull-down assay and luciferase reporter assay, we found that *STAT1C* dramatically decreased the DNA binding and transcription activity of STAT3, which confirmed previous findings that *STAT1C* downregulated STAT3 activation (**Figure 2.11C and D**) We then assessed how *STAT1C* transfection might affect the physical interaction between STAT1 and STAT3 using co-immunoprecipitation. As shown in **Figure 2.12** (right panel), by Western blots, transfection of *STAT1C* again resulted in 30-40% reduction in the expression of STAT3. Co-immunoprecipitation studies (left panel) showed that transfection of *STAT1C* substantially increased the STAT3-STAT1 binding in both EC1 and EC109 cells. Considering that the total STAT3 protein level was decreased after *STAT1C* expression, these co-immunoprecipitation data strongly support the concept that the STAT3 homodimers are decreased upon *STAT1C* transfection.

2.5. Discussion

STAT1 has been reported to regulate cellular differentiation and apoptosis through transcription-dependent as well as transcription-independent

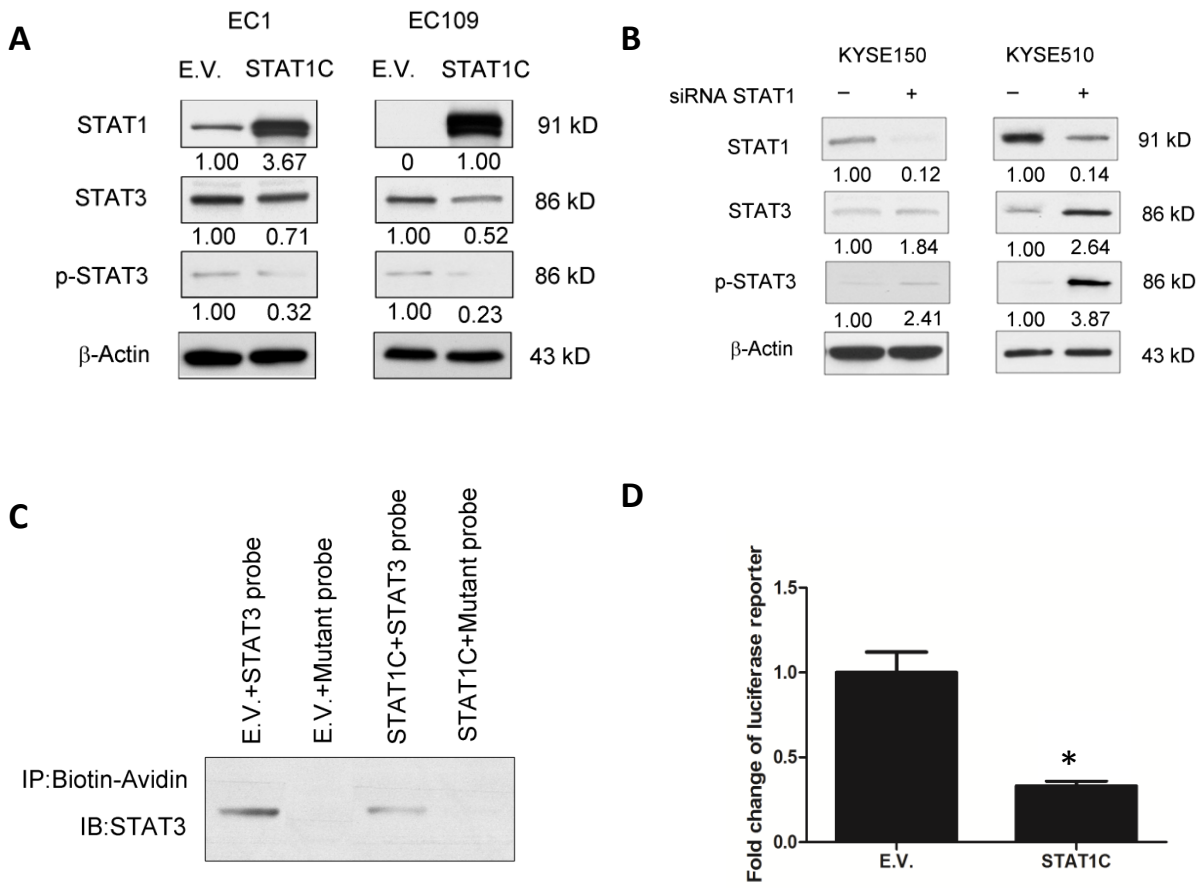

Figure 2.11 STAT1C decreases the expression, transactivation and DNA binding of STAT3. A. Western blot studies showed that gene transfection of *STAT1C* induced an appreciable decrease in total STAT3 and phospho-STAT3 in EC1 and EC109 cells. Cell lysates were harvested 48 hours after gene transfection. B. In contrast, both STAT3 and phospho-STAT3 were increased in response to siRNA knockdown of STAT1 in KYSE150 and KYSE510. C. Using biotin pull down assay, *STAT1C* decreases STAT3 DNA binding ability in EC1 cell line. D. By using luciferase reporter, *STAT1C* transfection dramatically decreases the transcription activity in EC1 cell line. (*$p<0.05$) (E.V.: empty vector)

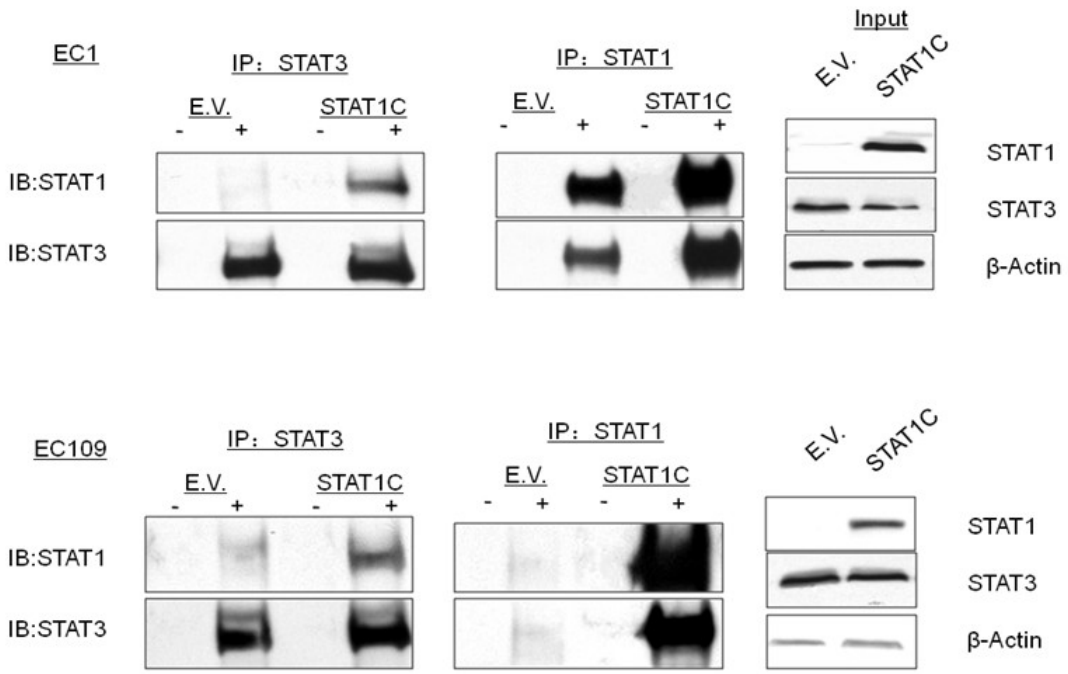

Figure 2.12 STAT1C increases STAT1-STAT3 heterodimer formation. Co-immunoprecipitation experiments revealed that gene transfection of *STAT1C* into EC1 and EC109 induced a substantial increase in STAT1:STAT3 heterodimer; at the same time, the total STAT3 protein level was decreased. Cell lysates were prepared 48 hours after gene transfection. (E.V.: empty vector)

mechanisms **[22]**. There is also evidence that STAT1 carries tumor suppressor functions **[23-25]**. Decreased or loss of STAT1 expression has been observed in many cancer types such as breast cancer, melanoma and leukemia **[2, 26-28]**; transfection of *STAT1* or *STAT1C* into cancer cells can arrest their growth by inducing apoptosis and cell-cycle arrest **[1, 9, 29]**. STAT1 appears to exert its tumor suppressor functions via multiple mechanisms, including upregulation of caspases, Fas, FasL, TRAIL and $p21^{waf1}$ **[22]**. The apoptosis-inducing function of STAT1 also has been linked to its role in IFN-γ signaling and the subsequent upregulation of several pro-apoptotic proteins including Bak and Bax **[2]**. Despite the fact that the tumor suppressor function of STAT1 is increasingly recognized, the significance of STAT1 in ESCC has not been clearly defined.

One of the key findings of this study is that the absence of STAT1 expression in ESCC significantly correlates with a worse clinical outcome. Of note, we would like to acknowledge that this observation was based on a comparison between 8 STAT1-negative and 66 STAT1-strong/weak tumors. This highly skewed pattern may have introduced some biases; thus, larger studies using other patient cohorts are needed to confirm this finding. Nonetheless, we do believe that STAT1 holds prognostic value, since we found prognostic significance when we compared STAT1-weak/negative cases and STAT1-strong cases in the cohort of

poorly or intermediate-differentiated tumors. Furthermore, STAT1 expression was also found to correlate with the depth of tumor invasion and tumor size. In this regard, tumor size is one of the determinants of the TNM clinical staging system for ESCC patients **[30, 31]**. To our knowledge, the prognostic significance of STAT1 in ESCC has never been previously described. Nevertheless, we are aware of a relatively small number of published studies that had found prognostic significance of STAT1 in other types of cancer, such as gastric cancer and melanoma **[26, 32]**.

Correlating with our clinical observations, our *in-vitro* studies have provided further support that STAT1 carries tumor suppressor functions in ESCC. Specifically, we found that transfection of *STAT1C* into ESCC induced apoptosis and cell-cycle arrest, with the effect on apoptosis being more pronounced than that on cell proliferation. On the other hand, siRNA knock-down of STAT1 in ESCC cells results in opposite effects. In parallel to our findings, we have identified two in-vitro studies in the literature that may have implicated a role of STAT1 in the biology of ESCC **[9, 33]**. In one of these two studies, IFN-γ and EGF were found to induce apoptosis in KYSE 70 and KYSE 590, two ESCC cell lines **[9]**. However, while the apoptosis induced by both cytokines was found to correlate with STAT1 activation, whether STAT1 activation is directly responsible for the occurrence of apoptosis in these cells was not clear. In this current study,

the use of *STAT1C* and STAT1 siRNA allowed us to pinpoint STAT1 as a key mediator of the induction of apoptosis and cell-cycle arrest in ESCC.

We attempted to delineate the mechanisms by which STAT1 induces apoptosis in ESCC. We found that some of the mechanisms are similar to those previously reported for other cancer types **[34]**. Thus, gene transfection of *STAT1C* in fibrosarcoma cell lines was found to induce caspase activation and caspase-dependent apoptosis **[13]**. As we found that STAT1C-induced apoptosis in ESCC correlates with the down-regulation of several pro-survival proteins such as BCL-2 and BCL-xL, previous studies also have shown that STAT1 can promote apoptosis by down-regulating BCL-2 and BCL-xL in multiple myelomas **[29]**.

We also have found evidence that STAT1 can modulate the expression of G_1 cell-cycle regulatory proteins such as $p21^{waf1}$ and cyclin D1 in ESCC. Upregulation of cyclin D1 has been shown to shorten the G_1 phase and reported to link to the development and progression of many types of cancer, such as breast cancer **[35]**, gastric cancer **[36]** and mantle cell lymphoma **[37]**. In ESCC, cyclin D1 expression detectable by IHC is associated with a worse prognosis **[38, 39]**. Thus, down-regulation of cyclin D1 induced by STAT1 may partly explain our observation that high STAT1 expression in ESCC is associated with a better clinical outcome.

Our observation that the down-regulation of cyclin D1 occurred as early as 6 hours after *STAT1C* gene transfection suggests that this biochemical change was a consequence of STAT1 up-regulation, rather than a 'by-product' of apoptosis.

In various cancer cell types, STAT1 has been previously reported to be an important mediator for NF-κB, which is known to play a critical role in carcinogenesis and chemoresistance in ESCC **[40, 41]**. The NF-κB signaling pathway was found to be constitutively activated in many ESCC cell lines; down-regulation of p65 has been shown to increase the sensitivity of ESCC cells to chemotherapeutic drugs **[17, 18]**. With this background, we tested if STAT1 is functionally linked to NF-κB in our experimental model. Our findings that modulation of STAT1 expression changed the expression level of phospho-p65, as well as the nuclear localization of p65/phospho-p65, and these findings support the concept that the STAT1 inhibits the growth of ESCC via its suppression of NF-κB signaling. Similar results have been described in cervical cancer, melanomas and fibrosarcomas **[16, 42-44]**.

STAT3, another member of the STAT family, has been shown to exert opposing biological effects of STAT1 **[20, 21, 45]**. In contrast with STAT1, STAT3 promotes survival, proliferation and motility of cancer cells, and induces immune tolerance **[46]**. Some of these opposing biological

effects are likely related to the cross-talk between STAT1 and STAT3. For instance, it has been shown that STAT3 can block the activation and function of STAT1 in human monocytic cells **[47]**. STAT3-knockout mouse embryonic fibroblasts exhibited prolonged IL-6-mediated STAT1 activation and induction of IFN-γ-inducible genes **[48]**. In this study, we found evidence that STAT1 interferes with STAT3 signaling in ESCC cells using at least two mechanisms. First, STAT1 down-regulates STAT3 as well as phospho-STAT3. To our knowledge, this is a novel finding. Second, co-immunoprecipitation studies showed that *STAT1C* transfection in ESCC cells substantially increased STAT1:STAT3 heterodimer. Since the total STAT3 protein level was decreased after *STAT1C* transfection, it is logical to assume that STAT3:STAT3 homodimers were dramatically decreased at the same time. We believe that these findings are linked to the tumor suppressive effects of STAT1C in ESCC, as previous studies have shown that the relative proportions of STAT1 homodimers, STAT3 homodimers and STAT1:STAT3 heterodimers dictate the cell fate **[21, 49, 50]**. Based on the literature, other mechanisms where STAT1 inhibits STAT3 signaling may also exist. For instance, it has been shown that STAT1 can compete with STAT3 for the common receptor docking sites or target DNA sequences **[2, 20]**. Of note, most of these STAT1-mediated effects on STAT3 take place in the cytoplasm, and this may correlate with our observation that cytoplasmic STAT1, rather than nuclear STAT1, was

prognostically important.

The role of STAT1 as a tumor suppressor is not without controversy. STAT1 overexpression has been demonstrated in several types of human cancer **[51-55]**. Some reports also have demonstrated that constitutive STAT1 signaling promotes tumor growth, increases resistance to chemotherapy and radiation and carries a worse clinical outcome in patients with glioblastoma multiforme **[56]**. The possible explanations for these rather contradictory findings may be related to the biological heterogeneity of cancer and STAT1 may function as a tumor suppressor or oncoprotein in a cell-type specific manner.

However, there are still some limitations in this paper. First, we didn't collect enough information during follow-up years, for example, radiotherpy or chemotherapy after surgery. Thus, so caution should be applied in extrapolating these findings from the patients with radiotherapy or chemotherapy. In addition, we should perform multivariate survival analysis to eliminate these factors to harmonize statistical bias. Second, the *in-vivo* study may further support our hypothesis. Third, it would be better to detect the transfect efficience when we performed transient transfection. Moreover, the tet-on system can be used to build stable STAT1 transfected ESCC cell lines.

2.6 Conclusion

In conclusion, our findings suggest that STAT1 is a tumor suppressor in ESCC. Loss of STAT1, which is frequent in ESCC, contributes to the pathogenesis of these tumors. We have provided evidence that STAT1 attenuates the tumorigenicity of ESCC by inhibiting the STAT3 and NF-κB signaling pathway, and therefore that activation of STAT1 may be a useful approach to treat ESCC.

2.7 References

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Chapter 3

Correlation of STAT1 with apoptosis and cell-cycle markers in esophageal squamous cell carcinoma.

This chapter has been modified from a previous publication:

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As first author, I designed and performed all the experiments described here in, except for the following: Zhang Y and Yun H performed some in-vitro experiment and IHC staining shown in **Figure 3.4**; I also wrote the manuscript

3.1 Abstract

We recently found evidence that STAT1 in esophageal squamous carcinoma (ESCC) cells exerts tumor suppressor function, and it regulates five key regulators of apoptosis or cell-cycle progression, including BCL-2, BCL-xL, survivin, cyclin D1 and p21. In this study, using immunohistochemistry, we also assessed the expression of these proteins in 62 primary tumors. The expression of these markers was heterogeneous, ranging 39 to 69% of the cohort. Significant correlation was found between STAT1 and three proteins (p21, BCL-xL and survivin), whereas only a trend was identified for cyclin D1 and BCL-2. We then correlated the expression of these proteins with several clinicopathologic parameters, including lymph node metastasis, depth of invasion, clinical stage and overall survival. Significant correlations were found between BCL-2 and deep invasion (p=0.033), survivin and lymph node metastasis (p=0.006), as well as cyclin D1 and clinical stage (p=0.014). Patients with p21-positive tumors had a significantly longer survival compared to those with p21-negative tumors (p=0.031). To conclude, our findings support the concept that STAT1 exerts its tumor suppressor effects in ESCC via modulating the expression of key regulators of apoptosis and cell-cycle progression.

3.2 Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most deadly cancers. This disease is highly prevalent in the Chaoshan area in China, with the annual average, age-standardized incidence rate being 10-fold higher than that of most places worldwide **[1]**. While it has been suspected that genetic and/or environmental factors may predispose the Chaoshan population to ESCC, the pathogenesis of ESCC remains to be elusive. Recently, we studied the biological significance of STAT1 in ESCC, since STAT1 has been shown to promote apoptosis and carry tumor suppressor functions in different types of cancers **[2]**. In support of the concept that STAT1 is a tumor suppressor in ESCC, we found that STAT1 expression is commonly lower in ESCC tumors (67 of 131, 51.1%), as compared to case-matched normal tissue; importantly, a relatively low level of STAT1 expression in ESCC was found to be significantly correlated with a worse clinical outcome, tumor invasion and tumor size **[3]**. In the same study, gene transfection of *STAT1C* (a constitutively-activated form of STAT1) into two ESCC cell lines (EC1 and EC109), resulted in significant apoptosis, and this biological change correlated with a marked reduction in the expression of several anti-apoptotic proteins and a cell-cycle facilitator (including BCL-2, BCL-xL survivin and cyclin D1) as well as an upregulation of $p21^{Waf1}$, a negative regulator of G1 cell-cycle progression **[4]**. With this background, we hypothesize that STAT1 may mediate its tumor suppressor function in

ESCC by modulating the expression of these anti-apoptotic proteins and cell-cycle regulators. While some of these markers have been previously studied in ESCC regarding their clinical significance (e.g. prognosis), their relationship with STAT1 expression in ESCC has not been explored. In this study, we use immunohistochemistry (IHC) to assess if the correlation between STAT1 and these markers also hold true in a cohort of patient samples. We also assessed if these markers correlate with various clinicopathologic parameters, including the overall survival.

3.3 Material and methods

3.3.1 Patient cohort

This study included 62 randomly selected patients with primary ESCC who underwent radical esophageal resection at the Shantou Cancer Hospital from 2003 to 2010. None of the patients received preoperative radiotherapy or chemotherapy. 47/62 (75.8%) were male and 15/62 (24.2%) were female. The median age was 57.8 years (range, 37-75 years). 70-month follow-up data was available for 37 patients; 31/37 (83.8%) died during the follow-up period (median, 29 months). The study was approved by the ethical review committees of the Medical College of Shantou University. All participants involved in our study were given written informed consents.

3.3.2 ESCC cell lines and culture conditions

Two ESCC cell lines (KYESE150 and KYSE510) were included in this study. KYSE150 and KYSE510 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum at 37˚C under 5% CO_2,

3.3.3 Immunohistochemistry

Envision-Labeled Peroxidase System immunohistostaining was performed as described previously **[5]**. Briefly, formalin fixed, paraffin embedded ESCC tumors were used for this study. All cases were retrieved from the file at the Department of Pathology, Shantou University Medical College. The diagnosis of these cases was based on the criteria established by the World Health Organization classification scheme. Immunohistochemistry to detect STAT1 expression was performed using a method similar to that described previously **[5]**. Briefly, formalin-fixed, paraffin embedded tissue sections of 4 μm thickness were deparaffinized in xylene and hydrated in graded ethanol (100% to 50%). Antigen retrieval was performed using citrate buffer (pH 6.0) microwaved in a pressure cooker for 20 minutes and left to cool for 20 minutes. After antigen retrieval, tissue sections were incubated with 10% hydrogen peroxide (H_2O_2) and methanol for 10 minutes to block endogenous peroxidase activity, followed by washing in running tap water for 5 minutes. Subsequently, the sections were incubated for 20 minutes in

antibody diluent (Dako, Mississauga, ON, Canada), followed by incubation overnight at 4°C with primary antibodies. The following antibodies were employed: anti-cyclin D1, anti-p21, anti-BCL-2 and anti-survivin were purchased from Fuzhou Maxim Biotechnology Company (Fuzhou, China). Anti-BCL-xL (1:300) was purchased from Cell Signaling Technology, Inc. (Danvers, America). The antibodies were diluted in antibody diluent (Dako) Immunostaining was visualized with a labeled streptavidin-biotin method (20 minutes in biotinylated link and 20 minutes in streptavidin horse radish peroxidase, both from Dako) using (3, 3- diaminobenzidine/ H_2O_2) DAB as a chromogen (Dako). Hematoxylin was used as a counter stain. Following staining, sections were dehydrated in graded ethanol (50%-100%), followed by xylene incubation. Coverslips were applied using permount solution (Fisher Scientific).

IHC staining was examined by two pathologists who were blinded to the clinical outcome and concordance between two pathologies was guaranteed. For the evaluation of STAT1 immunostaining, both the intensity and percentage of immunostained cells were described previously in chapter 1. The percentages of positive stained cells were assigned the following scores: 0 (< 5% positive cells), 1 (6% to 25% positive cells), 2 (26% to 50% positive cells), 3 (51% to 75% positive cells), or 4 (> 75% positive cells). The staining intensity was scored on a scale of 0 to 3 as follows: 0, negative; 1, buff; 2, yellow; and 3, brown. The

percentage of positive cells and the staining intensities were then multiplied to generate the immunoreactivity score for each case. Overall staining scores from 0 to 2, 3-6 and ≥ 7 were considered negative, weak and strong expression, respectively. The weak and strong expressions were considered positive. The expression of cyclin D1 and p21 proteins was considered positive when staining was observed in the cell nucleus. For survivin, BCL-2 and BCL-xL, staining in the cytoplasm was considered positive.

3.3.4 Antibodies and western blotting

Western blot analysis was performed using standard techniques as previously described **[6]**. The following antibodies were employed: anti-PARP (1:1000), anti-caspase 3 (1:1000), anti-survivin (1:1000), all of which were purchased from Cell Signaling (Danvers, MA, USA). Anti-BCL-xL (1:1000) and anti-β-actin (1:1000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Densitometric analysis was performed using the Image J analysis system (Bethesda, WA, USA); the values for the bands were normalized to those of the β-actin bands.

3.3.5 Plasmids and cell transfection

Mammalian expression plasmids for pEF-survivin and BCL-xL was purchased from Addgene (Cambridge, MA). For each experiment, 1×10^6

ESCC cells were transiently transfected with 10 μg of BCL-xL or survivin vector or the empty vector (Invitrogen, Burlington, Ontario, CA) in 6-well plates using the lipofectamine 2000 reagent (Invitrogen) as per manufacturer's suggested protocol.

3.3.6 Cell growth and viability assays

To assess cell growth, ESCC cells were transfected with plasmids and plated at a density of 20,000/ml of culture medium. Cell count, done daily for 4 days, was performed using trypan blue staining (Sigma-Aldrich) according to the manufacturer's protocol. ESCC cells were grown in 96-well plates to 50% confluence followed by plasmid transfection for 48 hours in full media. An empty vector transfection was used as a control. Cell viability was assessed using the MTS Proliferation Assay Kit (Invitrogen) as previously described **[6]**.The absorbance was recorded by a BioRad spectrophotometer at 3 days of cell culture. Triplicate independent MTS experiments were performed.

3.3.7 Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics 19.0 (SPSS, Chicago, IL, USA). Chi-square and Fisher's tests were used to evaluate the relationship between protein expression and clinical/pathological parameters. Survival was estimated by using

Kaplan-Meier curves. The correlation between protein expressions was obtained using a Pearson's chi-squared test. P< 0.05 was considered as statistically significant.

3.4 Results

3.4.1 The biological impact of BCL-xL and survivin in ESCC cell lines

To support that these STAT1 downstream targets are functionally important, we selected 2 of these 5 targets for functional *in-vitro* studies. As shown in **Figure 3.1**, we transfected *BCL-xL* and *survivin* into KYSE150 and KYSE510 cell lines. Western blot studies showed that the gene transfection for both vectors was relatively efficient, inducing a relatively high protein level of survivin (left panel) and BCL-xL (right panel), respectively. Correlating with the induced expression of survivin or BCL-xL, we found an appreciable reduction in the expression level of apoptotic proteins, including caspase 3 and PARP. We also performed MTS assay, and we found a significant increase in the number of viable cells after *BCL-xL* or *survivin* was transfected into both cell lines (**Figure 3.2A**). As shown in **Figure 3.2B**, using trypan blue to count the number of viable cells, we found that transfection of *BCL-xL* or *survivin* into KYSE150 and KYSE510 cells led to a significant increase in cell growth, as compared to cells transfected with the empty vector ($p<0.05$ in both cell lines). These findings have provided support that these STAT1

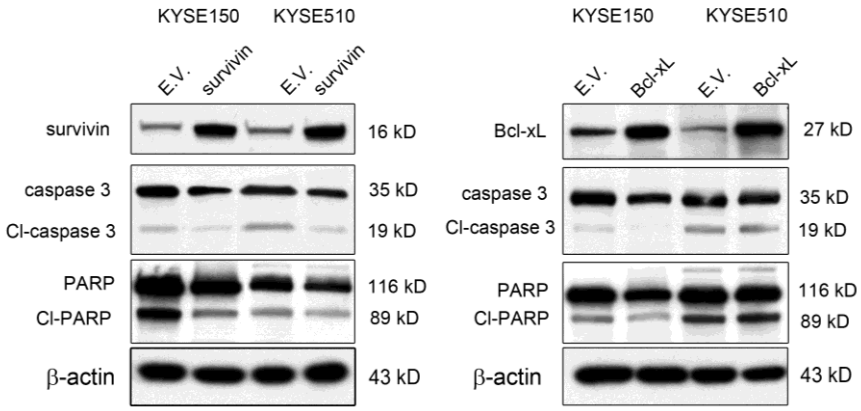

Figure 3.1 Apoptosis effects of BCL-xL and survivin in ESCC cell lines. Using Western blot analysis, the gene transfection of *BCL-xL* and *survivin* in KYSE150 and KYSE510 cells was shown to be effective, since the levels of *BCL-xL* and *survivin* were dramatically increased 2 days after *BCL-xL* and *survivin* transfection. (E.V.: empty vector)

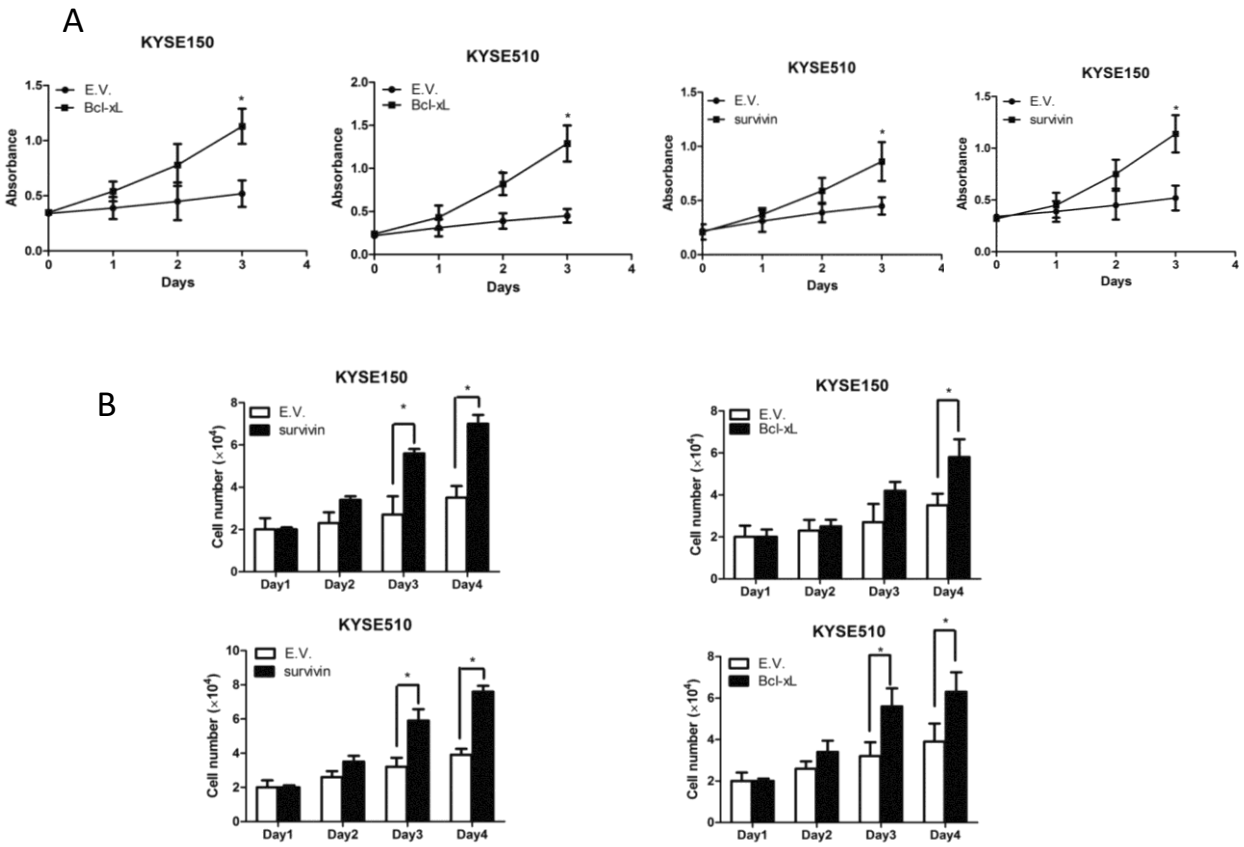

Figure 3.2 Cell growth effects of BCL-xL and survivin in ESCC cell lines. A. In both KYSE150 and KYSE510, transfection of *BCL-xL* and *survivin* induced a significant increase in cell growth, assessed by MTS assay. The cell numbers were assessed on 4 days after transfection. Triplicate experiments were performed independently and the results of a representative experiment are illustrated. B. Cell growth, as assessed by trypan blue cell counting, was found to significantly increase after *BCL-xL* and *survivin* transfection in KYSE150 and KYSE510 cells. Triplicate independent experiments were performed. (*$p<0.05$) (E.V.: empty vector)

downstream targets indeed carry biological significance in ESCC.

3.4.2 Immunohistochemical studies of the 5 markers and STAT1 in ESCC primary tumors

To validate our finding that the 5 markers are indeed downstream targets of STAT1 in ESCC tumors, we performed immunohistochemistry to assess the expression of these proteins and correlated the results with STAT1 expression in a cohort of ESCC primary tumors (n=62). For BCL-2, BCL-xL and survivin, the cytoplasmic immunoreactivity was evaluated and scored. For p21 and cyclin D1, the nuclear staining was evaluated and scored.

Of the 62 cases, BCL-2, BCL-xL and survivin were assessed positive in 24 (38.7%), 48 (77.4%), and 35 (56.5%) cases, respectively. For the two cell-cycle regulators, p21 and cyclin D1, positivity was found in 25 (40.3%) and 43 (69.4%) cases, respectively (**Figure 3.3**). In keeping with our *in-vitro* data, we found a significant inverse correlation between STAT1 and BCL-xL ($r=-0.27$; $p= 0.036$) as well as between STAT1 and survivin ($r=-0.29$; $p=0.025$). Also consistent with our *in vitro* findings, a significant positive correlation was found between STAT1 and p21 expression ($r= 0.55$; $p<0.001$). A trend for a positive correlation between STAT1 and BCL-2 ($p= 0.25$) or cyclin D1 ($p= 0.25$) was found (**Table 3.1**).

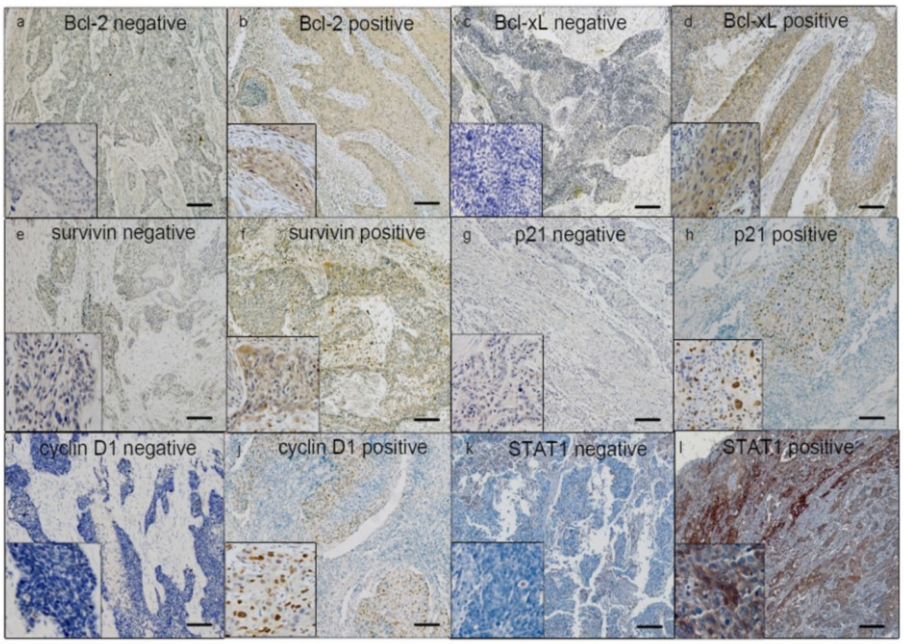

Figure 3.3 Immunohistochemistry for BCL-2, BCL-xL, survivin, p21 and cyclin D1. By immunohistochemistry applied to formalin-fixed paraffin-embedded tissues, all proteins were detectable in most ESCC tumors. The staining was predominantly cytoplasmic for BCL-2, BCL-xL and surviving and nuclear for p21 and cyclin D1. Based on the staining intensity, tumors in our cohort were categorized into positive or negative (a) Cytoplasmic negative expression of BCL-2 (b) Cytoplasmic positive expression of BCL-2 (c) Cytoplasmic negative expression of BCL-xL (d) Cytoplasmic positive expression of BCL-xL (e) Cytoplasmic negative expression of survivin (f) Cytoplasmic positive expression of survivin (g) Nuclear negative staining of p21(h) Nuclear positive staining of p21 (i) Nuclear negative staining of cyclin D1 (j) Nuclear positive staining of cyclin D1(k) Cytoplasmic negative expression of STAT1(l) Cytoplasmic positive expression of STAT1 (IHC stain, scale bar, 20 μm).

Table 3.1 Correlation between BCL-2, BCL-xL, survivin, cyclin D1, p21 and STAT1 protein expression in 62 ESCC samples

Proteins	BCL-2			BCL-xL			Survivin			p21			Cyclin D1		
	N	P	r_s	N	P	r_s	N	P	r_s	N	P	r_s	N	P	r_s
STAT1															
Negative/weak	18	15	-0.148	4	29	-0.267*	10	23	-0.285*	28	5	0.547*	8	25	-0.148
strong	20	9		10	19		17	12		9	20		11	18	

N: negative, P: positive

P value for Pearson's X^2 test; * $p<0.05$

3.4.3 The clinical significance of the 5 markers in ESCC

We then assessed whether the expression of BCL-2, BCL-xL, survivin, p21 and cyclin D1 correlates with various clinical and pathologic parameters, including gender, location and size of the tumor, lymph node metastasis, histologic grade, depth of tumor invasion/ clinical stage and overall survival. As shown in **Table 3.2**, we found that BCL-2 expression significantly correlated with the depth of tumor invasion ($p=0.033$). The expression of survivin and cyclin D1 significantly correlated with lymph node metastasis and clinical stage (both $p<0.05$). The expression of BCL-xL and p21 did not show significant correlation with any of the clinical parameters examined.

3.4.4 The prognostic value of the 5 markers in ESCC

Clinical follow-up data was available for 37 of the 62 patients included in this study, with a median follow-up of 29.0 months (range 1-70 months). Survival data was analyzed using the Kaplan-Meier method. The overall survival of patients with BCL-2-positive tumors (n=9) was nearly twice as long as those with BCL-2-negative tumors (n=28) (44.6 months versus 24.1 months, $p=0.044$). In contrast, the survival of patients with BCL-xL-positive tumors (n=27) was not significantly different from those with BCL-xL-negative tumors (n=10) (29.0 months versus 29.2 months, $p=0.915$). Similarly, the survival in the 14 patients with survivin-positive

Table 3.2 Clinical significance of BCL-2, BCL-xl, survivin, p21 and cyclin D1 expression in ESCC

		Patients		Protein Expression (negative/positive)									
Parameter		N	%	Bcl-2 (38/24)		Bcl-xL (14/48)		Survivin (27/35)		p21 (37/25)		Cyclin D1 (19/43)	
Age	≤58	30	48.4	19/11		8/22		13/17		20/10		6/24	
	>59	32	51.6	19/13	0.749	6/26	0.456	14/18	0.974	17/15	0.277	13/19	0.078
Gender	Male	47	75.8	27/20		9/38		21/26		28/19		14/33	
	Female	15	24.2	11/4	0.271	5/10	0.253	6/9	0.750	9/6	0.977	5/10	0.795
Tumor site	Upper	7	11.3	3/4		2/5		3/4		3/4		0/7	
	Middle	45	72.6	28/17		7/38		18/27		26/19		15/30	
	Lower	10	16.1	7/3	0.512	5/5	0.057	6/4	0.514	8/2	0.272	4/6	0.161
Differentiation	Poor	5	8.1	2/3		0/5		2/3		4/1		2/3	
	Moderate	34	54.8	20/14		8/26		13/21		21/13		14/20	
	Well	23	37.1	16/7	0.426	6/17	0.441	12/11	0.573	12/11	0.482	3/20	0.70
Tumor size	<5 cm	30	48.4	17/13		6/24		13/17		18/12		6/24	
	⩾5 cm	32	51.6	21/11	0.469	8/24	0.638	14/18	0.974	19/13	0.960	13/19	0.078
Depth of invasion	T1-T2	14	22.6	12/2		4/10		9/5		9/5		5/9	
	T3-T4	48	77.4	26/22	0.033*	10/38	0.542	18/30	0.075	28/20	0.690	14/34	0.64
Lymph metastasis	yes	33	53.2	20/13		8/25		9/24		18/15		6/27	
	no	29	46.8	18/11	0.906	6/23	0.739	18/11	0.006*	19/10	0.380	13/16	0.023*
Clinical stage	1–2	28	45.2	18/10		6/22		17/11		19/9		13/15	
	3–4	34	54.8	20/14	0.660	8/26	0.844	10/24	0.013*	18/16	0.233	6/28	0.014*

*p<0.05

Clinical stage is based on the TNM classification for esophageal cancer

Abbreviation: T1: Tumor invades lamina propria, muscularis mucosae, or submucosa; T2: Tumor invades muscularis propria; T3: Tumor invades adventitia; T4: Tumor invades adjacent structures.

tumors was not significantly different from the 23 cases of s urvivin-negative tumors (34.7 months versus 25.6 months, p=0.492). For p21, the overall survival in the positive (n=7) and negative groups (n=30) was 45.2 months and 23.1 months, respectively (**Figure 3.4**), with a significant difference between the two groups (p=0.031). The median survival rate for patients with cyclin D1-positive tumors (n=18; 29.0 months) was similar to that for patients with cyclin D1-negative tumors (n=19, 28.8 months) (p=0.87).

3.5 Discussion

STAT1, which has been reported to have tumor suppressor functions, is known to regulate cellular differentiation and apoptosis through transcription-dependent as well as transcription-independent mechanisms **[7]**. Decreased or loss of STAT1 expression has been observed in many types of cancer such as breast cancer, melanoma and leukemia **[8-11]**. One of our recent studies has revealed evidence that the expression of STAT1 is frequently decreased in ESCC, and this abnormality significantly correlates with a worse clinical outcome **[3]**. Correlating with this clinical observation, our prior *in-vitro* studies using two ESCC cell lines have provided evidence that STAT1 carries tumor suppressor functions in ESCC. Specifically, we found that gene transfection of *STAT1C* into ESCC cell lines effectively induced apoptosis,

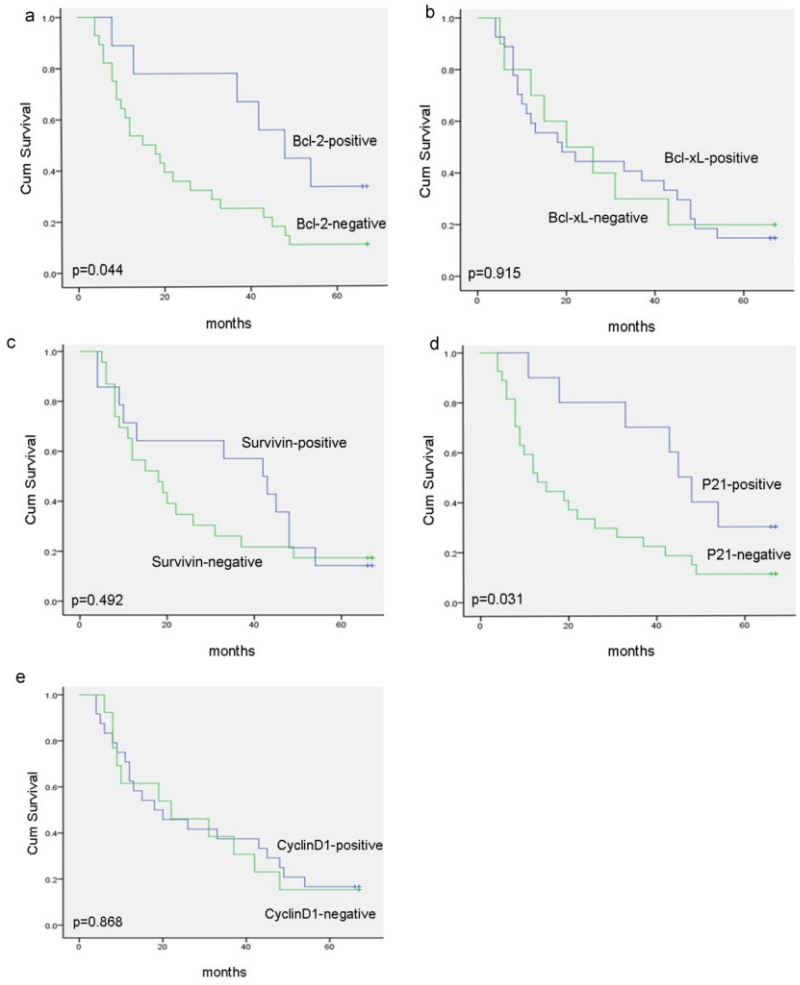

Figure 3.5 Kaplan-Meier curves of esophageal cancer patients in different positive and negative sub-groups. (a) BCL-2-positive patients have longer survival than negative subgroups. (b), (c), and (e) There is no significant correlation between overall survival and the expression level of BCL-xL, survivin or cyclinD1. (d) Significant correlation between overall survival and the expression level of p21 protein levels was found when the two groups were defined as p21-positive or p21-negative.

and this finding was associated with the substantial down-regulation of

several pro-survival proteins, such as BCL-2, BCL-xL and survivin, as well as modulation of two G1 cell-cycle regulatory proteins, $p21^{waf1}$ and cyclin D1.

In previous study, we validated the correlation between STAT1 and these five proteins in 4 ESCC cell lines. Importantly, these correlations hold true in patient samples for p21, BCL-xL and survivin. In addition, a trend was observed between STAT1 and BCL-2, as well as between STAT1 and cyclin D1, and the statistical significance may have been reached if more cases were included.

Of the five markers, two belong to the BCL family, namely BCL-2 and BCL-xL, both of which are known to play important roles in regulating apoptosis in ESCC **[12]**. We found that BCL-2 expression significantly correlated with a long survival in our study. While this finding may appear to be counter-intuitive, we have found another published report describing similar findings in a cohort of ESCC patient samples **[12]**. However, the correlation of BCL-2 expression and the survival of ESCC patients is not without controversy. We are aware of two reports describing that BCL-2 expression in primary-resected ESCC correlates with a worse clinical outcome in these patients **[13, 14].** Furthermore, two other reports suggest that BCL-2 expression is not related to tumor progression nor the prognosis in ESCC **[15, 16].** The reason for these discrepancies is likely

multi-factorial, but it may be related to the fact that caspases can cleave BCL-2 into a pro-apoptotic molecule. Thus, in the presence of caspase activation, BCL-2 is pro-death; in contrast, in the absence of caspase activation, BCL-2 is pro-survival.

BCL-xL has been found to be over-expressed in numerous types of cancer, including myelomas, lymphomas, hepatomas, gastric carcinomas and ovarian cancers **[17-21]**. This over-expression is often associated with decreased apoptosis in tumors, resistance to chemotherapeutic drugs and a poor clinical outcome. Down-regulation of BCL-xL by siRNAs was found to suppress cell growth and induce apoptosis in ESCC cells **[22]**. Moreover, ESCC patients with high BCL-xL expression were found to have a significantly shorter survival than those with low BCL-xL expression level **[23]**. Nonetheless, in this study we found that BCL-xL expression did not correlate with any of the examined clinical parameters or prognosis. Again, the relatively small number of patient samples included in this analysis may have contributed to the lack of statistical significance. Nonetheless, the possibility of technical difference (e.g. the choice of anti-BCL-xL and/or antigen retrieval methods) may have accounted for this discrepancy.

Survivin is a member of the inhibitor-of-apoptosis protein (IAP) family and has been reported to promote cell survival and correlate with a worse

clinical outcome in different types of cancers, such as breast and colorectal cancer **[24]**. Several studies suggest a correlation between the high expression of survivin and a short survival in ESCC patients **[25]**. Interestingly, the subcellular location of survivin appears to be important prognostically in ESCC, since nuclear expression was found to have a negative impact whereas cytoplasmic expression has no prognostic relevance **[26].** In the present study, we found that survivin expression level correlates with lymph node metastasis and late clinical stage, but has no correlation with the overall survival. The lack of prognostic significance is contradictory with the previous findings that over-expression of survivin in ESCC correlates with poor prognosis **[27-29]**. The reasons for this discrepancy may be due to the small sample size and a relatively short follow-up. Further studies with larger sample sizes and longer follow up may help to clarify this issue.

Both cyclin D1 and p21 proteins are involved in regulation of cell-cycle progression. Upregulation of cyclin D1 has been shown to shorten the G1 phase and is linked to development and progression of many types of cancer, such as breast cancer, gastric cancer and mantle cell lymphoma **[30-32]**. P21 is a cyclin-dependent kinase (CDK) inhibitor that directly inhibits the activity of the cyclin D1/CDK4 complex. In ESCC, cyclin D1 expression has been shown to be associated with a worse prognosis **[33]**. However, several studies demonstrated that cyclin D1 is an independent

prognostic factor in ESCC that patients with high cyclin D1 expression have a more favorable prognosis than those with low cyclin D1 expression **[34]**. High expression of p21 has been shown to correlate with better survival and predicted the response to chemoradiotherapy **[35]**. The role of p21 as a tumor suppressor is not without controversy. A previous report showed that p21 expression correlates with a poor clinical outcome in ESCC patients **[36, 37]**. Our study demonstrates that the expression of cyclin D1 is associated with lymph node metastasis and the clinical stage of ESCC, while p21 nuclear staining has no significant correlation with the survival. Although these results may not be persuasive because of the small sample size, several studies on ESCC also support our findings of a lack of significant correlation between p21 expression and different clinicopathological parameters among ESCC patients **[12, 13]**.

However, there are still some limitation in this paper that we didin't have enough information about the pateints' treatment after surgery, such as radiotherapy and chemotherapy. Multivariate survival statistical analysis should be used to eliminate these factors.

3.6 Conclusion

In summary, the present study primarily evaluated the clinical significance of five proteins, including BCL-2, BCL-xL, survivin, p21 and cyclin D1, in a

cohort of ESCC patients, by correlating their expression of these proteins with various clinical and pathological parameters. Our overall results support the concept that STAT1 exerts its tumor suppressor effects in ESCC via its modulation of a host of regulators of apoptosis and cell-cycle progression.

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Chapter 4

ERK promotes proteasomal degradation of STAT1 and high ERK expression correlates with a worse clinical outcome in esophageal squamous cell carcinoma

This chapter has been modified from a manuscript in preparation:

Zhang Y, Yun H, Lai R, Su M. ERK promotes proteasomal degradation of STAT1 and high ERK expression correlates with a worse clinical outcome in esophageal squamous cell carcinoma.

As first author, I designed and performed all the experiments described here, except for the following: Yun H performed some IHC staining shown in Figure **4.13**; I also wrote the manuscript.

4.1 Abstract

We recently reported that STAT1 is a tumor suppressor in esophageal squamous cell carcinoma (ESCC), and patients with $STAT1^{low}$ tumors had a significantly shorter survival. Here, we investigated the mechanism(s) that are responsible for the $STAT1^{low}$ phenotype in ESCC. We found evidence that the ubiquitin-proteasome pathway can efficiently degrade STAT1 in ESCC cells, as MG132 treatment rapidly and dramatically increased STAT1 expression in these cells. This process is not dependent on the phosphorylation of the two important STAT1 residues, Y701 and S727, as site-directed mutagenesis of these two sites did not affect STAT1 degradation. We also found that ERK promoted proteasomal degradation of STAT1, supported by the observations that pharmacologic inhibition of ERK resulted in a substantial increase of STAT1 whereas expression of constitutively active ERK further reduced the STAT1 protein level. In addition to suppressing STAT1 expression, ERK limited STAT1 signaling by decreasing the production of IFNγ. In parallel with our *in-vitro* study results, the expression of ERK inversely correlated with that of STAT1 in a cohort of ESCC tumors (n=131). In 74 patients with follow-up data, those with ERK^{low} tumors survived significantly longer than those with ERK^{high} tumors (p=0.04); patients with $ERK^{low}/STAT1^{high}$ tumors had the longest survival (p=0.001). To conclude, ERK is an effective negative regulator of STAT1 signaling in ESCC, by promoting its proteasomal

degradation and decreasing IFNγ production. Our data suggests that inhibition of ERK and/or restoration of STAT1 expression are useful therapeutic strategies for ESCC.

4.2 Introduction

Signal transducers and activators of transcription (STATs) are critical mediators of cytokine signaling. Upon cytokine stimulation, STATs become phosphorylated by specific receptor kinases, and then subsequently dimerize and translocate into the nucleus where they regulate gene expression **[1]**. STAT1 is an important mediator of gamma-interferon (IFNγ) signaling, which activates STAT1 by promoting its phosphorylation at tyrosine 701 (Y701) that is present in the C-terminal transcriptional activation domain of STAT1 **[2]**. STAT1α also can be phosphorylated at serine 727 (S727) in response to different stimuli or activators, including extracellular regulated protein kinases (ERK), IFNγ, lipopolysaccharide and ultraviolet irradiation **[3-5]**. STAT1 is frequently down-regulated in various types of human cancer, such as breast cancer, head and neck cancer, multiple myeloma and leukemia **[6, 7]**, and these findings are in line with the postulated role of STAT1 as a tumor suppressor.

Esophageal squamous cell carcinoma (ESCC) is the fourth leading cause of cancer deaths and the fifth most common diagnosed cancer in China

[8]. ESCC is particularly prevalent in the Chaoshan area where the age-standardized incidence is approximately 7-fold that of the world population **[9]**. By studying ESCC cell lines and tumor samples collected from the Chaoshan areas, we recently published that STAT1 is a tumor suppressor of ESCC and this protein is frequently down-regulated in ESCC; importantly, the $STAT1^{low}$ phenotype significantly correlates with a worse clinical outcome **[10]**. Nonetheless, the mechanisms underlying the down-regulation of STAT1 in ESCC have not been previously explored. With this background, the main purpose of this study is to delineate the mechanisms by which STAT1 is down-regulated in ESCC. In this study, we identified that ERK is a key regulator of STAT1, by means of promoting its proteasomal degradation and decreasing the production of IFNγ. The importance of ERK/STAT1 axis in ESCC is highlighted by the observation that the $ERK^{high}/STAT1^{low}$ phenotype significantly correlates with a worse clinical outcome in ESCC patients.

4.3 Materials and methods

4.3.1 Cell lines and patient samples

Three human ESCC cell lines, EC1, KYSE150 and KYSE510, were used in this study. They were maintained in RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1 × antibiotic mixture (Invitrogen, Carlsbad, CA, USA). All cells were

cultured at 37°C in a humidified incubator containing 5% CO_2.

We used the same cohort of patients as chapter 1 that 131 consecutive ESCC tumors were randomly collected from Shantou Tumor Hospital between 2005 and 2012. All patients underwent potentially curative surgery without preoperative chemotherapy or radiotherapy. In this cohort, 98 were men and 33 were women; the range of age was 36-78 years, with a median of 57 years. Follow-up data was available for 74 patients; most (58, 78.4%) died during the follow-up period (median survival, 31.4 months). Of the 131 ESCC tumors, 22 case-matched normal esophageal tissues adjacent to the tumors were included in the study. Written informed consents were obtained from patients, and the study was reviewed and approved by the institutional ethics committee.

4.3.2 siRNA, plasmid constructs and drugs

STAT1 siRNA and scramble RNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Transfection of siRNA was performed using Lipofectamine RNAimax (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Plasmids including eGFP-STAT1, eGFP-STAT1Y701F, eGFP-STAT1S727A, Flag-STAT1β were purchased from Addgene. Flag-tagged, constitutively-active STAT1 (or STAT1C) cloned into the backbone of pcDNA3.1 was a gift from Dr. Toru Ouchi (Roswell Park Cancer Institute) **[11]**. The constitutive active

MEK-1 (HA-ca-MEK) vector was a gift from Dr. Nathalie Rivard (Université de Sherbrooke) **[12]**. The proteasome inhibitor N-carbobenzoxyl-L-leucinyl-L-norleucinal (MG132) was purchased from Calbiochem (La Jolla, CA, USA) and 5-Aza-2′-deoxycytidine (5-Aza) was purchased from Sigma (St Louis, MO, USA). Mitogen-activated protein kinase kinase (MEK1) inhibitor U0126 was purchased from Cell Signaling (Danvers, MA, USA).

4.2.3 Co-immunoprecipitation, immunoprecipitation and Western blot analysis

To detect the interaction between ERK and STAT1 and evaluate the ubiquitination of STAT1, whole-cell extracts were prepared by lysing the cells in an immunoprecipitation buffer. A total of 2 μg of anti-STAT1 monoclonal antibody (Santa Cruz Biotechnology) was added to 500 μg of protein lysate isolated in cell lysis buffer (Sigma Aldrich, St Louis, MD, USA) and the samples were rotated overnight at 4 ^{o}C. Subsequently, 30 μl of protein G Plus/A beads (EMD Millipore, Billerica, MA, USA) was added to the samples and rocked overnight at 4^{o}C. The beads were then washed 3 times with cold phosphate-buffered saline followed by the final wash using cold cell lysis buffer or RIPA buffer. Antibodies reactive with human β-actin (1:1000), caspase-9 (1:1000), caspase-3 (1:1000), Bcl-2 (1:1000), Bcl-xL (1:1000), STAT1 (1:1000), phospho-STAT1 serine 727

(or p-STAT1 S727) (1:1000), phospho-STAT1 tyrosine 701 (or p-STAT1 Y701) (1:1000), phospho-ERK (or p-ERK) (1:1000), ERK (1:1000), Ubiquitin (or Ub) (1:1000), phospho-JAK2 (or p-JAK2) (1:1000), JAK2 (1:1000), Human influenza hemagglutinin (or HA) (1:1000) and Poly ADP ribose polymerase (PARP) (1:1000) were purchased from Cell Signaling.

4.3.4 Cell growth and Colony formation assay

To evaluate the effect of MG132 on the growth of ESCC cell lines, cell viability was determined by using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI) and trypan blue assay according to the manufacturer's protocol. Colony formation assay was performed as described previously **[10]**.

4.3.5 Immunohistochemistry (IHC)

IHC staining was performed using the Envision Labeled Peroxidase System (Dako, Carpinteria, CA). Consecutive 4 μm thick slices from each sample were deparaffinized in dimethyl benzene, rehydrated through a graded ethanol series and incubated with fresh 3% H_2O_2 for 10 minutes to quench endogenous peroxidase activity. After a rinse in phosphate-buffered saline (PBS), antigen retrieval involved microwave heating. Following incubation with 10 mmol/l citrate buffer (pH 6.0) for

20min, primary antibodies for STAT1 (Cell Signaling, 1:75), phospho-p44/42 MARP (ERK1/2,D13.14.4E) (Cell Signaling, 1:200) and p44/42 MARP (ERK1/2, 137F5) (Cell Signaling, 1:500) were incubated at 4°C overnight. After washing, corresponding secondary antibody (DAKO) was added for incubation at 37°C for 30 minutes before reaction with diaminobenzidine and counterstaining with haematoxylin. IHC staining was examined by two pathologists who were blinded to the clinical outcome and concordance between two pathologies was guaranteed. For the evaluation of STAT1 immunostaining, both the intensity and percentage of immunostained cells were described previously **[10]**. Tumors were considered $STAT1^{low}$ when they were weak/negative staining, whereas tumors were considered $STAT1^{high}$ if they had a strong staining

Scoring for the p-ERK and ERK immunostaining was performed similarly. Thus, the percentage of positive cells in each case was categorized as: 0 (<5% positive cells), 1+ (6% to 25% positive cells), 2+ (26% to 50% positive cells), 3+ (51% to 75% positive cells) or 4+ (> 75% positive cells). The staining intensity was categorized as 0 to 3+. The final scores were based on the multiplication of the percentage score and intensity score, which ranged from 0 to 12. Tumors were considered ERK^{low} or $p\text{-}ERK^{low}$ when they were assigned a score of <6, whereas tumors were considered ERK^{high} or $p\text{-}ERK^{high}$ if they had a final score of ≥6.

4.3.6 Quantitative RT-PCR

Using the RNeasy Mini Kit (QINGEN, Valencia, CA, USA), total cellular RNA was extracted from cells following the manufacture's protocol. Reverse transcription was performed using 1 μg of total RNA and Superscript reverse transcriptase (Invitrogen). Quantitative PCR was performed using SYBR Green (Invitrogen), and primer sets for IFNγ, IFN receptor and GAPDH were purchased from Invitrogen. Samples were processed on an ABI 9700 HT system (Applied Biosystems Inc., Foster City, CA). Results were analyzed using the SDS 2.2 software, and the relative expression levels of IFNγ were normalized to the GAPDH levels. Primer sequence for IFNγ is: Forward: 5′-TGACCAGAGCATCCAAAAGA-3′, Reverse: 5′-CTCTTCGACCTCGAAACAGC-3′. IFNγ receptor: Forward: 5′-TCTTTGGGTCAGAGTTAAAGCCA-3', reverse: 5′-TTCCATCTCGGCATACAGCAA-3′.

4.3.7 Immunofluorescence and confocal microscopy

Immunofluorescence was performed as previously described **[11]**. Cells were grown on cover slips coated with poly-L-lysine (Sigma Aldrich) in a 6-well plate and fixed with 3% paraformaldehyde in PBS (pH 7.4). Cell were rinsed three times with PBS, permeabilized with Triton X-100, washed again with PBS, and incubated with 200 μL of anti-STAT1 and

ERK antibody (1:50, Sigma Aldrich) overnight at room temperature in a humidified chamber. The cover slips were rinsed three times in PBS and incubated with secondary antibody conjugated with Alexa Fluor 488 or 568(Invitrogen) at a 1:250 dilution for 1 hour at room temperature. After three rinses in PBS, the coverslips were mounted on a slide using the mounting media (Dako). Cells were visualized with a Zeiss LSM 710 confocal microscope at the Core Cell Imaging Facility, Cross Cancer Institute, Alberta, Canada.

4.3.8 Flow cytometry

Apoptotic cell death of saffron was measured using the FITC-conjugated Annexin V/PI assay kit and flow cytometry. After treatment with scramble RNA or siRNA STAT1 for 24 hours, cells were treated with 10 μM MG132 for 24 hours, then collected by trypsinization, washed with ice-cold PBS twice, re-suspended in 500μl binding buffer, and stained with 5 μl of FITC-conjugated Annexin V (10mg/ml) and 5 μl of PI (50mg/ml). Cells were incubated for 5-15 minutes at room temperature in the dark, and analyzed by flow cytometry (Beckman Coulter, USA).

4.3.9 Enzyme-linked immunosorbent assay (ELISA)

The supernatant of cell suspension from harvested cells were collected, the level of IFN-γ was analyzed by ELISA using the commercially

available ELISA kits in a 96-well microplate (IFN-γ Assay kit; eBioscience, San Diego, CA, USA) at optical density of 450 nm, according to the manufacturer's protocal.

4.3.10 Statistical analysis

Data was expressed as mean ± standard deviation. The prognostic significance of the expression of various markers was analyzed using the Kaplan Meier's analysis. The correlation between p-ERK, ERK and other clinical parameters was evaluated using Chi square or Student's t-test. Differences among the treatment groups were assessed using ANOVA and the appropriate statistical software (SPSS, IBM, USA). A p-value of ≤ 0.05 was considered as statistically significant.

4.4 Results

4.4.1 MG132 elevates the protein level of STAT1 in ESCC cell lines

To investigate the mechanism(s) by which STAT1 is down-regulated in ESCC, we first questioned if the STAT1 gene is silenced via gene methylation. Thus, we treated two STAT1 low ESCC cell lines, EC1, KYSE150 and a STAT1high ESCC cell line, KYSE510, with 5-Aza of varying concentrations (1-10 μM) and durations (0-48 hours). To evaluate the efficiency of 5-Aza in ESCC cells, we used SOCS1 as a positive control **[13]**. By Western blots and quantitative RT-PCR, we found that the

protein level of SOCS1 was elevated upon different dose of 5-Aza treatment after 12 hours, indicating that 5-Aza can effectively block gene methylation in ESCC as reported in the previous paper **[13]**. However, we did not find any appreciable change in STAT1 expression nor phospho (p)-STAT1, suggesting that gene methylation does not play a role in suppressing STAT1 expression in ESCC **(Figure 4.1)**.

In view of one previous report that STAT1 can be degraded via the ubiquitin-proteosome pathway in mouse embryonic fibroblasts **[14]**, we tested if this mechanism contributes to the low expression level of STAT1 in ESCC. Thus, we treated EC1, KYSE150 and KYSE510 with varying concentrations (1-10 μM) of MG132 for 24 hours. By western blots, we found that the protein level of STAT1 was dramatically elevated in a dose-dependent manner, with STAT1 being detectable in both cell lines at a MG132 concentration as low as 1 μM **(Figure 4.2A)**. Furthermore, the STAT1 protein level increased in a time-dependent manner, with STAT1 being detectable as early as 3 hours (**Figure 4.2B**). With the exception of EC1, a cell line that did not express p-STAT1^{S727}, the STAT1 phosphorylation level at Y701 and S727 generally increased in parallel with the total protein level of STAT1. These findings suggest that there are mechanism(s) that constitutively activate STAT1 in ESCC cells at steady state. In support of this concept, transfection of *STAT1* into KYSE150 and KYSE510 led to the expression of p-STAT1 at Y701 and S727; EC1

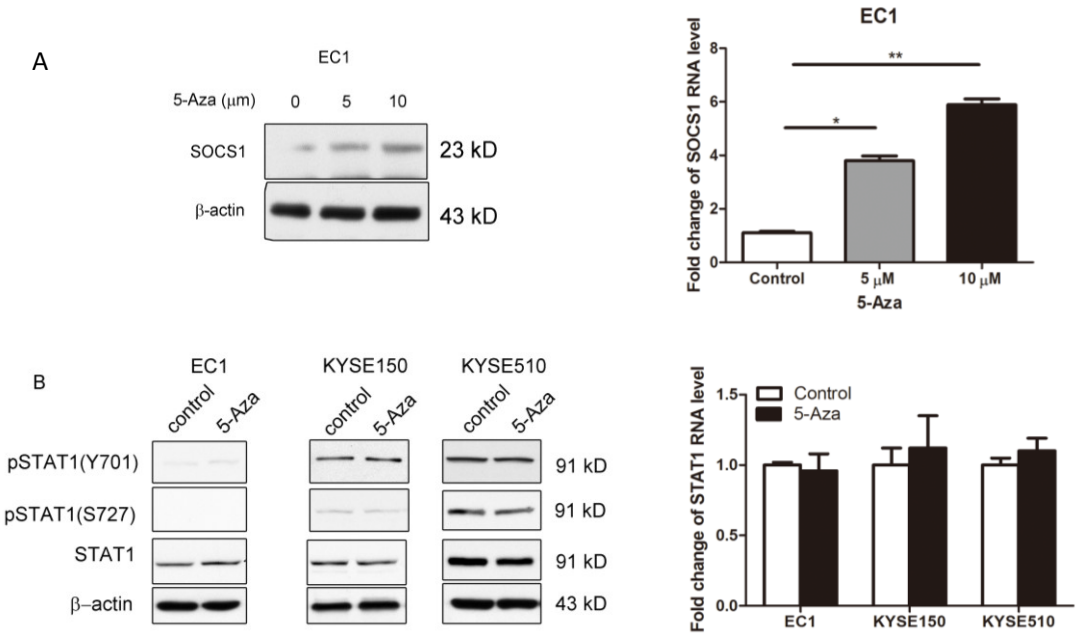

Figure 4.1 Expression of p-STAT1 and STAT1 has no change via 5-Aza in ESCC. A. ESCC cell line, EC1 was treated with different dose of 5-Aza for 24 hours, the realtime PCR and Westernblot were performed. The expression of SOSC1 was elevated upon 5-Aza treatment, indicating 5-Aza can effectively block gene methylation in ESCC. B. EC1 and KYSE150 cell lines were treated with 10 μM of 5-Aza for 24 hours, the expression of STAT1 was analyzed by realtime PCR and Western blot analysis. Similar results were observed in three independent experiments. (*p<0.05, **p<0.01)

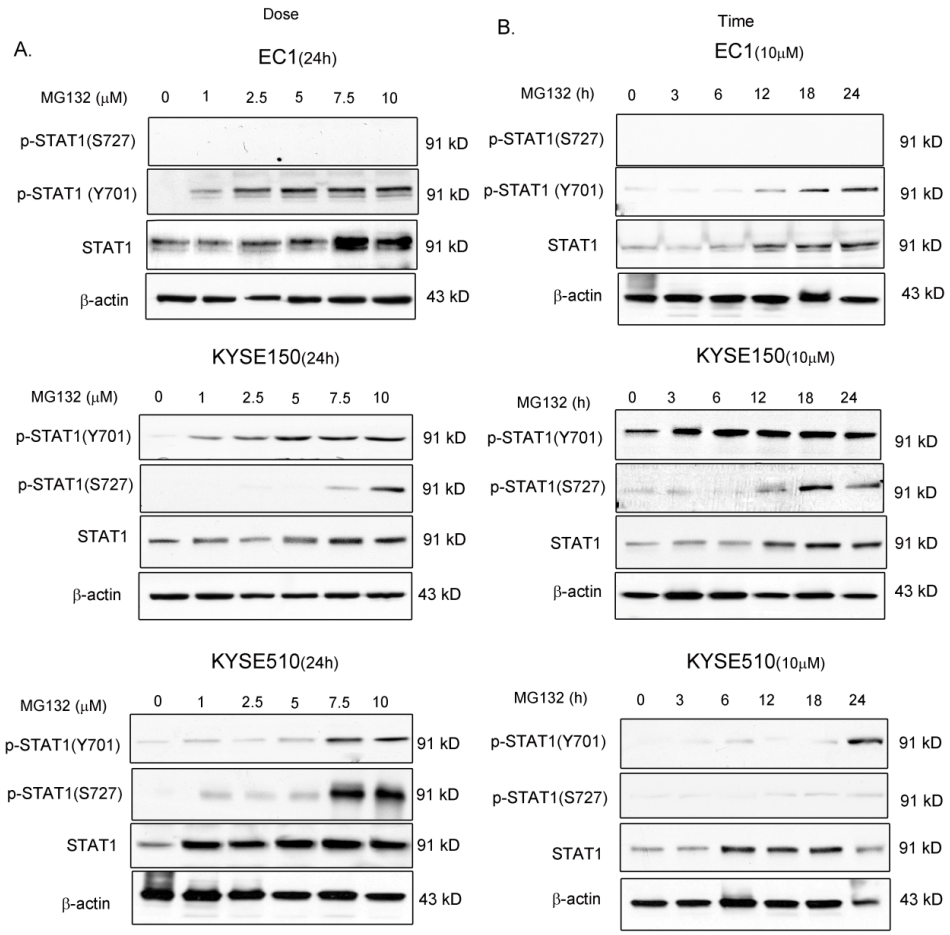

Figure 4.2 MG132 elevates protein level of p-STAT1 and STAT1 in ESCC cell lines. Western blot analysis demonstrates that the dose-dependent and time-dependent elevation of STAT1 induced by MG132. A. EC1, KYSE150 and KYSE510 cell lines were treated in the presence of 0-10 μM MG132 for 24 h. Total cell lysates were then prepared for immunoblot detection of p-STAT1, STAT1 and β-actin. B. Time course of MG132 effects on p-STAT1 and STAT1 protein levels. Both cell lines were treated with 10 μM MG132 for the indicated times, and cells were harvested for immunoblot analysis. Similar results were observed in three independent experiments.

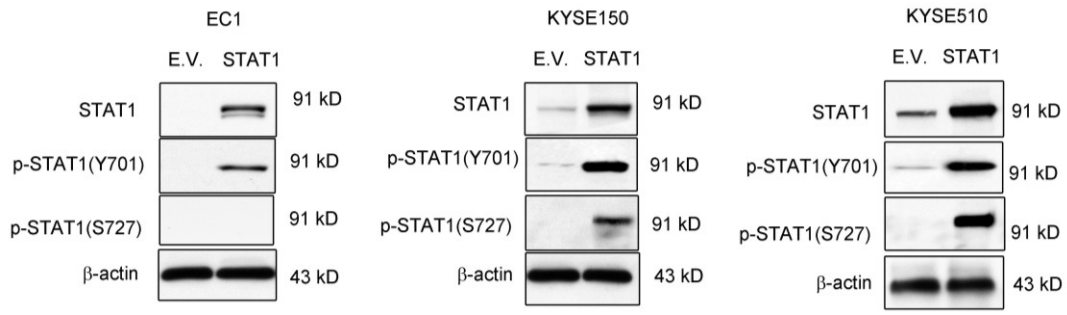

Figure 4.3 STAT1 transfection evelates protein level of p-STAT1 and STAT1. Using Western blot analysis, after 2 days of the STAT1 transfection in EC1, KYSE150 and KYSE510 cells, the levels of STAT1, p-STAT1 were dramatically increased. Similar results were observed in three independent experiments. Similar results were observed in three independent experiments. (E.V.: Empty vector)

remained to be negative for p-STAT1^{S727}, suggesting a difference in the constitutive STAT1 activating mechanism(s) in EC1 as compared to theother two ESCC cell lines (**Figure 4.3**).

4.4.2 ERK promotes proteasomal degradation of STAT1

In mouse embryonic fibroblasts, it has been described that ERK mediates phosphorylation of STAT1 at the serine 727 residue and increases proteasomal degradation of STAT1 **[14]**. Thus, we asked if the proteasomal degradation of STAT1 in ESCC is also regulated by the ERK signaling pathway. In keeping with this hypothesis, treatment of EC1 and KYSE150 with varying concentrations (0-10 μM) of U0126, a MEK/ERK inhibitor, increased the levels of STAT1 and p-STAT1 in a dose-dependent manner (**Figure 4.4A).** At the same time, the level of p-ERK decreased while the total ERK protein level was unchanged. As shown in **Figure 4.4B**, transfection of the HA-tagged, constitutively active MEK/ERK plasmid resulted in further reduction of STAT1 and p-STAT1 in EC1 and KYSE510. Since EC1 expresses a relatively low level of STAT1 at the steady state, the western blots were subjected to a longer exposure to reveal the reduction of STAT1 and p-STAT1. Immunoprecipitation and western blots were performed to detect STAT1 ubiquitination in both EC1 and KYSE 150 cells. As shown in **Figure 4.5A**, STAT1 ubiquitination was decreased in the presence of U0126 compared to the negative controls.

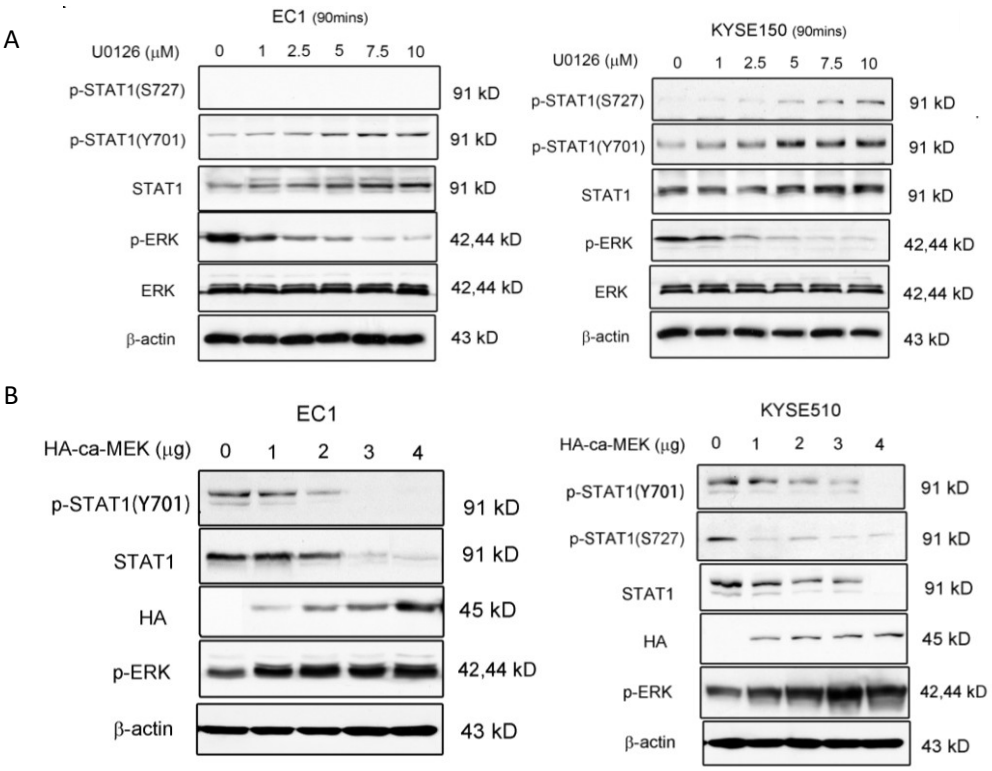

Figure 4.4 ERK activation promotes STAT1 degradation. A. EC1 and KYSE150 cell lines were treated with increasing doses of U0126 for 90 min. Western blot analysis of p-STAT1, STAT1, p-ERK and ERK in total cell lysates are shown. B. EC1 and KYSE510 cells were transfected with increasing amounts of a constitutive-active MEK expression (HA-ca-MEK) plasmid, and endogenous protein levels of p-STAT1 and STAT1 in the lysates were determined by immunoblotting. Similar results were observed in three independent experiments.

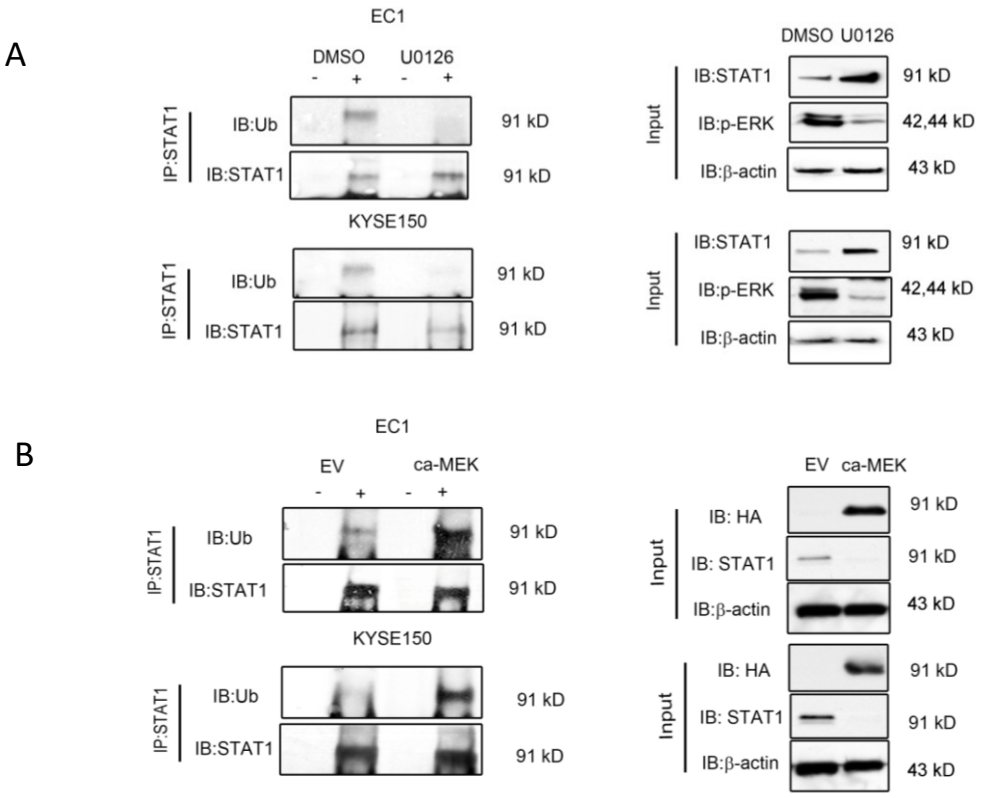

Figure 4.5 STAT1 ubiquitination is detected upon U0126 treatment and MEK transfection. A. Immunoprecipitation - immunoblotting analysis were performed to detect ubiquitination in EC1 and KYSE150 cells treated with or without U0126 for 2 h. B. HA-ca-MEK plasmid was transfected into both cell lines together with empty vector. STAT1 ubiquitination was detected by immunoprecipitation with anti-STAT1 antibody and immunoblotting with anti-Ub antibody. Similar results were observed in three independent experiments. (E.V.: empty vector)

Moreover, transfection of the constitutively-activated MEK1 plasmid increased STAT1 polyubiquitination, compared with the empty vector **(Figure 4.5B)**. Taken together, these results showed that ERK activation promotes polyubiquitination and proteasomal degradation of STAT1. To further investigate the mechanism underlying ERK-mediated STAT1 proteasomal degradation, we examined whether ERK binds to STAT1. Co-immunoprecipitation experiments were performed using EC1 and KYSE150 cells, with or without MG132 treatment. As shown in **Figure 4.6A**, immunoprecipitation of ERK pulled down STAT1 in both cell lines, and MG132 treatment appreciably increased the amount of STAT1 bound to ERK, probably due to the fact that the total amount of STAT1 increased in response to proteasomal inhibition by MG132. Reciprocal pull-down experiments showed essentially the same results, except that we did not see a MG132-induced increase in the amount of ERK bound to STAT1, probably due to the fact that the protein level of 'bioavailable' STAT1 was substantially lower than that of ERK. Moreover, the co-localization of ERK and STAT1 was confirmed by confocal microscopy (**Figure 4.6B**). To determine whether the phosphorylation status of STAT1 is required for the ERK-STAT1 interaction in ESCC cells, we transfected EC1 cells with wild-type STAT1, STAT1^{Y701F} or STAT1^{S727A}. All of the plasmids used were GFP-tagged. Results are illustrated in **Figure 4.6C**. ERK was pulled down along with STAT1 as well as two mutants of STAT1phosphorylation

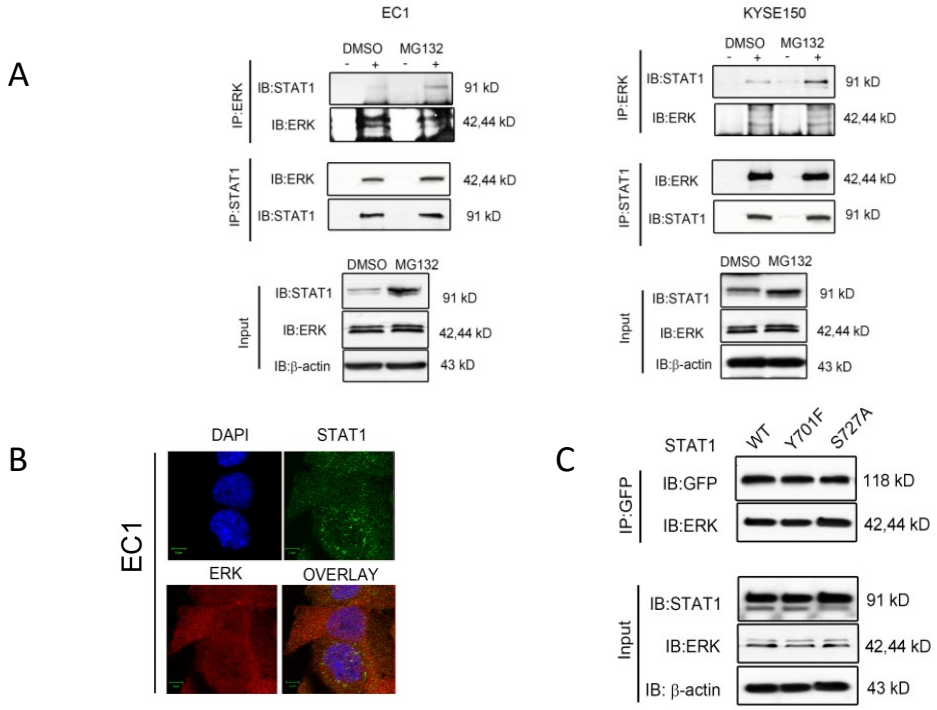

Figure 4.6 ERK binds to STAT1 independent of phosphorylation sites. A. The interaction of endogenous ERK and STAT1 was investigated by co-immunoprecipitation-immunoblot analysis in EC1 and KYSE150 cell lines with or without MG132 treatment. Co-immunoprecipitation was carried out with control IgG and anti-STAT1, or anti-ERK antibodies as indicated. Immunoprecipitated proteins were then analyzed by Western blot with anti-ERK and anti-STAT1 antibodies, respectively. B. Co-localization of ERK and STAT1 *in vitro*. EC1 cells were placed on coverslips and stained with the indicated antibodies (scale bar, 5 μM). C. GFP-STAT1 (WT), GFP-STAT1 (Y701F), or GFP-STAT1 (S727A) were transfected into EC1 cells. Co-immunoprecipitation was performed using GFP antibody and immunoblotting with ERK antibody.

sites at the residue Y701 or S727, two sites known to be important for the function of STAT1 **[15].**

4.4.3 ERK promotes polyubiquitination of STAT1 independent of STAT1 phosphorylation

As mentioned, one previous study using mouse embryonic fibroblasts has shown that ERK phosphorylates STAT1 at S727 and targets it for proteasomal degradation **[14]**. To determine whether the two phosphorylation sites of STAT1 (Y701 and S727) contribute to STAT1 polyubiquitination, we transfected plasmids encoding GFP-tagged STAT1 or the two STAT1 mutants (S727A and Y701F) into the EC1 cells, and the results are illustrated in **Figure 4.7A**. As expected, treatment of U0126 effectively decreased the amount of p-ERK, and this change correlated with a reciprocal increase in GFP and STAT1 proteins. The levels of both $STAT1^{S727A}$ and $STAT1^{Y701F}$ increased in response to U0126 treatments. In addition, silencing of ERK by inhibitor U0126 decreased the polyubiquitination of STAT1, either in the form of wild-type and mutants (**Figure 4.7B**). These data suggests that the degradation of STAT1 mediated by ERK is independent of the phosphorylation at Y701 or S727. These findings are in parallel with the co-immunoprecipitation experiments in which we showed that ERK can bind to the two STAT1 mutants.

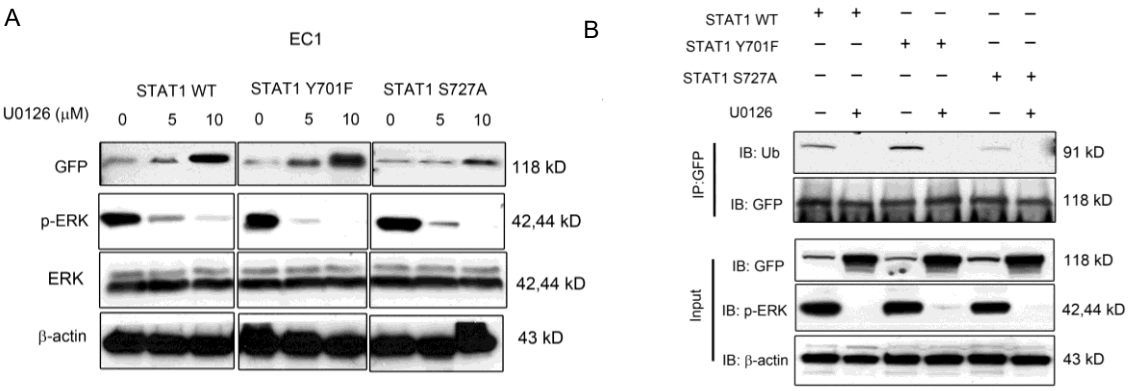

Figure 4.7 U0126 elevates STAT1 protein level and decreases STAT1 ubiquitination independent of phosphorylation sites. A.GFP-STAT1 (WT), GFP-STAT1 (Y701F), or GFP-STAT1 (S727A) were transfected into EC1 cells together with increasing amounts of U0126. The protein level of GFP in the lysates was determined by Western blot. Similar results were observed in three independent experiments. B. STAT1 ubiquitination was detected by immunoprecipitation with anti-GFP antibody and immunoblotting with anti-Ub antibody. Similar results were observed in three independent experiments. The CO-IP bands (IB: Ub and IB: GFP) were obtained from same cell lysis but two different gels.

STAT1β is a C-terminal-truncated version of STAT1 that lacks S727, but retains Y701 **[15]**. To further support the concept that phosphorylation of $STAT1^{S727}$ is not required for ERK-mediated STAT1 proteasomal degradation, we transfected Flag-tagged STAT1α and STAT1β plasmids into EC1 and KYSE150 cells with or without U0126. As shown in **Figure 4.8A**, we found that U0126 can increase both exogenous STAT1α and STAT1β in both cell lines; in addition, ERK can promote the polyubiquitination of both STAT1α and STAT1β **(Figure 4.8B)**. These results further support that active ERK can mediate STAT1 degradation that is independent of the phosphorylation of S727.

4.4.4 ERK is a negative feedback regulator for IFN-γ/STAT1 signaling

The ERK signalling pathway has been reported to play a crucial role in IFN-γ/STAT1 signaling in human macrophages **[16]**. We hypothesize that, in addition to decreasing STAT1 expression, ERK may further down-regulate STAT1 signaling by suppressing IFN-γ production. Our data is in support of this concept. Using quantitative RT-PCR, we found that inhibition of ERK by U0126 significantly increased the expression of IFN-γ and IFN-γ receptor in both ESCC cell lines **(Figure 4.9A)**. As shown in **Figure 4.9B**, similar results were found when we performed the ELISA to detect the active form of IFN-γ. As shown in **Figure 4.9C**, we also found evidence that IFN-γ up-regulates p-ERK, which appears to

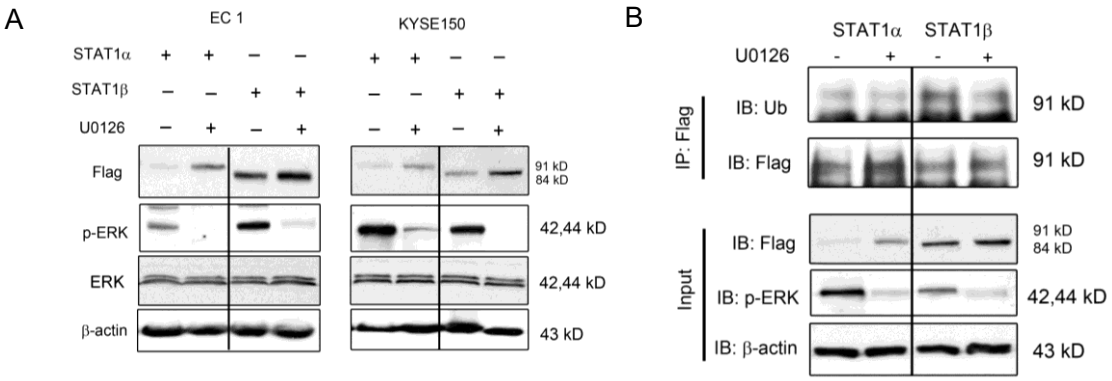

Figure 4.8 ERK promoted polyubiquitination of STAT1 independent of STAT1 serine 727 phosphorylation. A. Flag-STAT1α and Flag-STAT1β were transfected into EC1and KYSE150 together with or without U0126. The protein level of Flag in the lysates was determined by Western blot. B. Flag-STAT1α and Flag-STAT1β were transfected into EC1 with or without U0126.STAT1 ubiquitination was detected by immunoprecipitation with anti-Flag antibody and immunoblotting with anti-Ub antibody. Similar results were observed in three independent experiments.

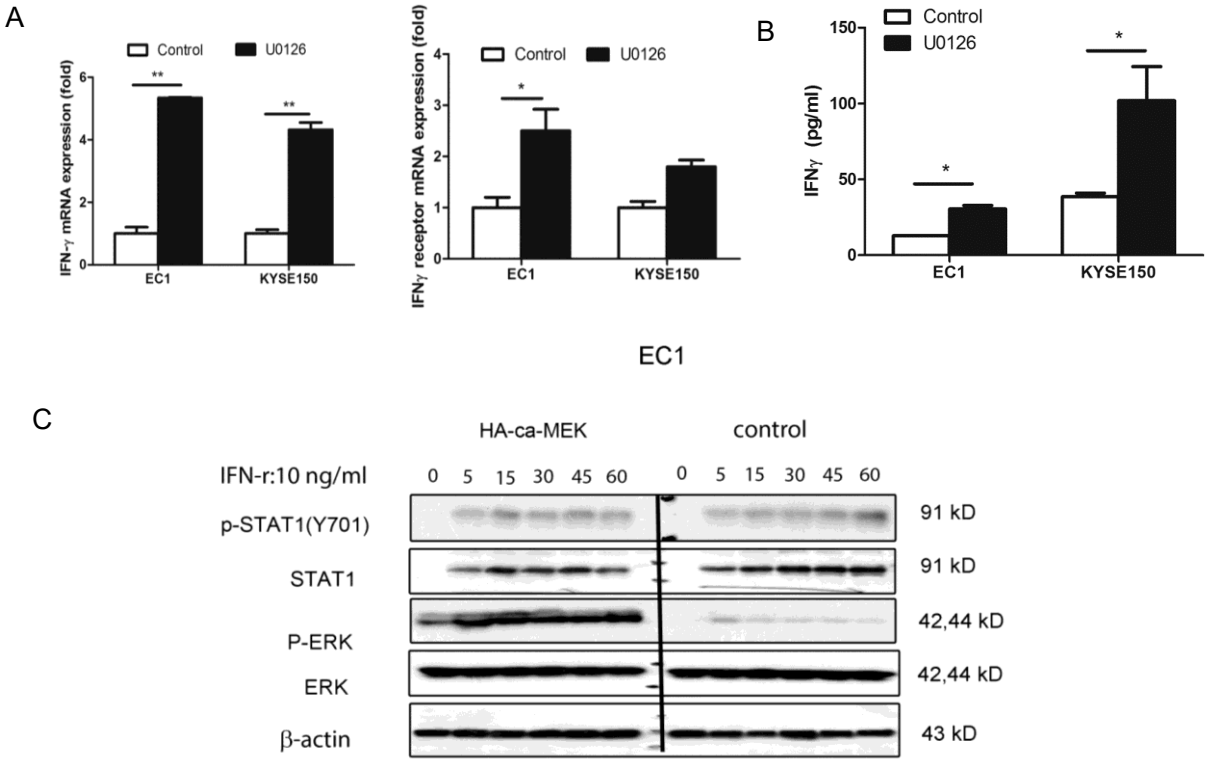

Figure 4.9 ERK is a negative feedback regulator for IFN-γ/STAT1 signaling. A. ESCC cell lines EC1 and KYSE150 were treated with U0126 for 3 hours, the IFN-γ and the receptor mRNA level was detected by qRT-PCR. B. ESCC cell lines EC1 and KYSE150 were treated with U0126 for 3 hours, the active form of IFN-γ was detected by ELISA. C. ESCC cell lines EC1 transfected with HA-ca-MEK or empty vector were treated with IFN-γ (10 ng/ml) for 0-60 minutes. The expression of p-STAT1, STAT1 was measured by Western blot. Similar results were observed in three independent experiments.

serve as a gatekeeper to prevent over-stimulation of STAT1, which is known to provide potent pro-apoptotic signal in ESCC cells **[10]**. To demonstrate the effectiveness of ERK in blocking IFN-γ-mediated activation of STAT1, we transfected ESCC cells with the constitutively active MEK vector before the addition of IFN-γ to the cell culture. As shown in **Figure 4.9C**, the expression of constitutively active ERK substantially dampened the up-regulation of p-STAT1 induced by IFN-γ. Moreover, we performed the colony formation to evaluate the biological effect of ERK on STAT1 in both cell lines. As shown in **Figure 4.10**, U0126 significantly diminished the clonogenic ability of STAT1 siRNA knockdown and constitutively-active MEK significantly diminished the anti-clonogenic ability of STAT1C ($p<0.05$). These data suggested that ERK could block the STAT1 function.

4.4.5 MG132-induced apoptosis in ESCC is STAT1-dependent

The observations that MG132 can induce effective apoptosis in various types of cancer, including ESCC, have been widely published **[17, 18]**. In view of our finding that STAT1 can induce effective apoptosis in ESCC **[10]**, and our current observations that proteasome degradation is a key pathway to down-regulate STAT1, we hypothesized that MG132 induces apoptosis in ESCC via a STAT1-dependent pathway. First, we confirmed that MG132 effectively induced apoptosis in ESCC cells, as evidenced by

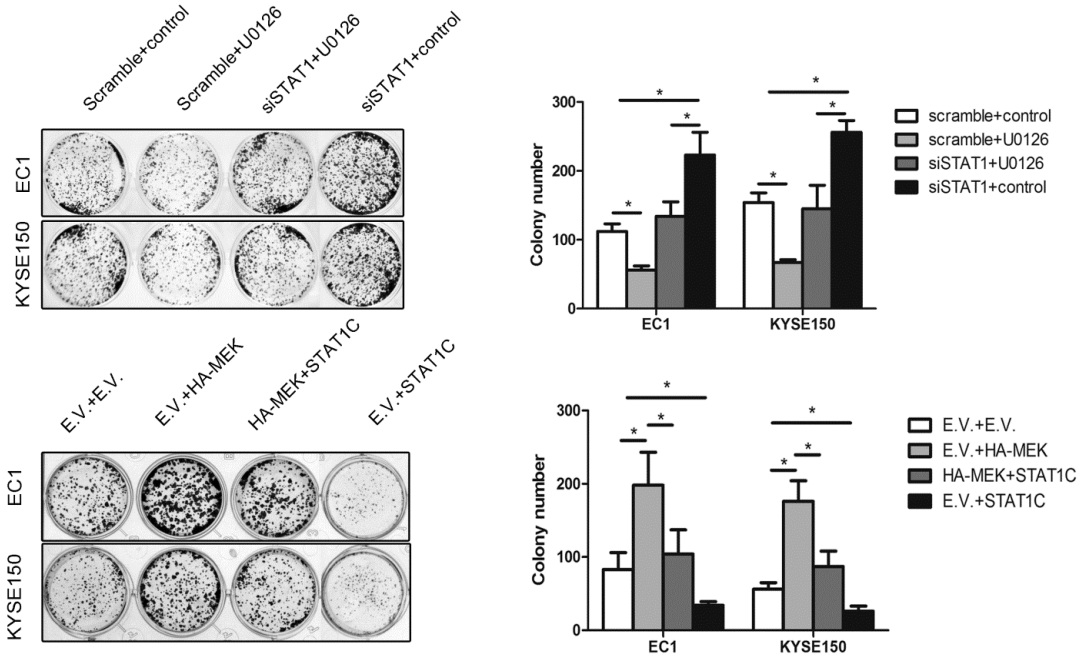

Figure 4.10 Functional studies of ERK/STAT1. The function study of ERK/STAT1 correlation is detected by using colony formation assay. We treated the cells with STAT1 siRNA transfection and U0126; similarly, we transfected of STAT1C and constitutive active MEK together to detect the ESCC colony formation ability. Similar results were observed in three independent experiments. (*p<0.05; **p<0.01)

the reduction of viable cells as well as enhanced the cleavage of caspase 3 and PARP expression in dose and time dependent manner of MG132treatment (**Figure 4.11**). Second, we found that siRNA knockdown of STAT1 significantly attenuated MG132-induced cell inhibition (at 5 μM) in EC1 and KYSE150 cells (**Figure 4.12A and B**), and these findings correlated with the lack of change in caspase 3, PARP and Annexin-V/propodium iodide (**Figure 4.12C and D**). Taken together, these observations suggest that MG132-induced apoptosis in ESCC is highly STAT1-dependent.

4.4.6 STAT1 inversely correlates with ERK/p-ERK in ESCC tumors

Results from our *in-vitro* studies led us to hypothesize that the expression of ERK/p-ERK may inversely correlate with that of STAT1 in ESCC tumors. Using immunohistochemistry applied to serial section of paraffin-embedded tumor samples, we assessed the expression of ERK, p-ERK and STAT1 in 131 tumors. The expression of these three markers detected by immunohistochemistry is illustrated in **Figure 4.13**. For p-ERK, 66 (50.4%) tumors were assessed high and 65 (49.6%) were low. For ERK, 91 (69.5%) tumors were high and 40 (30.5%) tumors were low. For STAT1, 64 (48.9%) were assessed high and 67 (51.1%) were low. As shown in Table 4.1, STAT1 inversely correlated with p-ERK and ERK ($p=0.015$ and $p=0.001$, respectively, Fisher‘s exact test). Analysis of our

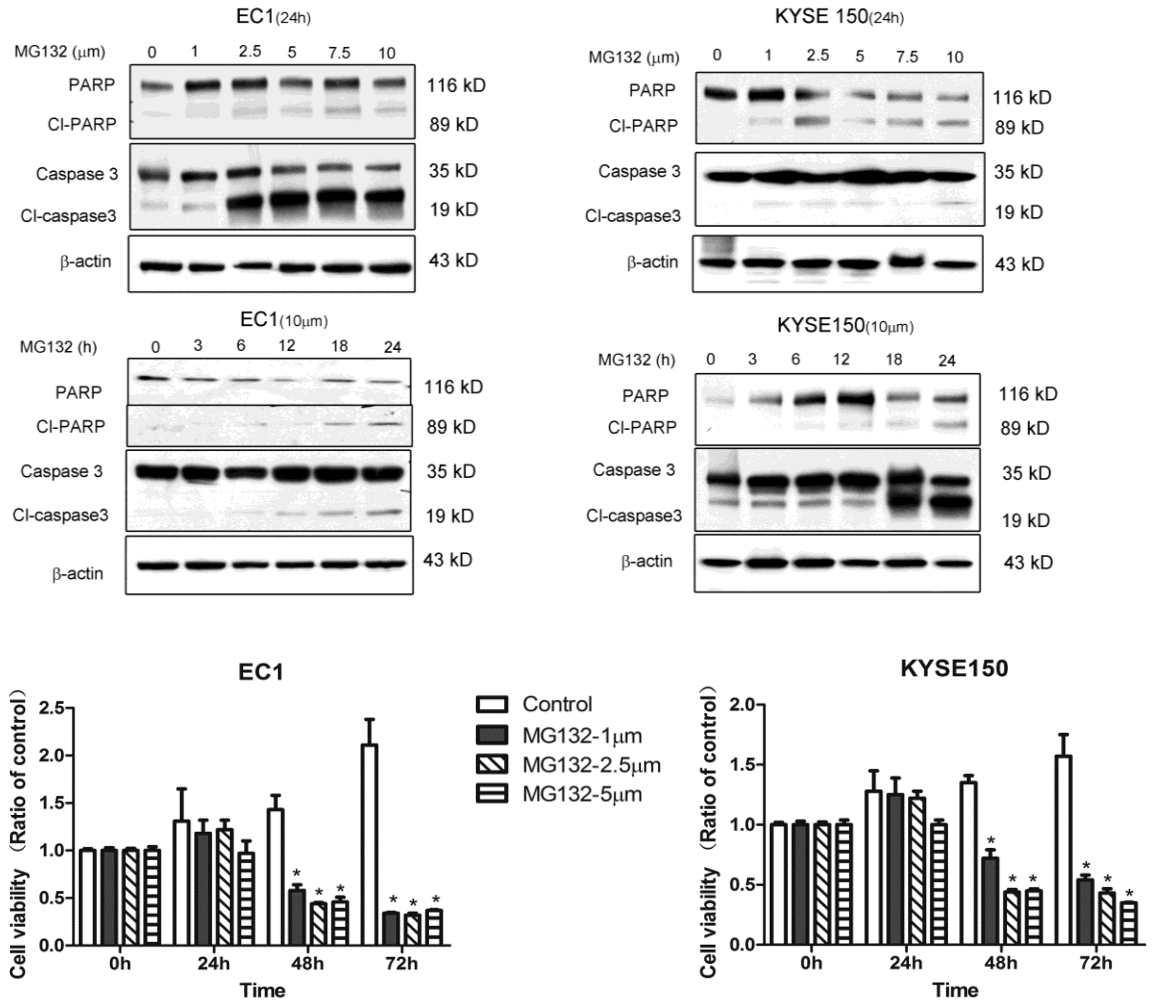

Figure 4.11 MG132 induces cell apoptosis in ESCC. A. Western blot analysis shows that MG132 treatment induces cleavage caspases 3 and PARP expression in both ESCC cell lines in a time- and dose-dependent manner. B. Cell viability assay by MTS assay indicates that MG132 concentrations exceeding 1-5 μM induce cell death in both EC1and KYSE150 cell lines. Values are expressed as means ± SD of three independent experiments. (*p<0.05)

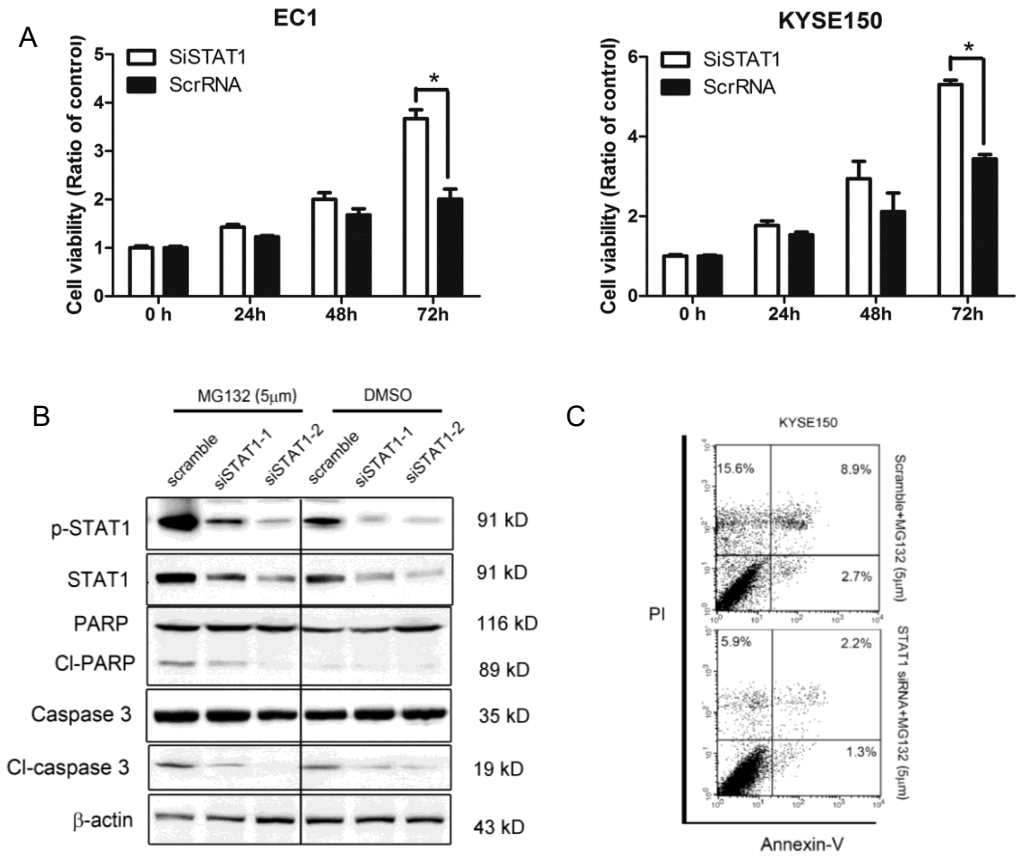

Figure 4.12 Silencing of STAT1 protein expression by siRNA attenuated the MG132-induced cell apoptosis. A. Cell viability assay using MTS shows that STAT1 siRNA attenuates 5 μM MG132-induced cell death in both EC1 and KYSE150 cell lines. STAT1 siRNA (siRNA) versus scrambled RNA (Scr RNA). B. Western blot analysis indicates that STAT1 siRNA reduces the expression of STAT1 and decreases 5 μM MG132-induced cleavages of PARP and caspase 3 compared to the scrambled RNA in the KYSE150 cell line. C. Apoptosis detected by flow cytometry also showed that STAT1 siRNA attenuates 5 μM MG132-induced cell death in KYSE150. Values are expressed as means ± SD of three independent experiments. (*p< 0.05)

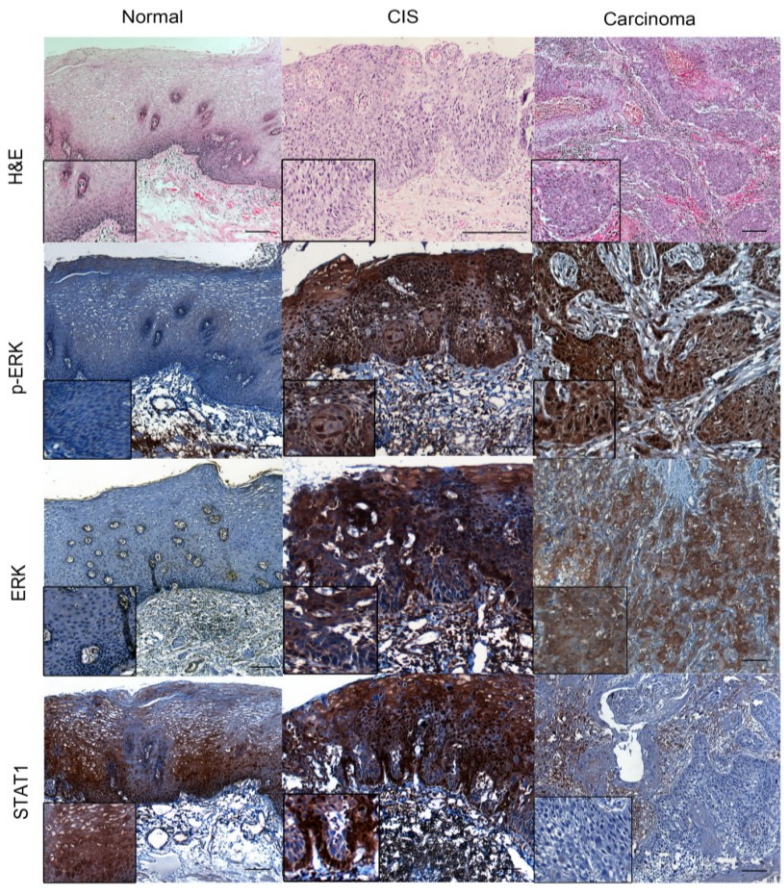

Figure 4.13 p-ERK, ERK and STAT1 expression in normal epithelial, carcinoma in situ (CIS) and ESCC patient samples. The esophageal normal epithelial, carcinoma in situ and carcinoma tissues were stained by Hematoxylin and eosin (H&E) and immunohistochemistry, variable levels of p-ERK, ERK and STAT1 were detected in normal esophageal, CIS and ESCC tumors examined. (IHC stain, scale bar, 50 μm).

Table 4.1 The correlation between STAT1 and p-ERK, ERK expression in ESCC

STAT1 expression level	p-ERK expression level			
	High	Low	Total	
High	25	39	64	
Low	41	26	67	
Total	66	65	131	P=0.015*

STAT1 expression level	ERK expression level			
	High	Low	Total	
High	34	30	64	
Low	57	10	67	
Total	91	40	131	P=0.001*

*p<0.05

Table 4.2 Correlations between p-ERK and ERK expression and various clinic pathologic parameters in 131 ESCC patients

Parameter		Case NO.	low/ high expression			
			p-ERK		ERK	
Age	**≤57**	66	38/28		20/46	
	>58	65	27/38		20/45	
Gender	**Male**	98	48/50		31/67	
	Female	33	17/16		9/24	
Tumor site	**Upper**	13	4/9		3/10	
	Middle	104	54/50		33/71	
	Lower	14	7/7		4/10	
Differentiation	**Poor**	12	4/8	*0.04	2/10	*0.012
	Intermediate	75	37/38		20/55	
	Well	44	30/14		18/16	
Tumor size	**>5cm**	82	40/42		25/57	
	<5cm	49	25/24		15/34	
Depth of invasion	**T1-T2**	103	55/48		28/75	
	T3-T4	28	10/18		12/16	
Lymph metastasis	**Yes**	68	31/37		18/50	
	No	63	34/29		22/41	
Clinical Stage#	**1**	6	4/2	*0.02	5/1	*0.031
	2	55	35/20		17/38	
	3	64	25/39		17/47	
	4	6	1/5		1/5	

*p<0.05

#**Clinical stage** is based on the TNM classification for esophageal cancer

Abbreviation: T1: Tumor invades lamina propria, muscularis mucosae, or submucosa; T2: Tumor invades muscularis propria; T3: Tumor invades adventitia; T4: Tumor invades adjacent structures.

This cohort of patients is the same as the patient cohort in Chapter 1.

data also indicated a significant correlation between ERK expression and p-ERK expression (Spearman coefficient, r=0.476; p=0.001).

4.4.7 The clinical significance of p-ERK/ERK expression in ESCC

In our previous study, we found that the expression of STAT1 is prognostically significant **[10]**. As summarized in **Table 4.2**, we correlated p-ERK/ERK expression with various clinical and pathologic parameters and found that both p-ERK and ERK significantly correlated with the clinical stage (p=0.02 and p=0.03, respectively, Chi square) as well as the histologic grade (p=0.04 and p=0.01, respectively, Fisher's exact test). We further performed the multivariate analysis to investigate the relevant prognostic factors of ESCC, as shown in **Table 4.3**, the poor differentiation and high p-ERK staining is considered as an independent risk factor that associated with worse outcome of ESCC patients (Relative risk=1.45 and 2.27, separately). The survival results are illustrated in **Figure 4.14A-B**. Patients with p-ERK^{low} or ERK^{low} tumors survived significantly longer that those with p-ERK^{high} or ERK^{high} tumors (39.5 months versus 29.0 months, p=0.04, 41.2 versus 28.9 months, p=0.03). We then analyzed the survival of these 74 patients categorized into 4 groups based on the differential expression of p-ERK, ERK and STAT1. As shown in **Figure 4.14C-F**, patients with tumors that are p-ERK^{low} and STAT1^{high} expression survived significantly longer than the other 3 groups

Table 4.3 Univariate and multivariate Cox proportional hazard analyses for cancer-specific survival

	Univariate		Multivariate	
	HR(95%CI)	**P value**	**HR(95%CI)**	**P value**
Age, years (<57 vs. >58)	0.94(0.40-2.23)	0.19		
Gender (male vs. female)	0.75(0.39-1.44)	0.32		
Tumor site (middle vs. others)	0.89(0.25-3.21)	0.50		
Differentiation (poor vs. others)	3.82(1.5-6.9)	0.001	1.45(0.45-3.43)	0.01*
Tumor size (>5cm vs. <5cm)	1.32(0.75-2.70)	0.03	0.78(0.45-1.46)	0.53
Depth of invasion (T1-2 vs. T3-4)	0.51(0.05-5.17)	0.10		
Lymph node metastasis (yes vs. no)	1.40(0.78-3.54)	0.02	0.65(0.34-1.33)	0.43
Clinical Stage (1,2 vs. 3,4)	0.31(0.24-1.83)	0.36		
pERK expression (High vs. low)	4.45(2.96-6.75)	0.001	2.27(1.67-4.32)	0.02*
ERK expression (High vs. low)	0.95(0.87-2.54)	0.24		
STAT1 expression (low vs. High)	1.43(0.53-3.43)	0.05	0.88(0.44-1.21)	0.23

*p<0.05

#**Clinical stage** is based on the TNM classification for esophageal cancer

Abbreviation: T1: Tumor invades lamina propria, muscularis mucosae, or submucosa; T2: Tumor invades muscularis propria; T3: Tumor invades adventitia; T4: Tumor invades adjacent structures.

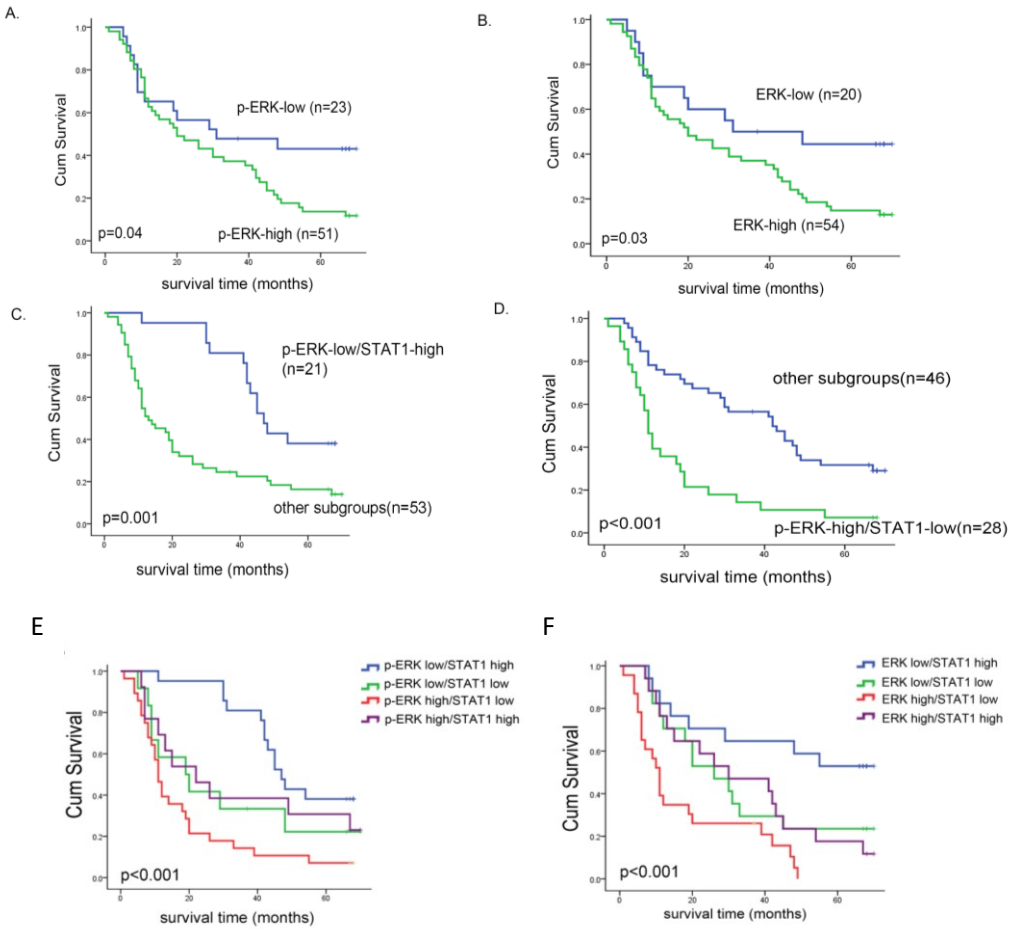

Figure 4.14 Kaplan-Meier curves of esophageal cancer patients in different p-ERK, ERK and STAT1 sub-groups. A-B. By Kaplan-Meier analysis, significant correlation between overall survival and the expression level of p-ERK or ERK was found, when the two groups were defined as high and low. C-F. By Kaplan-Meier analysis, significant correlation between overall survival and the expression level of p-ERK/ERK and STAT1 was found, when patients were divided into four sub groups based on different expression level of p-ERK/ERK and STAT1.

(50.1 months versus 21.4 months, p=0.001); patients with tumors that were p-ERK^{high}/STAT1^{low} carried the worst prognosis. Similar results were obtained when ERK was used instead of p-ERK.

4.4.8 The up-regulation of p-ERK/ERK and down-regulation of STAT1 are implicated in cancer progression of ESCC

To identify if the up-regulation of p-ERK/ERK and the down-regulation of STAT1 are implicated in ESCC cancer progression, we assessed the expression of these three markers in 22 cases of benign esophageal epithelial tissues adjacent to ESCC and 12 carcinoma *in situ* samples using immunohistochemistry. As illustrated in **Figure 4.15**, the expression of p-ERK as well as ERK were significantly lower in benign epithelial tissues (IS: 0.49 ± 0.21), as compared to CIS (IS: 5.47 ± 0.32, p<0.001). The expression of p-ERK in ESCC (n=131) was also higher than that of CIS tissues, although the difference did not reach statistical significance (p=0.10). Similar pattern was found with ERK expression, with gradual decrease from ESCC, CIS to benign esophageal tissues (IS: 7.59 ± 0.26 versus 3.83 ± 0.49 versus 0.52 ± 0.16; p<0.001). Lastly, the expression of STAT1 was significantly higher in benign esophageal tissues (IS: 194.10 ± 11.93) as compared to CIS (IS: 160 ±15.84) (p=0.04), whereas the STAT1 expression level in CIS was also higher than that of ESCC (IS: 106 ± 7.78), although this difference did not reach statistical significance.

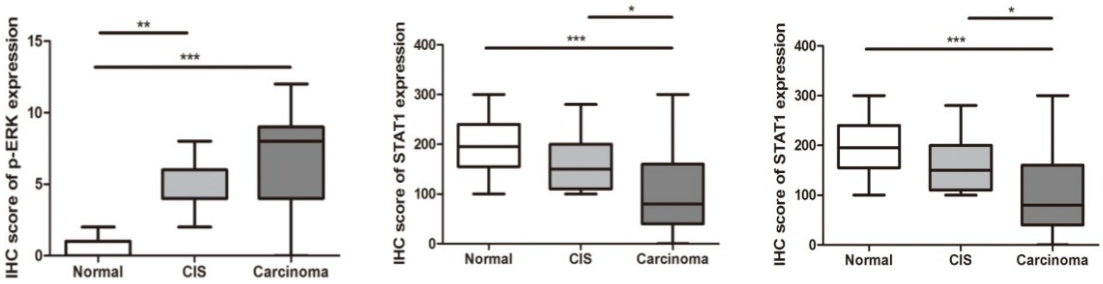

Figure 4.15 IHC score of STAT1, p-ERK and ERK in normal, CIS and cancer tissues. The boxplot shows that the IHC score of ERK and p-ERK are significantly lower in normal tissues than in CIS and ESCC; meanwhile, there is no significant difference between the ESCC and CIS for p-ERK. These expressions of STAT1 in the normal tissues and CISs were significantly higher than ESCC (*p<0.05, **p<0.01, ***p<0.001).

4.5 Discussion

STAT1, the first member discovered in the family of STAT proteins, was identified as the key mediator for type I and type II IFNs. Accumulating evidence suggested that STAT1 is a tumor suppressor in various cancer models **[1, 19]**. STAT1 was found to be frequently down-regulated in neoplastic cells, as compared to their adjacent benign tissues in breast cancer, colorectal cancer and liver cancer **[20]**. Our group also has recently published that STAT1 plays a tumor suppressor role in ESCC **[10]**, in which we found that 43 of 57 (75.4%) ESCC samples examined showed a down-regulation of STAT1 as compared to the case-matched, benign epithelia adjacent to the tumors. In the same study, we found that patients with $STAT1^{low}$ tumors had a significantly worse clinical outcome; gene transfection of *STAT1* into ESCC cells was found to effectively induce apoptosis, highlighting the biological importance of STAT1 in this tumor.

The main objective of the current study is to identify the mechanism(s) that are responsible for the low expression of STAT1 in ESCC tumors. One previous study has shown that reduced STAT1 expression in squamous cell carcinoma of the head and neck is due to gene methylation and silencing of the STAT1 gene **[21]**. Data from our experiments using 5-Aza does not support that gene methylation is the

key mechanism leading to the down-regulation of STAT1 in ESCC. Based on another study in which ERK was found to promote STAT1 proteasomal degradation in mouse embryonic fibroblasts, we tested if ERK is responsible for the down-regulation of STAT1 in ESCC. Our data supports this concept. To our knowledge, this current study represents the first example in which ERK is shown to be responsible for the down-regulation of STAT1, a tumor suppressor, in human cancers. In contrast with the mouse embryonic fibroblast model, ubiquitination and proteasomal degradation of STAT1 by ERK in ESCC cells are not dependent on STAT1 phosphorylation at Y701 or S727, two phosphorylation sites known to be functionally important for STAT1. However, we didn't detect both phosphorylation sites mutant in ERK-STAT1 binding.

We also found evidence that mechanisms that constitutively activate STAT1 may exist in ESCC cells, since p-STAT1^{Y701} increased along with total STAT1 in all 3 ESCC cell lines examined. We believe that this finding carries important therapeutic implications. In view of the fact that STAT1 phosphorylation at tyrosine 701 is required for its dimerization, nuclear translocation and DNA-binding **[22]**, a restoration of STAT1 expression in ESCC may be sufficient to induce apoptosis. The loss of STAT1 phosphorylation at serine 727 in EC1 but not the other 2 cell lines may have shed important insight into the biological heterogeneity of ESCC. Regarding p-STAT1^{S727}, it is believed to boost the transcriptional activity

of STAT1 and it has been implicated to enhance the anti-viral and anti-proliferation function of IFN-γ **[23, 24]**. While results from a study suggest that phosphorylation of STAT1^{S727} is dependent on IFN-γ-induced tyrosine phosphorylation of STAT1 **[25]**, a few other studies have provided contradictory conclusions **[26-29]**. This discrepancy may be linked to the observations that phosphorylation of STAT1^{S727} is mediated by different kinases stimulated by different signals **[30-37]**. In one study, phosphorylation of STAT1^{S727} in response to IFN-γ was found to be dependent on JAK2 **[38].** In this regard, we found that EC1, which lacks p-STAT1^{S727}, expressed a very low level of JAK2 (as compared to the other 2 ESCC cell lines) and no detectable p-JAK2. It is tempting to speculate that the relative lack of JAK2 may have contributed to the absence of p-STAT1^{S727} in EC1 (**Figure 4.16**).

While the involvement of ERK in promoting the proteasomal degradation of STAT1 has not been previously reported in human cancer, ubiquitination and degradation of STAT1 has been described in a number of previous studies. Through our literature search, we found that the ubiquitin-proteasome pathway was implicated in the down-regulation of STAT1 activation by Fin **[39]**. It also has been reported that ubiquitination of STAT1 can be modulated in response to viral and parasitic infection **[40, 41]**. Several E3 ligases are known to mediate the ubiquitination and degradation of STAT1.

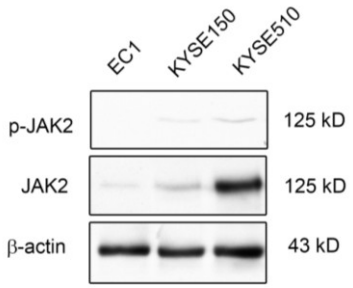

Figure 4.16 Expression of JAK2 in ESCC cell lines. By Western blot, the expression of p-JAK2 and JAK2 were detected in three ESCC cell lines, EC1, KYSE150 and KYSE510.

For example, both STAT-interacting LIM (SLIM) and smad ubiquitination-regulating factor 1 (Smurf1) promote STAT1 ubiquitination and degradation in mouse macrophage cells and HEK293 cells **[42-44]**. In parallel with our observation, SLIM and Smurf1-mediated STAT1 degradation was found to be independent of STAT1 phosphorylation. Furthermore, in mouse embryonic fibroblasts, F-box E3 ligase and βTRCP were reported to promote STAT1 proteasomal degradation that is dependent on STAT1 phosphorylation at S727 **[14].**

In addition to promoting the proteasomal degradation of STAT1, our data suggested that ERK dampens STAT1 activation by suppressing the production of IFNγ, which is known to be a potent activator of STAT1. IFNγ is used for treating cancers and viral infection, however, the therapeutic efficacy of IFNγ is highly variable among different types of cancer **[45, 46].** For example, while IFNγ appears to be effective in treating adult T cell leukemia and ovarian cancers, it is relatively ineffective for most patients suffering from chronic myeloid leukemia **[45]**. Moreover, IFNγ-treated melanoma patients were found to have a worse survival than those who did not receive IFNγ **[46]**. Based on the results of our study, one may hypothesize that the anti-cancer effects of IFNγ may be closely linked to the expression and activation status of STAT1 and ERK. If this turns out to be the case, one may hypothesize that IFNγ might be an effective anti-cancer drug for tumors that lack a high level of

expression and/or activation of ERK, and/or express a certain basal level of STAT1 that can be activated.

The MAPK-ERK signaling pathway is a major regulator in a wide range of cellular processes such as cell proliferation, differentiation, survival and motility **[47]**. ERK activation has been implicated in the pathogenesis and progression of various cancer types, such as cancers of prostate, kidney and colon **[48]**. ERK represents an attractive anti-cancer therapeutic target for the development of anticancer drugs. However, studies related to the ERK signaling pathway in ESCC are limited. In a cohort of patients from the Kazakh population, researchers have found that the expression of ERK is higher in ESCC samples compared to normal tissue, and this up-regulation significantly correlated with lymph nodes metastasis and histologic grade **[49]**. These results are compatible with our findings.

However, there are still some limitations in this paper. First, we didn't detect the mRNA level expression of STAT1 upon ERK inhibitor treatment or MEK transfection. Second, the *in-vivo* study may favor us to support our hypothesis. Third, no enough information was collected from patients during the follow-up years, for example, the radiotherapy or chemotherapy after surgery. Multivariate survival statistical analysis should be used to eliminate these factors.

In conclusion, we have demonstrated that ERK promotes proteaosomal

degradation of STAT1 and down-regulates STAT1 activation by suppressing IFNγ production. In conjunction with our previous publication that STAT1 is a tumor suppressor in ESCC, results from this study strongly support the concept that ERK promotes tumorigenesis of ESCC by suppressing the expression and activation of STAT1. This concept is supported by our clinical observation that the expression of ERK significantly correlates with a worse clinical outcome.

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Chapter 5

STAT1β enhances the tumor suppress effects of STAT1α in esophageal squamous cell carcinoma

This chapter has been modified from a manuscript in preparation:

Zhang Y, Yun HL, Zhang HF, Lai R, Su M. STAT1β enhances the tumor suppress effects of STAT1α in esophageal squamous cell carcinoma.

As the first author, I designed and performed all the experiments described here expect Westernblot shown in **Figure 5.12A**, which is done by Yun HL. Zhang HF helps to design the primer of STAT1β.

5.1 Abstract

While the tumor suppressor role of STAT1 is well established, a limited number of reports have described the function of STAT1β, a truncated isoform of STAT1. In previous reports, STAT1β is considered to be transcriptional inactive, thus inhibits STAT1α function. However, the biological function of STAT1β has never been reported in human cancer. In this paper, how STAT1β interacted with STAT1α and affected the biological function of STAT1 was studied in two esophageal squamous cell carcinoma (ESCC) cell lines. The prognostic significance of STAT1β was evaluated in a cohort of ESCC patients by immunohistochemistry and Western blot. Enforced expression of STAT1β induced and prolonged the tyrosine phosphorylation and nuclear translocation of STAT1α in ESCC cell lines. Our data suggested that STAT1β can interact with STAT1α and protected it from proteasome degradation, which may contribute to the process. Moreover, STAT1β increased DNA binding and transcription activity of STAT1. STAT1β sensitized ESCC cells to the anti-tumor drugs, for example, cisplatin and 5-flurouracil (5-FU). Using ESCC tumor samples analyzed by Western blot and immunohistochemistry, we found a downregulation of STAT1β in ESCC compared to the normal esophageal epithelial. The loss of STAT1β was related with lymph node metastasis, further invasion and worse clinical outcome in ESCC patient. Our data showed a new concept on the

potential contribution of STAT1β in cancer. STAT1β can enhance the tumor suppressor function of STAT1α in ESCC, which may provide a new therapeutic approach to ESCC patients.

5.2 Introduction

Signal transducers and activators of transcription1 (STAT1), a key mediator of interferon (IFN) signalling, regulates a variety of cellular activities such as apoptosis, cell proliferation and differentiation **[1]**. In response of extracellular stimuli, such as IFN-γ, activation of STAT1 is achieved by Janus kinase-mediated phosphorylation of its conserved tyrosine and serine residues present in the C-terminal transactivation domain, and this process results in STAT1 dimerization, nuclear translocation, DNA binding and eventually modulation of the expression of its target genes **[2]**. In a number of study models, STAT1 has been shown to carry tumor suppressor functions, and the evidence can be summarized as follows: 1) the pro-apoptotic effects of IFNs are largely mediated through STAT1 signaling **[2]**, 2) the constitutively active form of STAT1 can effectively induce apoptosis and inhibit cell growth **[3]**, 3) STAT1 is frequently down-regulated in various types of human cancer including breast cancer, head and neck cancer, multiple myeloma and leukemia **[4, 5]**. In our previous studies, our group has reported that STAT1 is an important tumor suppressor in esophageal squamous cell

carcinoma (ESCC); loss of STAT1 contributes to the pathogenesis of these tumors and correlates with a worse clinical outcome in ESCC patients **[6, 7]**.

STAT1β, a naturally short isoform of STAT1, lacks a 38-amino acid segment that includes the conserved STAT1^{S727} phosphorylation site and most of the C-terminal transactivation domain. STAT1β has not been extensively studied, although one report has described that STAT1β in human B-cells is transcriptionally inactive and it exerts a dominant-negative effect on STAT1α, the full-length STAT1 isoform **[8]**. Specifically, enhanced STAT1β was found to inhibit the phosphorylation, DNA binding, and transcriptional activity of STAT1 in human B cells **[8]**. However, in another study using B-cells, STAT1β was reported to induce cell death via a mechanism that is independent of p53 and STAT1α **[9]**. In a more recent published paper, it was found that STAT1β is transcriptionally active and capable of eliciting IFN-γ-dependent immunity against infection *in vivo* **[10]**. Nonetheless, the biological function and clinical significance of STAT1β in human cancers has never been examined, and whether STAT1β carries tumor suppressor function is unknown.

Using ESCC cell lines as a study model, we examined the biological and clinical significance of STAT1β. Our results support the concept that

STAT1β enhances the expression and tumor suppressor function of STAT1α, and this effect can be amplified by IFN-γ stimulation. Correlating with this concept, loss of STAT1β in ESCC tumors correlates with a significantly worse clinical outcome.

5.3 Materials and Methods

5.3.1 Cells and Patient samples

Human ESCC cell lines, EC1, EC109 and KYSE 150, were used in this study. They were maintained in RPMI 1640 or Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1 × antibiotic mixture (Invitrogen, Carlsbad, CA, USA). All cells were cultured at 37°C in a humidified incubator containing 5% CO_2.

We randomly collected 201 ESCC tumors at the Shantou Tumor Hospital between 2005 and 2012. All patients underwent potentially curative surgery without preoperative chemotherapy or radiotherapy. In this cohort, 150 were men and 51 were women; the range of age was 36-81 years, with a median of 57 years. Follow-up data was available for 130 patients; most (113, 86.9%) died during the follow-up period (median survival, 21.5 months). Of the 201 ESCC tumors, 12 case-matched normal esophageal tissues adjacent to the tumors were included in the study. Written informed consents were obtained from patients, and the study was

reviewed and approved by the institutional ethics committee.

5.3.2 Western blot analysis

Tissue and cell lyser was used to prepare frozen tumor samples and cell lines for Western blot analysis. Cell lines and tumor samples were lysed in RIPA buffer (Thermo scientific, Rockford, USA) containing protease inhibitor cocktail and Set II phosphatase inhibitor cocktail (EMD Millipore, , Billerica, MA, USA). Total tissue extracts were stored on ice for 20 minutes and then centrifuged at 13,000 rpm at 4°C for 15 minutes. Proteins from the supernatant were measured using a bicinchoninic acid (Thermo scientific). Equal amounts of cell lysate were separated by 10% sodium dodecyl sulfate - polyacrylamide gel electrophoresis and evaluated by Western blot analysis as described previously **[11]**. Antibodies reactive with human β-actin (1:1000), STAT1 (1:1000), phospho-Tyr 701 STAT1 (or p-STAT1) (1:1000), Flag (1:1000), Poly ADP ribose polymerase (PARP) (1:1000) were purchased from Cell Signaling (Danvers, MA, USA). STAT1α (1:1000) was purchased from Santa Cruz (Dallas, TX, USA). STAT1β (1:200) was purchased from SignalChem (Richmond, BC, CA).

5.3.3 Cell growth assay

The ESCC cell lines were transfected with STAT1β or empty vector

before the experiment. Then, 1×10^4 cells growing in each well of a 96-well microplate were treated with anti-tumor drug, cisplatin and 5-flurouracil (5-FU) for 0 to 8 days. On different time, the cells were incubated with 10-μl MTS reagent (Promega, MI, USA) for 1 hour. The increase in absorbance at 490 nm relative to the blank well control was measured using a microplate spectrophotometer.

5.3.4 Plasmid constructs and gene transfection

Plasmids including Flag-STAT1β and STAT1α were purchased from Addgene (Cambridge, MA, USA). For each experiment, 1×10^6 ESCC cells were transiently transfected with 10 μg of vector or the empty vector in 6-well plates using the Lipofectamine 2000 reagent (Invitrogen) as per the manufacturer's suggested protocol.

5.3.5 Co-immunoprecipitation and immunoprecipitation

To detect the interaction between STAT1α and STAT1β, whole-cell extracts were prepared by lysing the cells in an immunoprecipitation buffer. A total of 2 μg of anti-STAT1α monoclonal antibody (Santa Cruz Biotechnology) was added to 500 μg of protein lysate isolated in cell lysis buffer (Sigma Aldrich, St Louis, MD, USA) and the samples were rotated overnight at 4^oC. Subsequently, 30 μl of protein G Plus/A beads (EMD Millipore) was added to the samples and rocked overnight at 4^oC. The

beads were then washed 3 times with cold phosphate-buffered saline followed by the final wash using cold cell lysis buffer or RIPA buffer. Western blot analysis was then performed using standard techniques as previously described **[11]**.

5.3.6 Immunofluorescence and confocal microscopy

Cells were grown on cover slips coated with poly-L-lysine (Sigma Aldrich) in a 6-well plate and fixed with 3% paraformaldehyde in PBS (pH 7.4). Cells were rinsed three times with PBS, permeabilized with Triton X-100, washed again with PBS, and incubated with 200 µl of anti-p-STAT1 antibody (1:50, Sigma Aldrich) overnight at room temperature in a humidified chamber. The cover slips were rinsed three times in PBS and incubated with secondary antibody conjugated with Alexa Fluor 488 or 568 (Invitrogen) at a 1:250 dilution for 1 hour at room temperature. After three rinses in PBS, the coverslips were mounted on a slide using the mounting media (Dako, Mississauga, ON, Canada). Cells were visualized with a Zeiss LSM 710 confocal microscope at the Core Cell Imaging Facility, Cross Cancer Institute.

5.3.7 Colony formation assay

After STAT1β transfection, 500 cells/well were plated in six-well plates and incubated 10 days at 37°C. The cells were fixed with 4% buffered

formalin for 15 min and then stained with 1% crystal violet (Sigma Aldrich) for 30 minutes. The plates were gently washed with PBS and dried before microscopic evaluation. Cell clusters with >30 cells were considered as a colony.

5.3.8 Luciferase activity assay

STAT1 transcription activity was measured by STAT1 luciferase reporter. Luciferase activity was measured with the Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega) as described. Data were normalized for transfection efficiency by division of firefly luciferase activity with that of Renilla luciferase.

5.3.9 Subcellular fractionation and DNA binding assays

Nuclear and cytoplasmic protein of ESCC cells were extracted using the NE-PER protein extraction Kit (Thermo Scientific) according to the manufacturer's instructions. For Western blot analysis, tubulin and histone deacetylase 1 (HDAC1) were using as the cytoplasmic and nuclear loading control, respectively.

Oligonucleotide pull-down assays were performed with an annealed nucleotide comprising the STAT1 consensus site (5'-CATGTTATGCATATTCCTGTAAGTG-3) with a 5-biotin label. Nuclear extracts (50-100 μg) were incubated for 1 hour at 4°C with 1g

oligonucleotide in binding buffer. Sepharose–streptavidin (50 μL; Sigma) was added for 2 hours at 4°C. After 3 washes in PBS buffer, the complexes were resuspended in SDS sample buffer and processed for Western blotting as described above using anti-STAT1 and anti-pSTAT1 antibodies (Cell Signaling).

5.3.10 Quantitative RT-PCR

Using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), total cellular RNA was extracted from cells following the manufacture's protocol. The Human interferon regulatory factor 1 (IRF1) primer sequence is, Forward: 5'-3', ATGCCCATCACTCGGATGC; Reverse: 5'-3', CCCTGCTTTGTATCGGCCTG; Human chemokine (C-X-C motif) ligand 10 (CXCL10) primer sequence is, Forward: 5'-3', GTGGCATTCAAGGAGTACCTC; Reverse: 5'-3', TGATGGCCTTCGATTCTGGATT. STAT1α Forward: 5'-3': CCAATGGAACTTGATGGCCC; Reverse 5'-3': CAGAGCCCACTATCCGAGAC. Guanylate binding protein 2 (GBP2) Forward: 5'-3',CTATCTGCAATTACGCAGCCT, Reverse: 5'-3', TGTTCTGGCTTCTTGGGATGA; Intercellular adhesion molecule 10 (ICAM10) Forward: 5'-3',ATGCCCAGACATCTGTGTCC, Reverse: 5'-3',GGGGTCTCTATGCCCAACAA；Transporter associated with Antigen Processing 1(TAP1) Forward: 5'-3',

GCAAGACGACTTACTCTGGGT, Reverse: 5'-3': GGATCTGACACCACTGGACC.

5.3.11 Immunohistochemistry

Formalin fixed, paraffin embedded ESCC tumors were used for this study. All cases were retrieved from the file at the department of Pathology, Shantou University Medical College. The diagnosis of these cases was based on the criteria established by the World Health Organization classification scheme. Immunohistochemistry to detect STAT1β expression was performed using a method similar to that described previously **[12]**. Briefly, formalin-fixed, paraffin embedded tissue sections of 4 μm thickness were deparaffinized in xylene and hydrated in graded ethanol (100% to 50%). Antigen retrieval was performed using citrate buffer (pH 6.0) microwave in a pressure cooker for 20 minutes and left to cool for 20 minutes. After antigen retrieval, tissue sections were incubated with 10% hydrogen peroxide (H_2O_2) and methanol for 10 minutes to block endogenous peroxidase activity, followed by washing in running tap water for 5 minutes. Subsequently, the sections were incubated for 20 minutes in antibody diluent (Dako), followed by incubation overnight at 4°C with a rabbit polyclonal antibody reactive with anti-STAT1β (1:200 dilution, Cellchemo). The antibodies were diluted in antibody diluent (Dako). Immunostaining was visualized with a labeled

streptavidin-biotin method (20 minutes in biotinylated link and 20 minutes in streptavidin horse radish peroxidase, both from Dako) using (3, 3-diaminobenzidine/ H_2O_2) DAB as a chromogen (Dako). Hematoxylin was used as a counter stain. Following staining, sections were dehydrated in graded ethanol (50%-100%), followed by xylene incubation. Coverslips were applied using permount solution (Fisher Scientific, PA, USA).

IHC staining was examined by two pathologists who were blinded to the clinical outcome and concordance between two pathologies was guaranteed. For the evaluation of STAT1 immunostaining, both the intensity and percentage of immunostained cells were described previously in Chapter 1 **[6]**. For the evaluation of STAT1β immunostaining, the percentages of positive stained cells were assigned the following scores: 0 (< 5% positive cells), 1 (6% to 25% positive cells), 2 (26% to 50% positive cells), 3 (51% to 75% positive cells), or 4 (> 75% positive cells). The staining intensity was scored on a scale of 0 to 3 as follows: 0, negative; 1, buff; 2, yellow; and 3, brown. The percentage of positive cells and the staining intensities were then multiplied to generate the immunoreactivity score for each case. Overall staining scores from 0 to 2, 3-6 and ≥ 7 were considered negative, weak and strong expression, respectively. The weak and strong expressions were considered positive.

5.3.12 Statistical analysis

Data was expressed as mean ± standard deviation. The prognostic significance of the expression of various markers was analyzed using Kaplan-Meier's analysis. The correlation between STAT1β and other clinical parameters was evaluated using Chi-square or Student's t-test. Differences among the treatment groups were assessed using ANOVA and the appropriate statistical software (SPSS, IBM, USA). A p-value of ≤ 0.05 was considered as statistically significant.

5.4 Results

5.4.1 STAT1β increases and prolongs the tyrosine 701 phosphorylation and nuclear translocation of STAT1α

As shown in the left panel of **Figure 5.1A**, both ESCC cell lines (EC1 and KYSE150) had no detectable expression of STAT1 or p-STAT1^{Y701} after transfection of an empty vector. At 12 hours after the addition of varying doses of IFN-γ, there was also no appreciable change in the expression of STAT1 and p-STAT1^{Y701}. In contrast, with *STAT1β* transfection, we found detectable STAT1α expression that was best recognized by using the specific anti-STAT1α antibody. At 12 hours after the addition of increasing amount of IFN-γ, the total STAT1α as well as p-STAT1^{Y701} (both STAT1α and STAT1β) levels increased in a dose-dependent manner (**Figure 5.1A**). Time course experiments revealed similar results. As shown in the middle panel of **Figure 5.1B**, with *STAT1β* transfection,

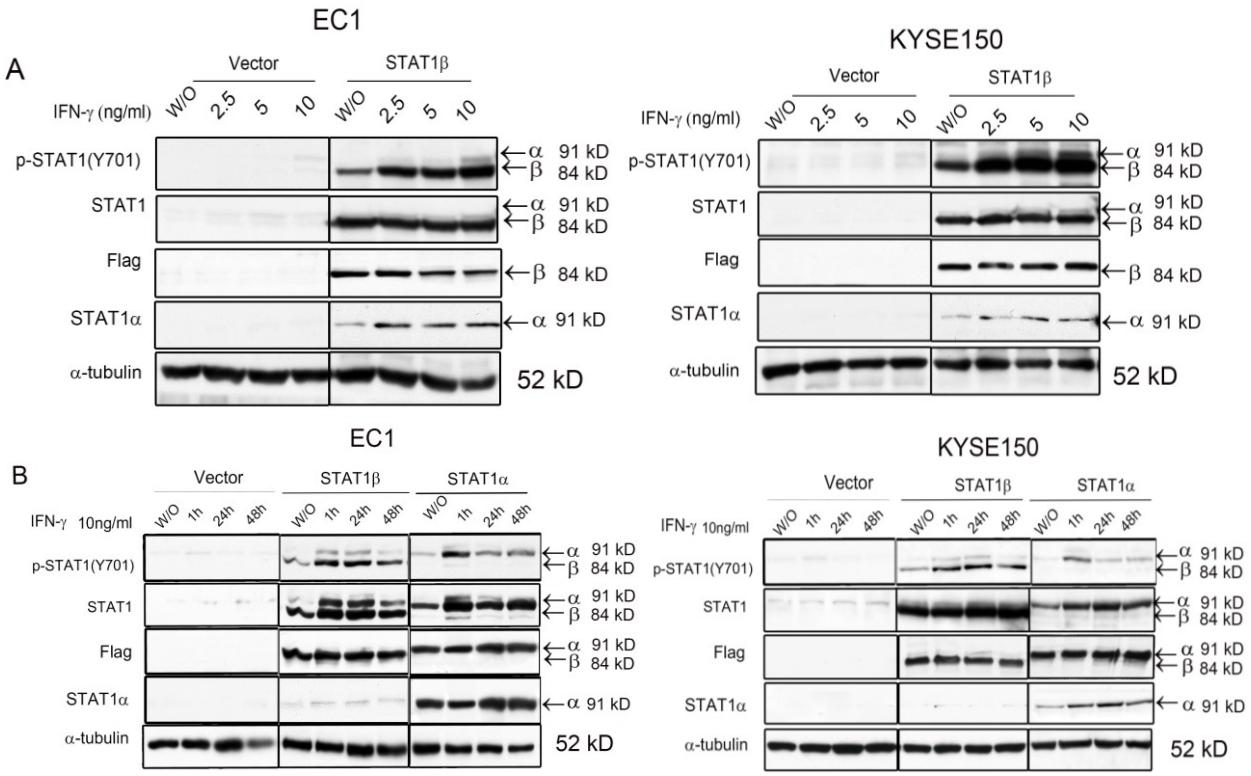

Figure 5.1 STAT1β increases the tyrosine 701 phosphorylation of STAT1α in ESCC cells. A. ESCC cell lines, EC1 and KYSE150 were stimulated with IFN-γ for the does indicated or left untreated (w/o) after empty vector or Flag-tagged *STAT1β* transfection. Total-protein extracts were used for detection of p-STAT1 (Y701), total STAT1, flag and STAT1α by Western blotting. B. Both cells were transfected with empty vector, *STAT1β* or *STAT1α* and then stimulated with IFN-γ (10 ng/ml) for the time indicated or left untreated (w/o). Total-protein extracts were used for detection of p-STAT1, total STAT1, flag and STAT1α by Western blotting. Similar results were observed in three independent experiments.

the addition of 10 ng/ml IFN-γ resulted in a rapid and dramatic increase in STAT1α as well as p-STAT1^{Y701} (both STAT1α and STAT1β). Importantly, the enhancement of p-STAT1α by STAT1β was almost as potent and sustained as by *STAT1α* transfection.

To further substantiate our finding that STAT1β increases the expression and phosphorylation of STAT1α, we performed confocal microscopy using EC109, another ESCC cell line that has no detectable STAT1 and p-STAT1^{Y701}. As shown in **Figure 5.2**, we found that no detectable nuclear p-STAT1^{Y701} at 1 hour or 24 hours after IFN-γ stimulation. In contrast, with STAT1β transfection, strong nuclear p-STAT1^{Y701} signals were detectable at 1 hour, and the signal was less intense at 24 hours. Similar findings were found STAT1α transfection was performed, although the p-STAT1^{Y701} signals were slightly more intense than that resulted from STAT1β transfection. These results correlated well with the Western blot results illustrated in **Figure 5.1**.

We then performed immunoprecipitation. As shown in **Figure 5.3**, tyrosine phosphorylation of STAT1α in EC1 cells was increased in the presence of STAT1β but not the empty vector. Importantly, tyrosine phosphorylation of STAT1α was largely abrogated when the STAT1βY701F mutant instead of wild-type STAT1β was used for transfection. This finding highlights the importance of the

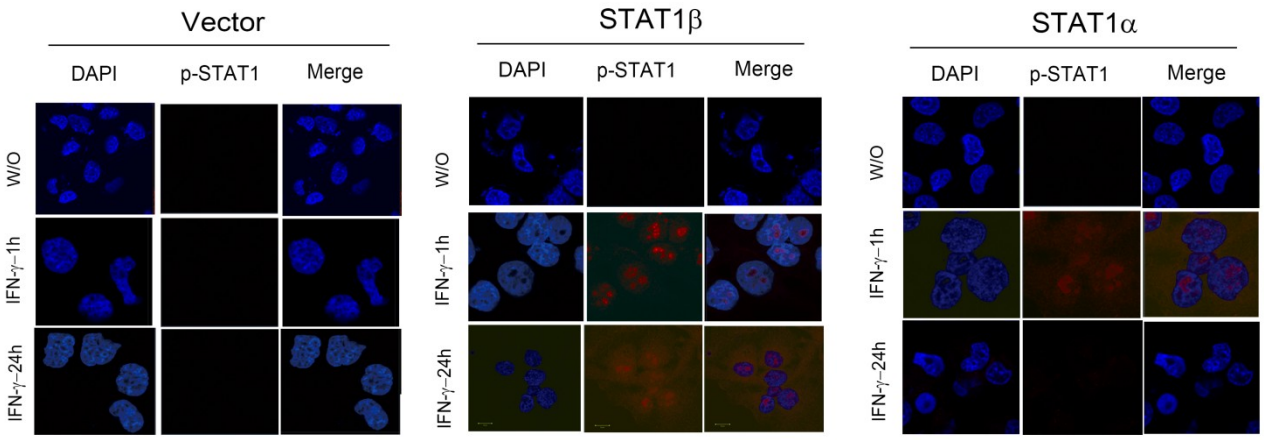

Figure 5.2 STAT1β prolongs the tyrosine 701 phosphorylation and nuclear translocation of STAT1α. EC109 was stimulated with IFN-γ (10 ng/ml) for the times indicated after empty vector or Flag-tagged STAT1β transfection. Phosphorylation of Tyr701 STAT1 was detected with Alexa Fluor 568 labeled secondary antibody (red). DAPI (100 ng/ml) was used for nuclear staining (blue). Fluorescence signals were analyzed with a Zeiss LSM 710 confocal microscope. Similar results were observed in three independent experiments.

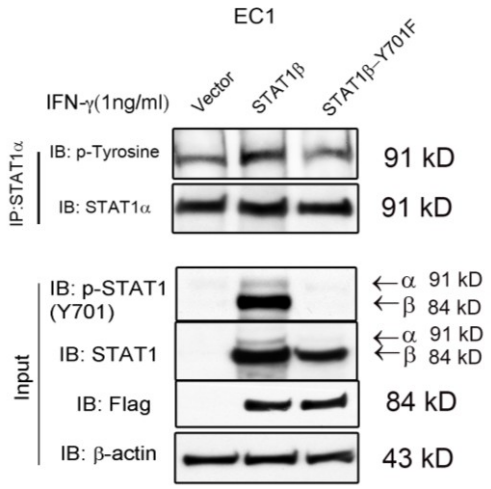

Figure 5.3 STAT1β increases the tyrosine phosphorylation of STAT1α. Tyrosine phosphorylation of STAT1α of EC1 was detected by immunoprecipitation and Western blot after empty vector or Flag tagged *STAT1β* or *STAT1β*^{Y701F} plasmid transfection. The data are representative of three independent experiments. Similar results were observed in three independent experiments.

activation/phosphorylation of STAT1β in potentiating the expression and phosphorylation of STAT1α.

5.4.2 STAT1β interacts with STAT1α and protects STAT1α from ubiquitination in ESCC

To investigate the mechanisms by which STAT1β enhanced STAT1α expression and phosphorylation, we asked if STAT1β increased the expression of *STAT1α* mRNA. By quantitative RT-PCR, we found a significant decrease in STAT1α mRNA after *STAT1β* transfection in both ESCC cell lines; in comparison, the $STAT1\beta^{Y701F}$ mutant did not have any appreciable effect (**Figure 5.4**). Since the relatively low STAT1 expression in ESCC is attributed to its degradation via the ubiquitin-proteasome pathway (chapter 4), we tested if this pathway is also involved in the upregulation of STAT1α mediated by STAT1β. In keeping this hypothesis, we performed immunoprecipitation using an antibody reactive with STAT1α. As shown in **Figure 5.5**, we found that transfection of *STAT1β* almost completely abrogated STAT1α ubiquitination; consistent with our previous data, Y701F mutation of *STAT1β* abrogated this effect.

To further investigate the relationship between STAT1α and STAT1β, we performed co-immunoprecipitation and Western blot experiments. By co-immunoprecipitation experiments (**Figure 5.6A)**, in the absence of

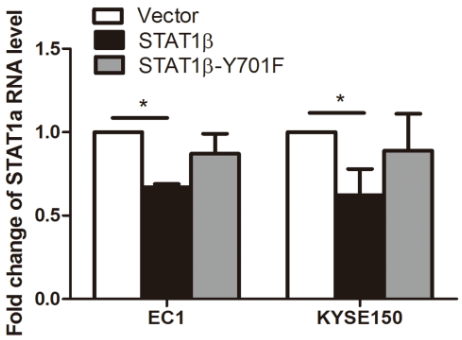

Figure 5.4 STAT1β decreases STAT1α mRNA level in ESCC cell lines. STAT1α mRNA expression was detected by real-time PCR after transfection of empty vector or Flag tagged *STAT1β* or *STAT1β*^{Y701F} plasmids. The values were normalized to the GAPDH and calculated relative to untreated empty vector cells. Mean values standard errors from at least three independent experiments are shown. (*p<0.05)

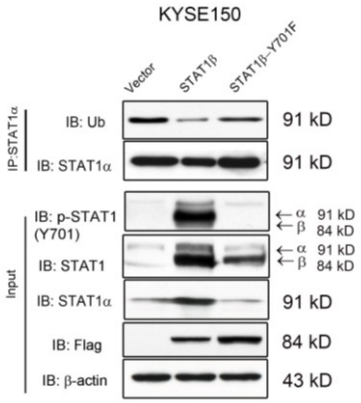

Figure 5.5 STAT1β protects STAT1α from ubiquitination in ESCC.

Immunoprecipitation-immunoblotting analysis and Western blot analysis for STAT1α and ubiquitination in KYSE150 cells treated with empty vector or Flag tagged *STAT1β* or *STAT1β*^{Y701F} plasmids. Similar results were observed in three independent experiments.

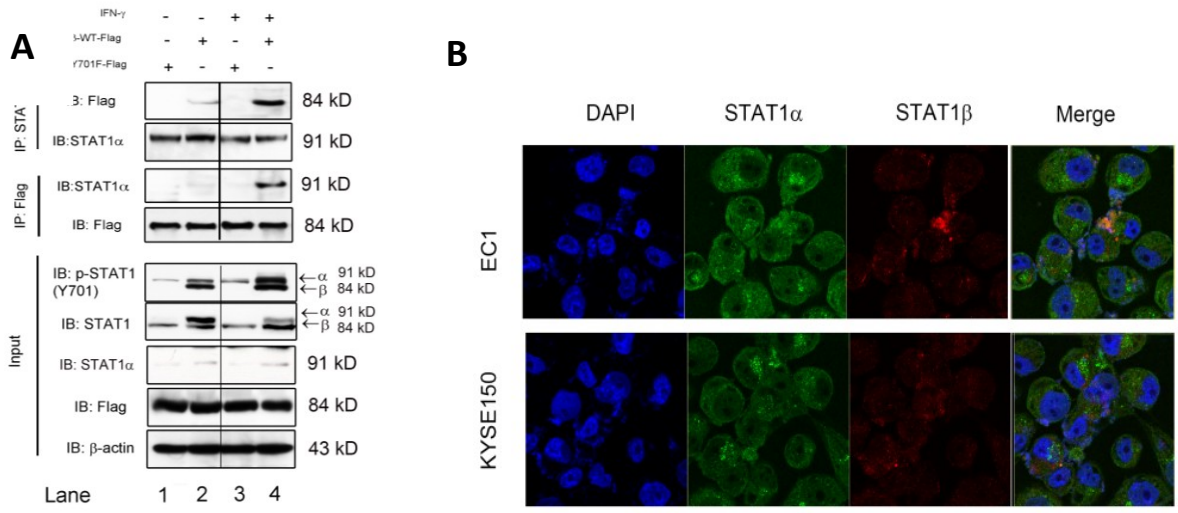

Figure 5.6 STAT1α binds to STAT1β in ESCC cell lines. A. The interaction of STAT1α and STAT1β was investigated by immunoprecipitation-immunoblotting analysis in EC1 cells with or without IFN-γ stimulation. Co-immunoprecipitaion was carried out with control IgG and anti-Flag or anti-STAT1α antiboodies as indicated. Immunoprecipitated proteins were analyzed by Western blot with anti-STAT1α and anti-Flag, respectively. B. Co-localization of STAT1α and STAT1β was confirmed with immunofluorescence. EC1 and KYSE150 were placed on the coverslips and stained with indicated antibodies (scale bar 5 μm). Similar results were observed in three independent experiments.

IFN-γ (lane 1 and 2), we found evidence of physical binding between Flag-tagged *STAT1β* and STAT1α in KYSE150 cells. In contrast, there was no detectable physical binding between Flag-tagged $STAT1\beta^{Y701F}$. This effect was amplified when IFN-γ was added (**Figure 5.6A,** lane 3 and 4). The physical interaction between these two STAT1 isoforms was further supported by our confocal microscopy results (**Figure 5.6B**). Taken together, our results suggest a model in which STAT1β protects STAT1α from proteosomal degradation, thus increasing the total protein level of STAT1α.

5.4.3 STAT1β enhances the DNA binding ability and transcription activity of STAT1α

Using a STAT1 luciferase reporter, we assessed the effect of STAT1β on the transcriptional activity of STAT1 in ESCC cell lines. As shown in **Figure 5.7A**, *STAT1β* transfection significantly increased the transcriptional activity of STAT1 ($p<0.05$), as compared to that of empty vector. Again, transfection of the $STAT1\beta^{Y701F}$ mutant yielded only a minimal increase, as compared to that of empty vector ($p=0.71$). Similar observations were observed when the cells were stimulated with IFN-γ, although the effects were more profound.

To test whether the increased transcriptional activity of STAT1 mediated by *STAT1β* transfection was caused by an increase in STAT1-DNA

binding, we performed a pull-down experiment using a biotinylated probe containing the STAT1 DNA-binding consensus sequence. As shown in **Figure 5.7B**, upon IFN-γ stimulation, STAT1-DNA was markedly promoted by *STAT1β* transfection, as compared to that of empty vector or *STAT1β*^{Y701F}.

To confirm the effect of STAT1β on the gene transcription effects of STAT1, we performed quantitative RT-PCR. ESCC cells were transiently transfected with empty vector, wild type *STAT1β* or *STAT1β*^{Y701F}, and the mRNA expression levels of several known STAT1 downstream targets (including *IRF1, TAP1, CXCL10, GBP2* and *ICAM10*) were analyzed **[12]**. The results are illustrated in **Figure 5.7C**. Compared to the transfection of the empty vector, *STAT1β* transfection at 24 hours significantly increased the mRNA expression of all five target genes examined. Again, enforced expression of the STAT1^{Y701F} mutant did not increased the expression of these 5 genes appreciably.

5.4.4 STAT1β enhances the tumor suppressor function of STAT1α

To evaluate the biological effect of STAT1β in ESCC cells, we performed colony formation assay using EC1 and KYSE150. As shown in **Figure 5.8A**, STAT1β significantly decreased the soft-agar colony formation ability of both cell lines stimulated with IFN-γ ($p<0.05$), whereas STAT1β^{Y701F} did not show similar tumor suppressor effects. To support the

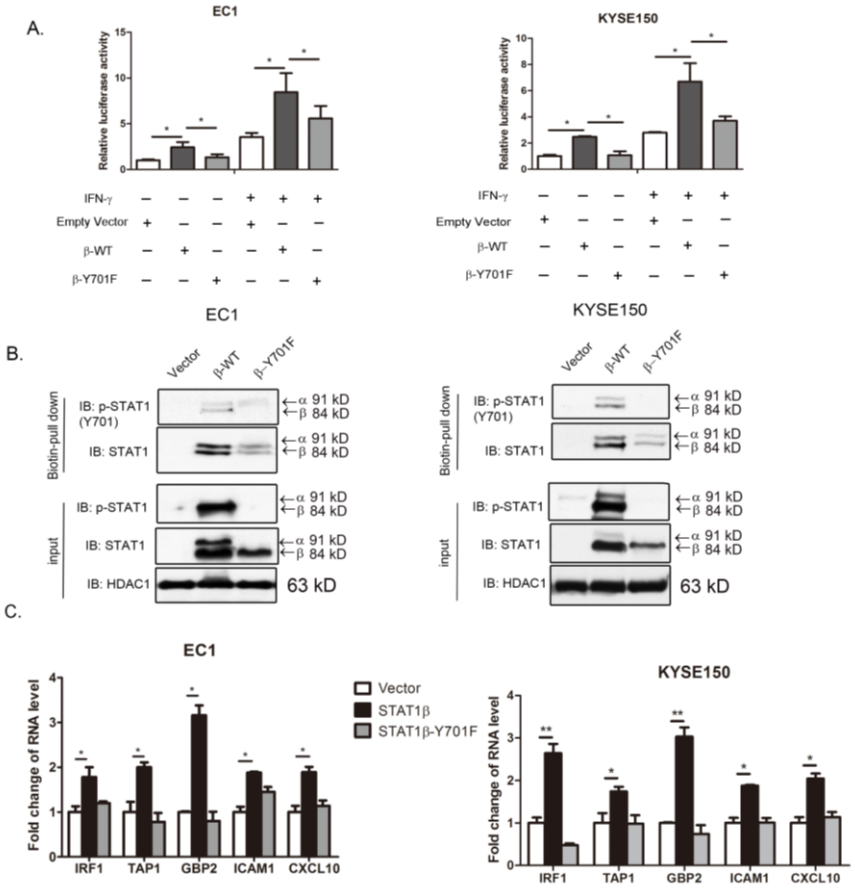

Figure 5.7 STAT1β enhances the transcription activity and DNA binding of STAT1 in ESCC cell lines. A. Both EC1 and KYSE150 cell lines were transfected by empty vector or Flag tagged *STAT1β* or *STAT1β*^{Y701F} plasmid upon IFN-γ stimulation. Then after 48 hours, the transcription activity of STAT1 was detected by luciferase reporter assay. B. DNA binding ability of STAT1 was detected in both cell lines with biotin probe by Western blot assay. C. Total RNA was extracted and used for RT-qPCR analysis for the genes indicated. GAPDH was used for normalization, and expression levels were calculated relative to empty vector transfection cells. Triplicate experiments were performed and results from a representative experiment are shown (*p< 0.05; **p< 0.01).

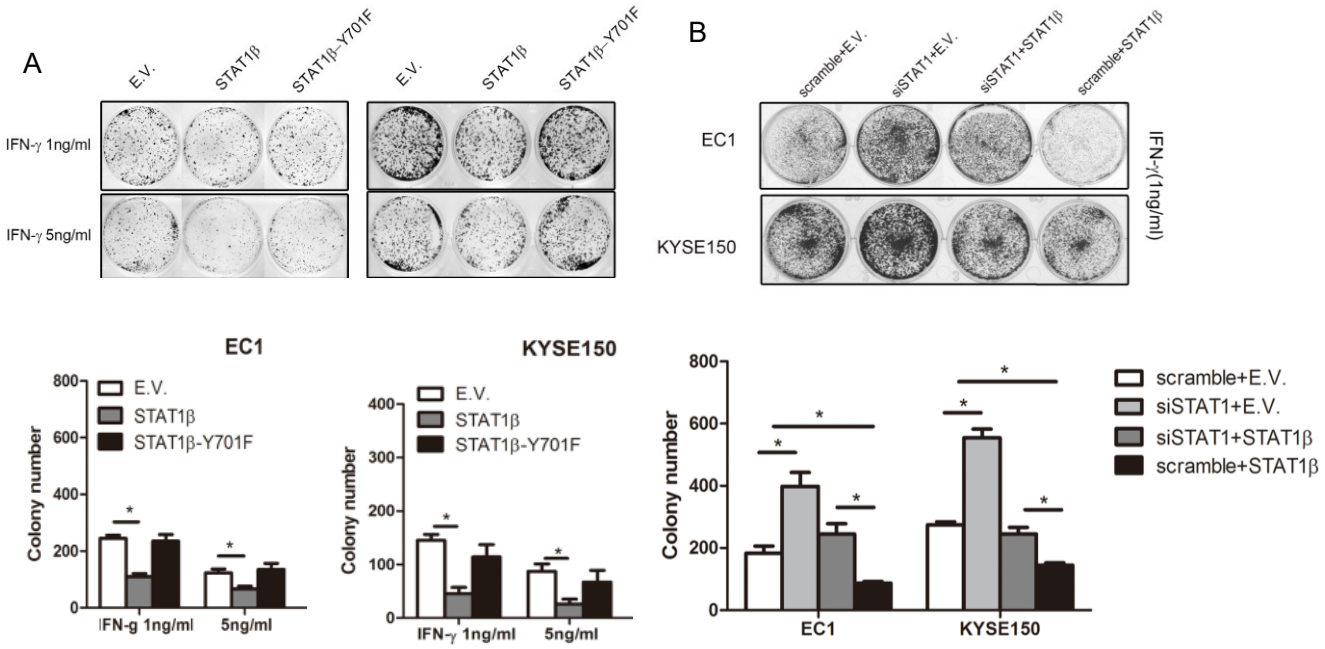

Figure 5.8 Biological functions of STAT1β in ESCC. Transfection of *STAT1β* into EC1 and KYSE150 cells led to a significant reduction in the number of colonies formed, as compared to cells transfected with empty vector upon different dose of IFN-γ stimulation after 10 days.. Triplicate experiments were performed and the results of a representative experiment are shown (*p<0.05). B. In both cell line, siRNA knockdown of STAT1 induced a significant increase in colony formation and transfection of *STAT1β* significantly diminished the clonogenic ability of these two cell lines (*p<0.05). Similar results were observed in three independent experiments.

functional role of STAT1β, we performed colony formation assay in the presence of IFN-γ (1 ng/mL) stimulation, and the results are shown in **Figure 5.8B**. Upon STAT1 siRNA knockdown, both EC1 and KYSE150 showed a significant increase in the number of colonies. However, enforced STAT1β expression significantly attenuated the biological effect of STAT1 siRNA knockdown (lane 3). Furthermore, enforced expression of STAT1β in the absence of STAT1 siRNA knockdown (lane 4) brought the number of colonies to the lowest level in both cell lines.

By western blot studies, we found that the gene transfection of *STAT1β* led to the expression of cleaved PARP in ESCC cells stimulated with IFN-γ (**Figure 5.9A**). *STAT1β* transfection synergized with 5-FU (5-fluorouracil) and cisplatin in decreasing colony formation (**Figure 5.9B**) as well as the number of viable cells by MTS assay (**Figure 5.9C**).

5.4.5 The prognostic significance of STAT1β in ESCC patients

By Western blot, we detected the expression of p-STAT1^{Y701} and total STAT1 in 12 ESCC tumors as well as their case-matched, benign esophageal epithelial tissues. As illustrated in **Figure 5.10A**, all 12 cases showed an appreciable decrease in the expression of the STAT1α isoform and p-STAT1α^{Y701} (the upper bands). Most (8/12, 66.6%) tumors showed lower expression of p-STAT1β^{Y701} and STAT1β (the lower bands); only 2

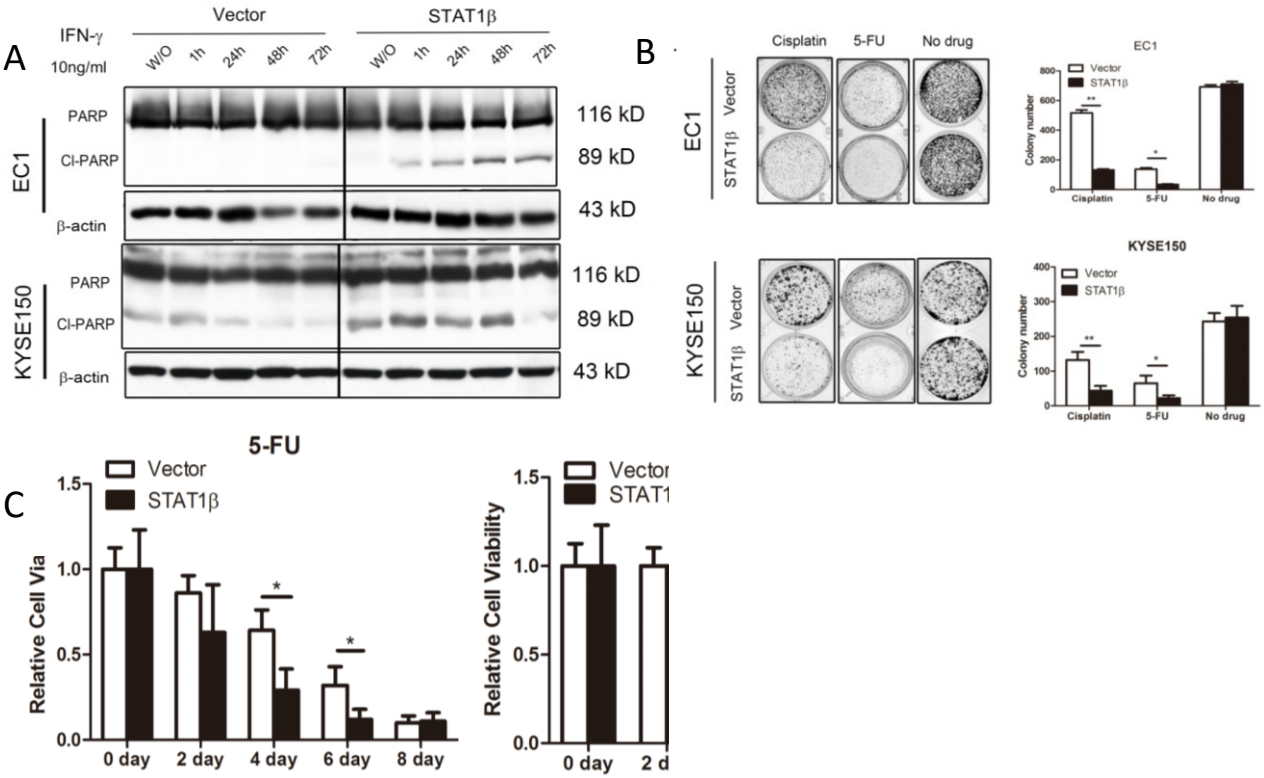

Figure 5.9 STAT1β promotes cell death and chemosensitivity in ESCC cell lines. A. Using Western blot analysis, the gene transfection of *STAT1β* in EC1 and KYSE150 cells induced cleave PARP increased after 2 days. B. The chemosensitivity to 5-flurouracil and cisplatin of ESCC cells was assessed by colony formation after transfection of *STAT1β* for 10 days. C. Cell growth, as assessed by MTS assay, was found to be significantly decreased after *STAT1β* transfection in EC1 and KYSE150 cells after 4 days upon IFN-γ. Triplicate experiments were performed and results from a representative experiment are shown (* $p<0.05$).

(16.7%) cases showed a slight increase and another 2 (16.7%) cases showed no appreciable difference.

We then performed immunohistochemistry (IHC) using 33 ESCC tumors and the case-matched, benign esophageal tissues. As illustrated in **Figure 5.10B and 5.10C**, STAT1β immunoreactivity was found in 29 (87.9%) cases of benign esophageal epithelial tissues and only 19 (57.6%) ESCC tumors. Using our IHC scoring method, we found that ESCC tumors had a significantly lower STAT1β expression than the benign epithelia, in keeping with the concept that STAT1β is a tumor suppressor and its expression is frequently lost during the carcinogenesis of ESCC.

In view of its tumor suppressor function, the prognostic significance of STAT1β was evaluated in a cohort of 201 ESCC tumors. We found that STAT1β was not detectable in 94 (46.8%) cases, weakly expressed in 47 (23.3%) and strongly expressed in 60 (29.9%) cases. By comparing the STAT1β staining intensity (negative/weak or strong) and total STAT1 staining obtained from one of our previous studies **[6]**, we found a significant positive correlation ($R=0.765$, $p<0.0001$, illustrated in **Table 5.1**). The correlation of STAT1β and various clinical parameters was also assessed and the results are summarized in **Table 5.2**. We found that negative/weak STAT1β staining significantly correlates with the depth of

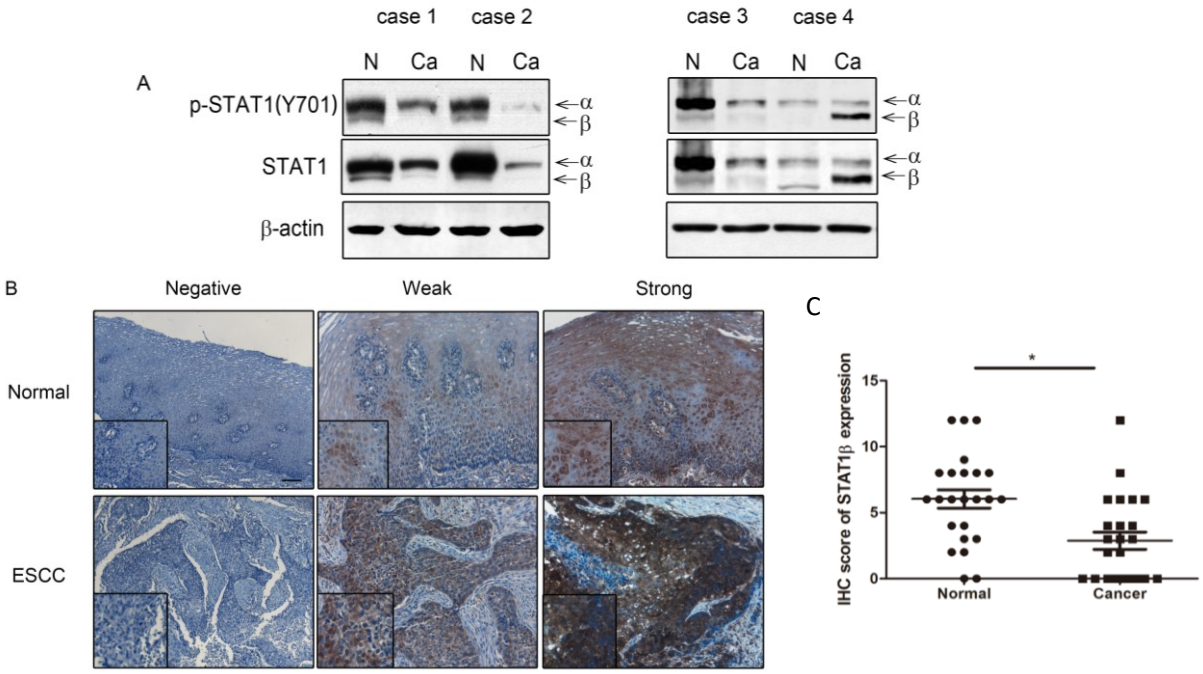

Figure 5.10 Expression of STAT1β in ESCC patient samples. A. By Western blots, STAT1β expression in ESCC tumors was examined. Compared to the benign esophageal tissue harvested at the surgical margins in the same specimens (labeled as N) cancerous tissues (labeled as Ca) often expressed a lower level of STAT1β (case 1-3). Some cases can be categrized into subset that expression of STAT1β in normal tissues is lower than cancer tissues (case 4). B. By IHC applied to formalin-fixed paraffin-embedded tissues, variable levels of STAT1β were detectable in esophageal epithelial and ESCC tissues. The staining was predominantly cytoplasmic. Based on the staining intensity, normal epithelial and tumors in our cohort was categorized into STAT1-negative, STAT1-weak or STAT1-strong (IHC stain, scale bar, 20 μm). C. IHC score of STAT1β was illustrated that the expression of STAT1β is higher in normal tissues compared to the cancer tissues.

Table 5.1 Correlation of STAT1 and STAT1β in 131 ESCC patient samples

STAT1β expression[#]	STAT1 expression		Result
	Negative/Weak	Strong	
Negative/Weak	67	17	
Strong	0	47	*R=0.765

*p<0.05

[#] These 131 patients is the same cohort as the patients in Chapter 1.

Table 5.2 Correlation STAT1β expression with clinical parameters in 201 ESCC cases

Parameter		Case number 201	STAT1β Expression by IHC		Result
			Negative/Weak	Strong	
Age	**≤57**	94	62	31	p=0.356
	>58	107	79	29	
Gender	**Male**	150	100	50	p=0.071
	Female	51	41	10	
Tumor site	**Upper**	15	8	7	p=0.190
	Middle	177	128	49	
	lower	9	5	4	
Differentiation	**Poor**	20	15	5	p=0.405
	Intermediate	95	68	27	
	Well	76	48	28	
Tumor size	**>5cm**	72	55	17	p=0.198
	<5cm	129	86	43	
Depth of invasion	**T1-T2**	45	21	24	p<0.001*
	T3-T4	156	120	36	
Lymph metastasis	**Yes**	96	75	22	p=0.045*
	No	105	67	38	
Clinical stage	**1**	22	11	11	p=0.026*
	2	76	53	23	
	3	91	71	20	
	4	12	6	6	

*p<0.05

#**Clinical stage** is based on the TNM classification for esophageal cancer

Abbreviation: T1: Tumor invades lamina propria, muscularis mucosae, or submucosa; T2: Tumor invades muscularis propria; T3: Tumor invades adventitia; T4: Tumor invades adjacent structures.

invasion ($p<0.001$), lymph node metastasis ($p=0.045$) and a high clinical stage ($p=0.026$). Clinical follow-up data was available in 130 of 201 cases included in this study (median follow-up, 21.5 months, and range 5-92 months). As shown in **Figure 5.11A**, patients with expression of total STAT1β^{strong} (n=43) had a significantly better clinical outcome compared to the STAT1β$^{negative/weak}$ (n=87, $p=0.025$). Moreover, we analyzed the survival of 74 patients categorized into 3 sub groups based on the differential expression of STAT1β and STAT1, STAT1^{strong}/STAT1β ^{strong}, STAT1^{strong}/STAT1β $^{negative/weak}$ and STAT1 $^{negative/weak}$/STAT1β $^{negative/weak}$. As shown in **Figure 5.11B**, STAT1^{strong}/STAT1β ^{strong} patients (n=27) have significantly longer survival time than the STAT1^{strong}/STAT1β $^{negative/weak}$ patients (n=7) and STAT1 $^{negative/weak}$/STAT1β $^{negative/weak}$ patients (n=40).

5.5 Discussion

We have previously demonstrated that STAT1 is a tumor suppressor in ESCC **[6, 7]**. Similar to STAT3 and STAT4, STAT1 has two isofoms, namely STAT1α and STAT1β. STAT1α is the full-length isoforms; it is considered as the physiological active form of STAT1, which has the transcription activity and is known to form complexes with other transcription factors to modulate gene expression in normal cells **[10]**. STAT1α has been reported to mediate various cellular activities, including cell growth inhibition, cell-cycle arrest and apoptosis **[6]**. In comparison,

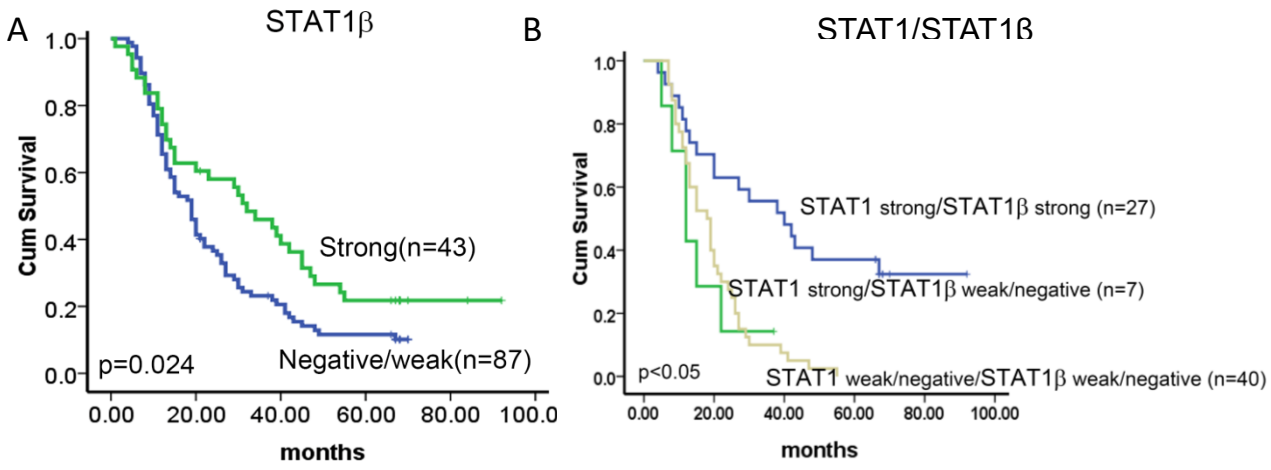

Figure 5.11 Kaplan-Meier curves of esophageal cancer patients in different STAT1β or STAT1/STAT1β expression subgroups. A. By Kaplan-Meier analysis, we found significant correlation between overall survival and the expression level of STAT1β, when the two groups were defined as STAT1-strong and STAT1-weak/negative. B. 74 ESCC patient samples were divided into 3 $STAT1^{strong}/STAT1\beta^{strong}$ (n=27), $STAT1^{strong}/STAT1\beta^{negative/weak}$ (n=7), and $STAT1^{negative/weak}/STAT1\beta^{negative/weak}$ (n=40) subgroups by the expression of STAT1 and STAT1β. $STAT1^{strong}/STAT1\beta^{strong}$ patients have a significant longer survival time than other two subgroups patients. This patient cohort (n=74) is the same as the patient cohort in Chapter 1.

the function of STAT1β has not been extensively studied. STAT1β is the truncated form of STAT1; it lacks a 38-amino acid segment that includes most of the transactivation domain and the functionally important residue, serine 727 **[13]**. Correlating with its truncated structure, it has been shown that STAT1β can bind to the promoter of genes, such as IRF1, LMP2 and TAP1, but it is transcriptionally inactive **[14, 15].**

As mentioned previously, STAT1β is believed to exert dominant negative effect on STAT1α, due to its lack of the transactivation domain and serine 727. This concept came from a study of human B cells, in which enforced expression of STAT1β was found to inhibit STAT1α activation by decreasing its phosphorylation at tyrosine 701, DNA binding, and transcriptional activity, as well as protecting the cells from fludarabine-induced apoptosis **[8]**. In keeping with this concept, infection with *M. tuberculosis* and *L. Mexicana* has been found to increase the expression and phosphorylation of STAT1β, leading to an inhibition of STAT1 signaling **[16, 17]**. In another study, there is evidence that the ratio of STAT1α/STAT1β may affect the sensitivity of cells to viral infection **[18]**. The mechanism of how STAT1β inhibits STAT1α is incompletely understood. One possible explanation is that STAT1β competes with STAT1α for the same DNA binding sites, but is incapable of activating the gene expression **[8, 16, 17]**. Another possible explanation is that STAT1β may compete with STAT1α for the same receptor sites, thus interfering

with STAT1α activation.

Results from a more recent study challenge the view that STAT1β is simply an inhibitor of STAT1α. Specifically, STAT1β was found to induce death in human B cell that is independent of p53 or STAT1α **[9]**. In a recently published paper, it was reported that STAT1β is transcriptionally active in response to IFN-γ; IFN-γ-induced tyrosine phosphorylation and promoter binding of STAT1 was prolonged in the absence of STAT1α **[10]**. In the same study, it also has been reported that STAT1β can induce the expression of many gene targets of STAT1 upon IFN-γ stimulation **[10]**.

Our findings from this current study also challenge the view that STAT1β is an inhibitor of STAT1α. For the first time, we found that STAT1β is effective in increasing the expression of STAT1α and potentiate its activation/phosphorylation mediated by IFN-γ. Indeed, we found that STAT1β substantially enhances the DNA binding and transcription activity of STAT1α in ESCC cells and STAT1β can modulate the expression of known STAT1 gene targets including IRF1, TAP1 and GPB2. Probably through these effects on STAT1α, STAT1β exerts tumor suppressor effects in ESCC. It is clear that our findings contradict those of a few previous studies of STAT1β, as discussed above. While the explanations for this discrepancy are not clear at this time, we consider that cell-type specificity is likely a contributing factor. Researchers also revealed that

prolonged phosphorylation of STAT1 after STAT1β transfection and this effect is related to a reduction of SOCS1, which is a negative regulator of the JAK-STAT1 pathway **[20]**. However, in our study, we did not found a significantly change of SOCS1 expression in ESCC cells after STAT1β transfection.

Our findings also led us to believe that the key mechanisms underlying the effects of STAT1β on STAT1α is related to its binding to STAT1α, thereby protecting it from being degraded via the ubiqutin-depedent pathway. In support of the concept that STAT1β potentiates the tumor suppressor effect of STAT1α, we found that the loss of STAT1β expression significantly correlates with a worse clinical outcome in a large cohort of ESCC patients. We have summarized our hypothetical model in **Figure 5.14**.

Similar to STAT1β, both STAT3β and STAT4β have been reported to be transcriptionally active and able to carry their unique functions **[21]**. There are at least two mechanisms underlying the transcription activity and biological function of STAT1β. One mechanism is that the beta isoforms of STATs can interact with other transcription factors that provide a C-terminal TAD. For example, STAT3β can cooperate with c-Jun to activate α-macroglobulin promoter **[22]**. Thus, gene transfection of *STAT3β* results in increasing STAT3α transcription activity **[23, 24]**.

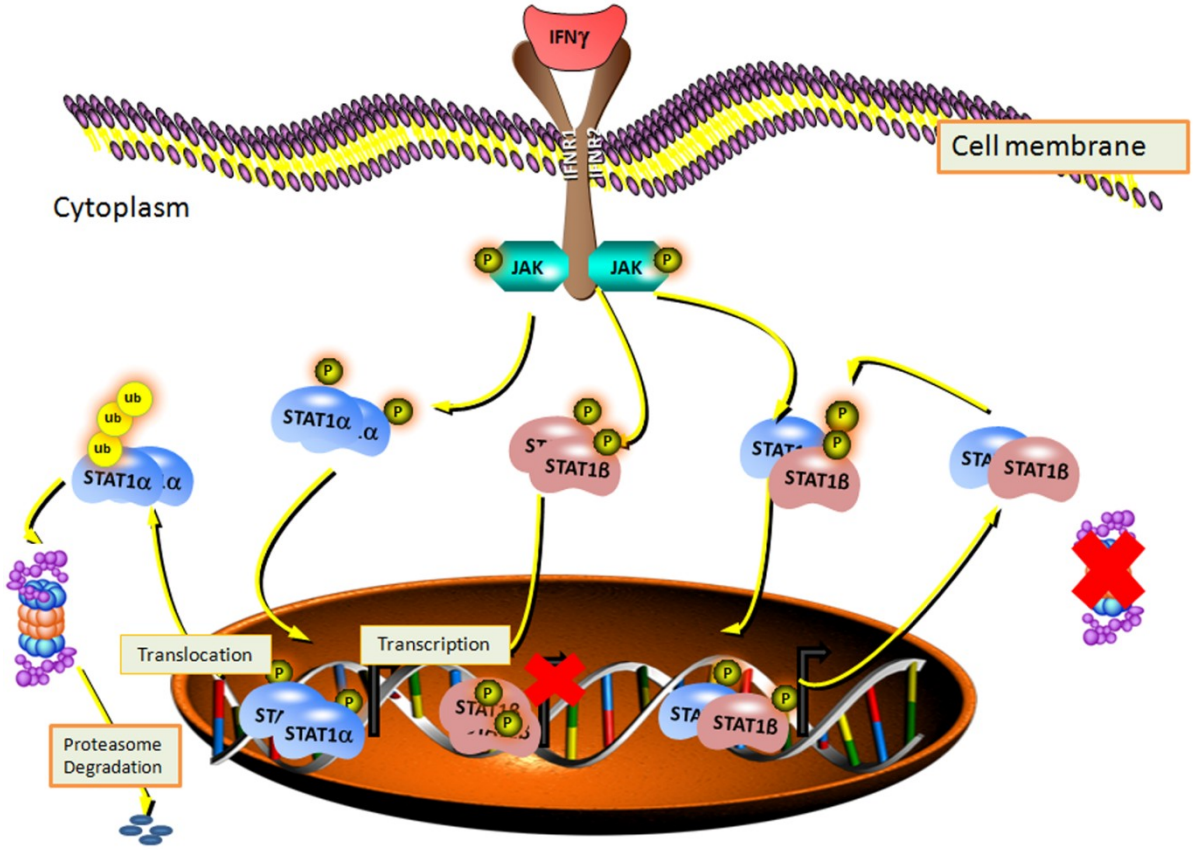

Figure 5.14 Schematic diagram of STAT1β interacts with STAT1α.

Upon IFN-γ stimulation, STAT1 can form three different dimers, α:α and β:β homodimers and α:β heterodimers. Those dimers can be phosphorylated and translocate into the nuclear. Once STAT1 releases from the target gene promoter, it returns to the cytoplasm and degraded by proteasome. However, the STAT1β can bind to STAT1α to protect it from the degradation, which can prolong the half-life of STAT1α to increase the transcription activity.

Another mechanism is that STAT1β still has the most of important functional domain. For example, the TAD contains tyrosine 701 phosphorylation residue which is essential for STAT1 various functions. The N-terminal domain can interact with CBP to exert a low affinity for it transcriptional activation **[24.25]**. The SH2 domains can participate in the dimerization of STAT1 to bind to the receptors **[26]**. The interaction of coiled-coil domain and the DNA binding domain can form antiparallel STAT1 dimers which translocate into nucleus and induce target genes expression **[27]**. Although STAT1β lacks the serine 727 phosphorylation site, it is likely that STAT1β can mediate some transcription activity **[24]**.

In our paper, we firstly used ESCC as a model to determine the biological function and clinical significance of STAT1β. The cells transfected with *STAT1β* were more sensitive to IFN-γ treatment and anti-tumor drugs, such as, 5-FU and cisplatin. Moreover, the analysis of ESCC patient samples further supported our hypothesis that STAT1β is a tumor suppressor in ESCC. We found that STAT1β protein level is significantly lower in ESCC tissues compared to the case-matched normal esophageal epithelial. Moreover, STAT1β expression was strongly related to the total expression of STAT1, which supported our hypothesis that STAT1β may stabilize the STAT1α. Another key finding of our study is that low STAT1β expression was significantly correlated with a worse clinical outcome, tumor invasion and lymph node metastasis in ESCC pateints.

This is the first report about the prognostic significance of STAT1β expression in human cancers.

However, we still have some limitation in this study. First, we didn't clearly demonstrate the elevation of luciferase activity, DNA binding of STAT1 and its downstram gene expression after STAT1β transfection was due to overexpressed STAT1β or enhanced STAT1α upon *STAT1β* transfection. It would be more convicing and reliable to generate stable cell lines that only have the STAT1β isoform expression. Second, knockdown STAT1α combined with enhanced STAT1β can be used to detect biological function of STAT1β in ESCC. Third, the *in-vivo* studies would favor to support our hypothesis. More detailed experimentation will be required to understand function of STAT1β isform.

In conclusion, for the first time, we investigated the biological function and clinical significance of STAT1β in human cancer, ESCC. STAT1β enhances the tumor suppressor function of STAT1 by increasing the expression and activation of STAT1α in ESCC cells. Our reulsts also indicated that STAT1β is downregulated in the ESCC carcinogenesis, which correlated with worse clinical outcome. Our study challenges the concept that STAT1β is a simply dominant negative regulator of STAT1, which may provide a new therapeutic target for treating ESCC patients.

5.6 Reference

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Chapter 6

General Discussion

6.1 Thesis overview

In this thesis, I have extensively evaluated the biological function and clinical significance of STAT1 in ESCC. I identified that STAT1 is a tumor suppressor in ESCC by regulating downstream genes expression and interacting with STAT3.

STAT1 carries a variety of cell biological function to inhibit tumorigenesis. Several previous studies have demonstrated that STAT1 is lost in various cancer types, such as breast cancer, head and neck squamous cell carcinoma **[1, 2]**. Enhanced STAT1 activation induces cell apoptosis and cell-cycle arrest by inducing its downstream molecules, such as cyclin D1, caspases, BCL-2 **[3]**. In addition, STAT1 activation is essential for tumor immunosurveillance and angiogenesis inhibition to enhance its anti-tumor function **[4, 5]**.

However, a small number of cancer studies demonstrated that STAT1 carries oncogenic function. For example, inappropriate activation of STAT1 were observed in a variety of malignant cells from breast cancer, melanoma, lymphoma and leukemia, suggesting that STAT1 may promotes tumor development under specific conditions **[6]**. In addition, activated STAT1 was reported to promote tumor transformation by interfering with the anti-tumor immune responses at different levels, such as, suppressing T cell function and inducing ionizing radiation **[7, 8]**. In

conclusion, despite most studies indicated STAT1 plays a tumor suppressor role in many cancer models, there are still some contradictory findings supported that STAT1 is a tumor promoter in some cancer types **[9]**.

Based on the literature review, I found very limited studies demonstrated the function of STAT1 in esophageal cancer **[10, 11]**. However, the biological function and clinical significance of STAT1 in ESCC has never been directly or comprehensively demonstrated. I hypothesized that STAT1 is a tumor suppressor in ESCC via distinct mechanisms, including the downstream gene expression regulation and STAT3 activation inhibition. In this thesis, I used a constitutively active form of STAT1 (STAT1C) to detect its biological function in ESCC. Then I detected that ERK is the key mediator to downregulate STAT1 in ESCC through proteasome degradation pathway. At last, the biological function of STAT1β was explored in ESCC. In contrast with previous published papers, we found that STAT1β interacted with STAT1α to protect it from ubiquitination, thus enhanced the tumor suppressor function of STAT1α. These findings supported our hypothesis that STAT1 is a tumor suppressor in ESCC, which provides the new therapeutic aspect to treat ESCC.

6.2 The importance of STAT1 in ESCC

In previous published papers, STAT1 function as a tumor suppressor in ESCC were supported by the observations that IFN-γ or EGF induced apoptosis in ESCC cell and this process correlated with STAT1 activation **[10,11]**. However, in my studies, I detected STAT1 function in ESCC by using a constitutively active form of STAT1, *STAT1C,* which is direct and convincing.

In Chapter 2 and 3, I detected the biological function and clinical significance of STAT1. As shown in previous chapters, the tumor suppressor role of STAT1 was supported for various reasons, First, I showed that enhanced STAT1 promotes cell death and inhibits cell proliferation in ESCC cell lines which is direct evidence. Second, enhanced STAT1 regulated several pro-apoptosis, anti-survival and cell-cycle biomarkers, such as BCL-2, BCL-xL, cyclin D1. The correlation of these biomarkers and STAT1 were also confirmed in a cohort of ESCC patient samples. Moreover, we found that STAT1 can directly inhibited STAT3 activation in ESCC, including its phosphorylation, DNA binding and transcription activity. This is the first report to talk about enhanced STAT1 inhibit STAT3 activation in esophageal cancer models.

In Chapter 4, I explored the machnisms of STAT1 downregulation in ESCC. First, I found that the proteasome inhibitor MG132 elevated the protein level of total STAT1 and p-STAT1, which revealed that STAT1 is

downregulate in ESCC due to ubiquitin-dependent degradation. Then, ERK inhibitor elevated protein level of STAT1 was found in both cell lines, indicating that ERK is a key mediator to downregulate STAT1 in ESCC. Similar findings were reported in MEF cell lines **[12]**. ERK signaling pathway has been report to be aberrant activate in tumors **[13]**. Our findings revealed that one of mechanisms that ERK plays as a tumor promoter is to downregulate STAT1, a well-known tumor suppressor. Furthermore, the patient samples study also confirmed our *in-vitro* findings that p-ERK and ERK expression was significantly correlated with STAT1 expression. The patients with p-ERK ^{strong}/STAT1$^{weak\ or\ negative}$ have the worst clinical results compared to other patients, meanwhile, p-ERK $^{weak\ or\ negative}$/STAT1 ^{strong} patients have the longest survival time.

6.3 Different roles of STAT1 isoforms in ESCC

The *STAT1* transcript generates two isoforms, STAT1α and STAT1β, a truncated form of STAT1. In Chapter 5, I identified the biological function and clinical significance of STAT1β. I found that STAT1β can interact with STAT1α to protect it from ubiquitination. The correlation of these two isoforms and the initial characterization of STAT1β have been described in a limited number of papers [**14-16].** These studies have shown that STAT1α and STAT1β have significantly different biochemical and biological properties. STAT1β was considered as a dominant negative

form of STAT1 by inhibiting STAT1α activation, including its DNA binding, phosphorylation and transcription activity in human B cells **[14]**. STAT1β can induce B cell apoptosis but the mechanism is indepedent of p53 and STAT1α **[15]**. However, in a recent published paper, it is found that STAT1β is not transcription inactive. Although STAT1β is not able to perform all the functions of STAT1α, it still induces majority of genes upon IFN-γ treatment **[16]**. Until now, the biological function of STAT1β has never been demonstrated in cancers. My thesis is the first one to talk about the STAT1β function in ESCC and revealed the new concept of STAT1α and STAT1β interaction.

6.4 Clinical significance of STAT1 in ESCC

In my thesis, I used a large cohort of ESCC patient samples to confirm our *in-vitro* findings. These samples were randomly collected from Chaoshan area which has the highest incidence of EC worldwide.

In Chapter 2, I found that STAT1 is downregulated in ESCC compared to the case-matched normal esophageal epithelial. The low expression of STAT1 was correlated with poor differentiation, lymph metastasis and poor survival in ESCC patients. In Chapter 3, I also found that the down-regulation of STAT1 was implicated in ESCC carcinogensis that the expression of STAT1 in benign esophageal epithelial tissues is significantly higher than carcinoma in situ tissues and case-matched

ESCC. These findings supported my hypothesis that STAT1 is a tumor suppressor and loss of STAT1 is contributor to ESCC tumorigenesis.

The prognostic of STAT1 in cancer has been reported in very limited papers with contradictory findings. Most studies stated that high expression of STAT1 predicted a better prognostic of patients in colorectal carcinoma and breast cancer **[17-19].** However, some papers reported that cancer patients with high STAT1 expression had a worse prognostic outcome, such as sarcoma **[20].** These distinct findings may due to the different cancer types and unique characters of patient groups.

In Chapter 5, I detected STAT1β expression in a large cohort of ESCC and found that STAT1β expression was significantly correlated with the expression of STAT1. STAT1β was lost in ESCC compared to the case-matched benign esophageal tissues. The expression of STAT1β significantly inversely related with depth of invasion, lymph node metastasis and worse clinical stage. The $STAT1\beta^{weak/negative}$ expression ESCC patients has worse clinical survival outcome compared to the $STAT1\beta^{strong}$ expression patients. This is the first prognostic study of the STAT1β that supported our *in-vitro* findings that STAT1β plays a tumor suppressor role in ESCC.

6.5 Limitations and future studies

The different studies in this thesis each have their own limitation and possible future directions. I have demonstrated them in the discussion part of different chapters. The key points are summarized as follows:

In Chapter 2, it would be interesting to detect STAT1 function in ESCC cells upon anti-tumor drug treatment, such as 5-FU and cisplatin. Thus, future study can be done by using colony formation assay and MTS assay to compare the chemoresistence of ESCC transfected with *STAT1C* or empty vector. The potential problem is that the chemotherapy drug is very toxic, so the concentration of the drug and the time length of treatment should be varied to get the best results. In addition, it would be more convincing to do some *in-vivo* studies. For example, to determine the function of STAT1 in ESCC tumorigenicity, severe combined immunodeficiency (SCID) mice will be injected by tet-on *STAT1* stable ESCC cell lines and feed the mice with different dose of doxycycline. The volume of tumor will be evaluated once a week and the number of macro metastases in the lung or lymph will be counted after 8 weeks.

In Chapter 3, the limited number of patients samples, i.e. 62 patients of ESCC, hindered the statistical analysis of certain categories. Some statistical analysis of immunohistochemistry markers in this group did not get a significant p-value due to the small size of samples. Thus, a larger size of ESCC patient samples should be used to identify the correlation of

biological markers and STAT1 in ESCC. Moreover, it would be interesting to plan further studies to identify that STAT3 expression in the same cohort of patient samples and analyze the correlations of STAT1 and STAT3 to confirm the *in-vitro* studies demonstrated in the Chapter 2.

In Chapter 4, I demonstrated that ERK promotes STAT1 ubiquitination and degradation in ESCC. However, I haven't detected the exact mechanisms that ERK promote STAT1 degradation, for example, some certain E3 ligases. The *in-vivo* study may favors to support the conclusion. The SCID mice can be injected the conditional stable ESCC cells with overexpressed ERK and empty control, the expression of STAT1 and ERK should be detected to confirm our hypothesis.

In Chapter 5, although I demonstrated the interaction of STAT1α and STAT1β, the function of STAT1β has not been comprehensively illustrated. It would be more interesting to detect the downstream genes of both STAT1α and STAT1β by using microarray assays. In addition, the conditional stable STAT1β ESCC cell lines would favor to explore the cross regulation of STAT1α and STAT1. The *in-vivo* study can also be proposed to support our *in-vitro* findings.

Overall, in all my studies, I didn't get enough information during the follow-up years, for example, the radiotherapy or chemotherapy after surgery. So, the statistical bias may affect the survival results. Multivariate

survival statistical analysis should be used to eliminate these factors.

6.6 Closing remarks

Overall, this thesis provided new insight into biological function and clinical significance of STAT1 in ESCC. To this point, I have identified the STAT1 expression in ESCC patient samples and its correlation with clinical parameters and survival data. Then, the *in-vitro* studies, I used the constitutively active form of STAT1 to detect the biological function of STAT1 in ESCC. Furthermore, I found that ERK is the key regulator to promote ubiquitin-depedent degradation of STAT1. The expression of ERK and STAT1 in a cohort of patient samples confirmed my *in-vitro* studies. In the last chapter, I explored the biological function and clinical significance of STAT1β in ESCC, which is the first report in cancer.

I believe that my work in this thesis can extend our knowledge about STAT1 function in cancer. This understanding of the STAT1 signaling pathway may provide us a new concept on the STAT1 in ESCC. The work shed the light on the further studies identifying JAK/STAT signaling pathway functions in ESCC, encouraging researchers to explore more specific methods and drugs to treat ESCC by targeting STAT1.

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