

## ABSTRACT

Title of Dissertation: IDENTIFICATION OF ESE-1 AS A NOVEL  
MOLECULAR TARGET OF  
CHEMOPREVENTIVE AGENTS FOR  
COLON CANCER PREVENTION

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Epithelial specific ETS-1 (ESE-1) is one of the E26 transformation-specific transcription factor superfamily and could be considerable interest as a possible target for regulating diverse types of human cancer. Despite its clinical importance, the reported biological role of ESE-1 on cancer development and progression are still controversial and its underlying mechanisms of tumorigenesis remains unclear. The objectives of this dissertation are to elucidate the role of ESE-1 in tumorigenesis. With the evidence in cancer phenotypes, the underlying mechanisms of ESE-1 in colon cancer was also investigated. ESE-1 knockout mice increased azoxymethane (AOM) - induced and dextran sulfate sodium (DSS)-promoted formation of aberrant crypt foci (ACF) compared to wild type mice. Overexpression of ESE-1 suppressed anchorage-independent growth and migration/invasion in human colon cancer cells and while knockdown of ESE-1 reversed anti-cancer activity. Full length ESE-1 was abundantly found in the nucleus, and internal deletion of nuclear localization sequence 2 (NLS2)

decreased the amount of nuclear ESE-1. Three lysine residues (<sup>318</sup>KKK<sup>320</sup>) in the NLS2 were critical for nuclear localization of ESE-1 and mediates tumor suppressive activity of ESE-1 through reduced  $\beta$ -catenin transcriptional activity. We identified two anti-cancer natural compounds, epigallocatechin-3-gallate (EGCG) and patchouli alcohol as ESE-1 inducers. Both EGCG and patchouli alcohol increased expression of ESE-1 protein and mRNA in human colon cancer cells. Patchouli alcohol showed reduced the number of tumors and tumor load in *Apc<sup>min/+</sup>* colon cancer animal model although protein expression level of ESE-1 did not show significant difference. These findings suggest a potential use of ESE-1 as a novel and potential molecular target of natural anti-cancer phytochemicals for colon cancer prevention.



IDENTIFICATION OF ESE-1 AS A NOVEL MOLECULAR TARGET OF  
CHEMOPREVENTIVE AGENTS FOR COLON CANCER PREVENTION

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## List of Abbreviations

ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)

ACF, aberrant crypt foci

AOM, azoxymethane

APC (Apc), adenomatous polyposis coli

ASK1, apoptosis signal-regulating kinase 1

CHOP, CCAAT-enhancer-binding protein homologous protein

CK1, casein kinase 1

Cox-2, cyclooxygenase-2

CRC, colorectal cancer

DNMT1, DNA methyltransferase 1

DSS, dextran sulfate sodium

Dvl, dishevelled protein

E1AF, adenovirus E1A factor

EGCG, epigallocatechin gallate

EGF, epidermal growth factor

Elf-1, E74-like factor-1

Elf3, E74-like factor 3

Elk-1, ETS Like-1 protein

EMT, epithelial to mesenchymal transition

EndMT, endothelial to mesenchymal transition

ER, Endoplasmic reticulum

ER $\alpha$ , estrogen receptor  $\alpha$

ERK, extracellular signal-regulated kinases

ERT, ETS-related transcription factor

ESE-1, epithelial Specific ETS-1

ESX, epithelial-restricted with serine box

ETS, E26-transcriptional

FzD8, frizzled receptor 8

Grp78, glucose-regulated protein 78 kDa

GSK3 $\beta$ , glycogen synthase kinase 3  $\beta$

H2AX, H2A histone family member X

IBD, inflammatory bowel disease

IRE $\alpha$ , inositol-requiring enzyme  $\alpha$

JNK, c-Jun N-terminal kinase

LRP5/6, low-density lipoprotein receptor-related protein 5/6

LEF, lymphoid enhancer factor

MAPK, mitogen-activated protein kinase

MMP-9, matrix metalloproteinase 9

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Net, new ets transcription factor

PA, patchouli alcohol

PFA3, polyomavirus enhancer activator 3

PI3K, phosphoinositide 3-kinase

PNT domain, pointed domain

PTEN, phosphatase and tensin homolog

PU.1, purine-rich sequences 1

R-PG, recombinant protein G Protein

Sap-1, SRF accessory protein-1

TAZ, Yes-associated protein (YAP) homolog transcriptional co-activator

TCF4, T-cell factor 4

TCGA, The Cancer Genome Atlas

TET1, ten-eleven translocation methylcytosine dioxygenase 1

TERT, telomerase reverse transcriptase

TNBC, triple negative breast cancer

TRAF2, TNF Receptor Associated Factor 2)

TXA2, Thromboxane A2

uPA, urokinase-type plasminogen activator

UPR, unfolded protein response

VEGF, vascular endothelial growth factor

XBP, x box protein

# Chapter 1: Literature Review and Current Problem

## 1.1 Introduction

Cancers are defined as diseases in which abnormal cells divide in an uncontrolled manner and invade other tissues. Cancer cells can spread to other parts of the body through the blood and lymphatic systems.

Cancer is the one of the most life-threatening chronic diseases worldwide. In the United States, cancer is the second most common cause of death, and colon cancer in particular is the third leading cause of death. Despite its prevalence, a holistic understanding of the mechanisms underlying colon cancer remains lacking. In addition, the complexity of cancer makes identification of the exact cause of a specific cancer difficult. To understand cancer mechanisms, research over several decades has examined factors causing cancer and revealed that inherited genetic factors and occupational, environmental, medical and other exposures contribute to cancer risk, because of the complexity of cancer (1).

Cancer progress is aggressive, and the survival rate is low if patients are diagnosed in late stages. Therefore, prevention and treatment of cancer in early stages are crucial. Chemoprevention is one way to cure cancer in early stages. Because cancer occurs as a result of abnormal and uncontrolled cell growth, chemopreventive agents might lower the risk of cancer in less aggressive ways compare to traditional chemotherapy.

Targeted therapy is new paradigm in chemoprevention research. Targeted therapy targets the cancer's specific genes or proteins, or the tissue environment that

contributes to cancer growth and survival. The basic strategy of targeted therapy is more focused on accuracy than traditional chemoprevention. Targeted cancer therapies are drugs/treatments designed to address the genetic modifications necessary for growth and progression of cancer, thus preventing cancer cell survival, growth and spreading. Molecular targets for targeted therapies or drugs are specific alterations that distinguish cancer cells from normal cells. Unlike traditional cancer therapies, targeted therapies work within specific types of cancer through blocking specific survival signaling pathways as well as certain targets responsible for maintaining the tumor environment.

Therefore, to examine more precise mechanisms for targeted therapy, this dissertation focuses on the identification of the biological function of the tumor suppressor gene ESE-1 in colon cancer and of dietary/plant-based compounds that may target ESE-1.

## 1.2. Cancer and human health

### 1.2.1. Cancer statistics

Colorectal cancer is the third most frequently diagnosed cancer and also the third leading cause of death in both men and women, among all cancer types in the United States (2). Overall, colorectal cancer incidence has been decreasing for several decades, owing to changes in risk factor exposures and the use of screening. However, both the incidence and mortality rates of colorectal cancer have increased in people younger than age 55, thus indicating that colorectal cancer remains a world-wide problem. In detail, the incidence rates have increased up to 1.8% annually among those



younger than age 55. The death rate declined by 2.7% every year among individuals ages 55 and older, but increased by 1% every year among adults younger than age 55, from 2007 to 2016 (2). These statistics indicate that colon cancer is not a chronic disease due to aging only in older people. The rate of increasing diagnosis of colon cancer in young age groups indicates a requirement for timely evaluation of persistent symptoms in all patients. Because early-stage colorectal cancer typically does not have symptoms, screening is usually necessary for early detection of this cancer. Consequently, both early detection and advance prevention are necessary in colon cancer. Overall, the 5-year relative survival rate for colon cancer is 65%; only 39% of patients are diagnosed with localized disease, and their 5-year survival rate is 90% (3).

#### 1.2.2. Cancer development and hallmarks of cancer

There are four steps in cancer development: initiation, promotion, progression and metastasis. In the initiation step, cancer initiators are metabolized to active forms by drug-metabolizing enzymes. The active initiators directly react with DNA, thus resulting in DNA alteration, including point mutation, deletion, insertion, reversion and chromosomal rearrangement. DNA mutations are irreversible and result in abnormal cell growth driven by cancer promoters, and the same genetic mutations are also transferred to daughter cells in the promotion stage.

Cancer promoters bind receptors on the cell surface and consequently affect intracellular pathways that increase cell proliferation. The resultant cancers are known as benign tumors because their growth rate remains slow, and the cancer cells still adhere to neighboring cells and do not cause invasion and/or metastasis.

Transformation of a benign tumor into malignant neoplasia occurs in cancer progression, in which the cancer cells exhibit rapid growth and aggressive and invasive properties that cause metastasis. The final step in cancer development is metastasis, in which cancer cells spread from one organ/part to another in the body. Metastasis occurs with angiogenesis, thus generating new blood vessels to supply nutrients. Metastatic cells lose adhesion to neighboring cells; they are locomotive and degradative, and they completely escape control by the immune system.

Hallmarks of cancer have been proposed and categorized as follows: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (4). The first four of the six hallmarks are in the original tumor. Cancer cells are characterized by self-sufficient growth signals, which cause cells to grow even in the absence of growth signals (5). Stimulatory signals, such as diffusible growth factors, extracellular matrix components and cell-to-cell adhesion/interaction molecules are essential for cell proliferation. Many oncogenes in cancer cells induce normal growth signaling in various ways: tumor cells produce diverse growth signals and consequently reduce their dependence on stimulation from the tissue microenvironment. Most cancer cells acquire the ability to synthesize growth factors, thus creating positive feedback signaling (6). The second hallmark is the loss of sensitivity to anti-growth signals: cancer cells avoid signals that halt growth. The third hallmark is anti-apoptosis: cancer cells evade apoptosis, programmed cell death, in normal environments. By escaping apoptosis, cancer cells acquire unlimited replicative potential: all malignant cancer cells are able to maintain their telomeres and thus eventually generate more mutations.

The other two hallmarks, which are angiogenesis and tissue invasion/metastasis, occur outside the original tumor site. Because cancer cells are unable to survive without nutrients or blood supply (~100 microns distance), the formation of new blood vessels must be initiated to support their growth, in a process known as angiogenesis. Some primary tumor cells leave their original locations and move to surrounding tissue areas or even travel to distant sites in the body, thus forming new colonies and secondary tumors; this invasion/metastasis ultimately results in death rather than the primary tumor.

### 1.3. Colon cancer development

Genetic events in colorectal tumorigenesis have been suggested. Sequential events have been proposed from initiation to promotion (normal epithelium to carcinoma). Adenomatous polyposis coli (APC) is a tumor suppressor gene whose mutation initiates colorectal tumorigenesis (7). APC protein regulates expression of many oncogenic target genes through modulating expression of  $\beta$ -catenin and T cell transcription factor 4 (TCF4) and their physical interaction. Mutant APC genes in colorectal cancer result in malfunctions in this activity. For example, wild type APC genes have tumor suppressive effects via inactivation of  $\beta$ -catenin (phosphorylated  $\beta$ -catenin) causing proteasomal degradation of  $\beta$ -catenin (8); in contrast, accumulation and translocation of  $\beta$ -catenin into the nucleus acts on target genes that are primarily oncogenes.

Regulation of cyclooxygenase-2 (Cox-2) is also involved in relatively early stages of colorectal tumorigenesis after promotion (pre-malignant adenoma stage). Cox-

2 promotes carcinogenesis via the conversion of arachidonic acid into prostaglandins and thromboxanes with malonaldehyde synthesis and hydrogen peroxide production. Cox-2 products activate serpentine receptors coupled to heterotrimeric G-proteins (R-TXA2 and R-PG), which are connected to oncogenic signaling elements (9). Therefore, Cox-2 participates in polyp formation in early stages of cancer by promoting cancer cell proliferation, anti-apoptosis mechanisms, angiogenesis, cell invasion and metastatic processes. Cox-2 related mechanisms have been established as a targeted therapy in human tumorigenesis (9).

K-Ras mutations are common in colon carcinogenesis (10), and approximately 35% of carcinomas and adenomas show K-Ras mutations in the colon (11). Interestingly, higher frequencies of those mutations are associated with adenoma size and changes in dysplastic severity, thus indicating that Ras mutations are common in early stages of colorectal tumorigenesis. These mutations might lead to increases in polyp size, owing to a selective growth advantage, in what may be an essential prerequisite for malignant transformation (11).

p53 genes are commonly mutated, but those genetic events rarely occur in adenomas. Both p53 genetic mutations and allele loss are more frequent in the transition from benign to malignant growth (12).

In summary, APC, Cox-2, K-Ras, PI3K and p53 are associated with colon cancer, and the first three gene mutations are all classified as early drivers in both adenomas and carcinomas (13); the most commonly mutated genes are APC (87.5%), KRAS (43.8%), TP53 (43.8%) and PIK3CA (25.0%). The roles of these mutations in cancer are supported by previous studies.

## 1.4. Signaling pathways related to carcinogenesis

### 1.4.1. Wnt/ $\beta$ -catenin signaling

Canonical Wnt signaling is widely known in carcinogenesis. Wnt ligand is a glycoprotein that binds Frizzled receptors, thus forming a large complex with LRP5/6 on the cell surface. The activated complex of Wnt receptor stimulates removal of GSK-3 $\beta$  from a regulatory APC/Axin/GSK-3 $\beta$ -complex. Without the Wnt ligand (off-state),  $\beta$ -catenin is phosphorylated by CK1 (14), and the APC/Axin/GSK-3 $\beta$ -complex carries out ubiquitination and proteasomal degradation  $\beta$ -catenin of through the  $\beta$ -TrCP/Skp pathway (15,16). In the presence of Wnt ligand (on-state), the co-receptor LRP5/6 is transferred to a complex with Wnt-bound Frizzled. Sequential phosphorylation, poly-ubiquitination and polymerization activate Dishevelled (Dvl), thus removing GSK-3 $\beta$  from APC/Axin.  $\beta$ -catenin is stabilized in the cytosol and translocated to the nucleus thorough Rac1 and other factors. Nuclear  $\beta$ -catenin then binds LEF/TCF transcription factors, thereby removing co-repressors and recruiting co-activators to Wnt target genes. In addition,  $\beta$ -catenin interacts with other transcription factors and regulates specific target genes. The  $\beta$ -catenin binding proteins include AXIN1, Androgen receptor, CBY1, CDH1, GSK3B, MUC1, PCAF, PSEN1, SMAD7, SLC9A3R1 and VE-cadherin. These data indicate a significant role of these binding proteins in regulating the transactivation of  $\beta$ -catenin.

#### 1.4.2. MAPK/ERK pathway

The MAPK/ERK pathway (Ras-Raf-MEK-ERK pathway) is a sequence of reactions occurring after cells receive a signal from a receptor on the cell surface. The binding of a signaling molecule to the receptor on the cell surface initially triggers the signal, and the pathway ultimately results in changes in protein expression associated with cellular events, such as cell division. Many proteins are involved in the pathway, including mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/ERK) which activate/inactivate proteins through phosphorylation.

MAPK signaling affects the hallmarks of cancer. The p38MAPK pathway is considered to result in tumor suppression in different cell lines and *in vivo* mouse knockout models (17,18). The role of p38MAPK as a tumor suppressor results from the inhibitory effects of p38 $\alpha$  and p38 $\beta$  isoforms on G0, G1/S and G2/M cell-cycle-checkpoint controls (19), thus resulting in growth arrest and apoptosis (17,20,21) or cellular senescence (22,23). These findings suggest that inactivation of the p38MAPK pathway promotes cellular transformation through regulation of cell proliferation and survival. p38MAPK suppresses tumorigenesis and shows inhibitory effects against malignant phenotypes. Overexpression of p38MAPK in rhabdomyosarcoma cells results in inhibition of cell proliferation and induction of terminal differentiation (24). In addition, the anti-tumorigenic effect of p38MAPK might also modulate cell invasion (25). p44/42MAPK and ERK are also MAPK/ERK pathway proteins.

As previously described, mitogen signaling plays an important role in cell cycle progression, especially in regulating cell cycle entry and proliferation. The ERK pathway is involved in integrating external signals from mitogens (such as EGF) into

signaling events inducing cell growth and proliferation in mammalian cells (26). Mitogens and growth factors activate canonical receptor tyrosine kinases including EGFR, after receptor dimerization and subsequent small GTPase Ras activation (27). Downstream events in the MAPK cascade (Raf-MEK-ERK) eventually lead to phosphorylation and ERK activation. Phosphorylated (activated) ERK induces phosphorylation of its many downstream targets that regulate cell proliferation (28). However, ERK has a dual function: activation and overexpression have been reported in cancer (29,30). Anti-proliferative effects, apoptosis and autophagy through sustained ERK activity in specific cellular environments have also been reported (31). These findings indicate that different strategies for cancer therapies might be considered, depending on the cellular events present.

#### 1.4.3. ER stress pathways in cancer

As described in the previous section, cancer cells immortally divide; therefore, controlling the growth of tumor cells may be a key chemopreventive agent. ER stress has dual effects on cancer: ER stress can enhance the growth of tumors but can also affect cytotoxicity, thus resulting in apoptosis. Under ER stress, cancer cells induce expression of Cox-2 via NF- $\kappa$ B pathways, in an anti-apoptotic manner. However, ER stress also activates pro-inflammatory NF- $\kappa$ B through CHOP, and IL-8 is retained in epithelial cells (32,33). Apoptosis can also be induced by ER stress pathways. The caspase-12 cysteine protease family members are involved in ER-stress-mediated apoptosis with the ER membrane, but they are not induced by other non-ER factors (34).

There are three representative ER-stress pathways in cancer: Grp78, PERK and IRE $\alpha$ . Grp78 is an ER chaperone protein that is active and highly expressed in different types of cancer cells (35,36). In cancer systems, Grp78 regulation plays a key role in cell apoptosis, proliferation, invasion, inflammation and immunity (37). Grp78 phosphorylates FAK and increases invasion by phosphorylation of p190PhoGAP and inhibition of Rock kinase (38). Translocation of Grp78 occurs in colorectal cancer on the cell surface, and Grp78 cooperates with beta 1-integrin in promoting cell-matrix adhesion (39). Translocation of Grp78 occurs in colorectal cancer on the cell surface, and Grp78 cooperates with beta 1-integrin in promoting cell-matrix adhesion (38). Insulin-like growth factor binding protein-3 triggers the primary localization of Grp78 in the ER. During ER stress, however, some Grp78 is also localized to the cytosol (40), nucleus and mitochondria (41).

Second, PERK/eIF2 $\alpha$  participate in the regulation of tumor initiation and survival. PERK is a trans-ER membrane serine/threonine protein kinase with an N-terminal ER luminal domain and a cytoplasmic C-terminal protein kinase domain (42). PERK phosphorylates Nrf2 transcription factor 89 and eIF2 $\alpha$ . Phosphorylated eIF2 suppresses translation of most transcripts (43) although it induces the translation of certain mRNAs such as ATF4 (44). Phosphorylated Nrf2 is released from an E3 ligase complex and localized to the nucleus, where Nrf2 generates enzymes that remove intracellular ROS (45). Therefore, PERK is essential to maintaining cellular redox homeostasis and decreasing ROS-generated genotoxic stress. PERK may be a regulator of cancer cell growth. Environmental stress downregulates protein synthesis through



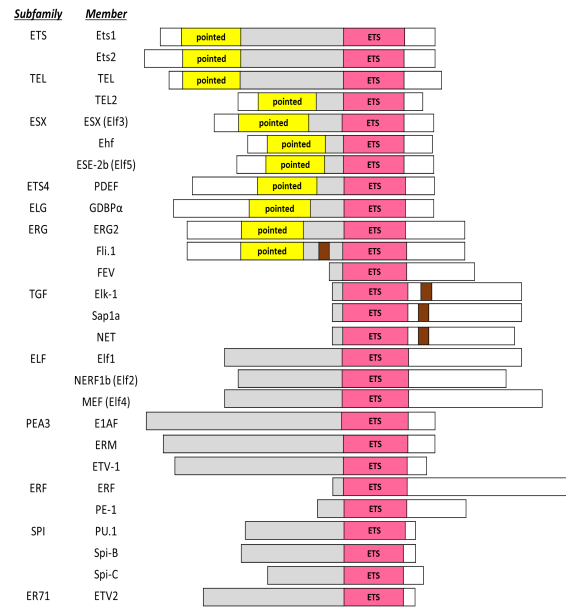
inhibition of PERK and phosphorylation of eIF2 $\alpha$ , thus regulating ATF4, which is associated with cancer cell proliferation and survival (46).

Finally, IRE1 $\alpha$ /XBP in cancer play a major role during ER stress. Under ER stress, IRE1 $\alpha$  is activated via oligomerization and autophosphorylation, thus leading to activation of IRE1 $\alpha$  endoribonuclease, which cleaves and begins splicing XBP1 mRNA (47). Interestingly, the unfolded protein response results in cell apoptosis under prolonged and uncompensated stress (48). Another pathway has also been suggested: IRE $\alpha$  is phosphorylated (active) and subsequently binds TRAF2 and activates ASK1, thus eventually resulting in activation of JNK and p38, causing ER-stressed related cell death (49,50). In addition, the IRE1 $\alpha$  and TRAF2 pathway plays a key role in mitochondria-independent apoptosis by activation of procaspase-4 (51).

## 1.5. E26 transcriptional factors (ETS) proteins and ESE-1 (Epithelial specific ETS protein-1)

### 1.5.1. General Characteristics and biological function of ETS proteins

ETS transcription factors are members of a family with homology and sequence similarity in their conserved DNA binding domains. Approximately 30 ETS proteins have been identified on the basis of structural ETS domain similarity, and ETS proteins play crucial roles in regulating physiological and pathological processes, including differentiation, embryogenesis,



**Fig. 1.1. Structure of ETS family proteins**

(52-54). Many ETS family proteins are downstream targets of signal transduction pathways that alter their DNA binding, transcriptional activity, interaction with cellular partners, subcellular localization and/or protein stability (55). ETS family members are further sub-classified into several types on the basis of sequence similarity of the ETS domain and the presence of further conserved domains (Fig 1). The overall conserved sequence is usually associated with similar molecular and biological functions of ETS-domain protein sub-family members.

The ETS transcription factors have homology and sequence similarity in their DNA binding domains and are characterized by a highly conserved 85-amino acid

motif called the ETS DNA binding domain, which regulates binding to purine-rich DNA sequences with a core GGAA/T motif and additional flanking nucleotides (56). Most ETS family proteins have their ETS domain, a winged helix-turn-helix structural motif, located in the C-terminal region. However, some ETS family proteins, such as the TCF subfamily, have the ETS domain in the N-terminal regions. Beyond the conserved ETS domain, a subgroup of ETS family proteins contain another developmental conserved domain (PNT domain) in their N-terminal regions; the helix-loop-helix structure of this domain is used for protein-protein interactions (57). Some ETS family proteins such as Fli-1 possesses two available domains for transcriptional activation at their N-and C-terminal regions, although the activation at the C-terminal region is not as strong as that at the N-terminal domain (58). The specific domain and amino acid sequences influence binding affinity and specificity: the ETS domain and the flanking amino acid sequences affect binding affinity (59), and the modification of a single amino acid in the ETS domain alters its DNA binding specificity (60). DNA sequences flanking the GGAA/T central core sequence also change the DNA binding affinity of ETS family proteins. Flanking DNA sequences as well as purine-rich core DNA sequences are major determinants of the primary binding of specific ETA family proteins.

The expression patterns of ETS family proteins differ. Several ETS family proteins, including Elk-1, Sap-1, Net and Net-b, which are part of the TCF superfamily, are ubiquitously expressed (61). However, some ETS family proteins are expressed in a tissue-specific manner. For example, PFA3/E1AF are primarily expressed in epithelial cells, such as those in the mammary gland, epidermis and brain, but not in

hematopoietic cells (62). Elf-1 is expressed in hematopoietic cells and developing epithelial tissues, but ESE-1/ESX which belongs to the same Elf subfamily, is specifically expressed only in epithelial cells and may be involved in epithelial cell differentiation (63). PU.1 is expressed exclusively in B-cells, macrophages and neutrophils (64).

In addition, each ETS protein has unique biological activity in different types of chronic diseases, owing to the specific environment of each type of cell. The up- or downregulation of specific ETS family proteins is associated with chronic human diseases including diabetes (65), inflammation (66), obesity, and chronic atrial fibrillation (67). The deregulation of the ETS domain proteins by either inappropriate expression or expression as fusions with other proteins is thought to be crucial in disease pathogenesis and generation. Several ETS domain proteins have been reported to be associated with cancer, as described in the next section.

#### 1.5.2. ETS proteins and cancer

ETS-domain proteins act as either activators or repressors of transcriptional factors, and many of them are targeted to promoters by specific DNA-protein and protein-protein interactions. Many ETS-domain proteins are targets of signal transduction pathways and respond to a variety of extracellular stimulations.

ETS-domain transcriptional factors have distinct biological functions in regulating cellular proliferation and differentiation during both embryogenic development and the adult stage. In addition, the deregulation of either the expression

or function of ETS-domain transcription factors by genetic modification often leads to tumorigenesis (55).

Therefore, ETS family proteins play a major role as oncogenes or tumor suppressor genes in diverse tissue areas. As shown in Table 1, diverse subfamilies of ETS proteins show pro-oncogenic or tumor suppressor effects in different cancers, through various mechanisms. Most ETS family proteins have been identified as oncogenes, as shown in Table 1, but some of them have also been reported to be tumor suppressor genes. For example, ETS-1 has oncogenic function in some tissue areas (68-72), but has tumor suppressor effects in colon cancer (73,74). As indicated in Table 1, ETS-2, TEL, ESE-3, ESE-2, PDEF and Fli1 also show either oncogenic or tumor suppressor effects in different tissues/cell lines.

Interestingly, some ETS proteins have been reported to have both pro-tumorigenic and tumor suppressor roles in the same tissue. For example, oncogenic (75) and tumor suppressor (76) effects of ESE-1 have been reported in non-small cell lung cancer cells. As also shown in Table 1, the role of ESE-1 in cancer still remains unclear, owing to conflicting results.

Table. 1.1 Role of ETS family proteins in cancer

| Subfamily | Member | Tissue/cell line | Mechanisms                                                                                                                                                                        | Role             |
|-----------|--------|------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
| ETS       | ETS-1  | breast           | Promotion of invasion by MMP-9 (77), angiogenesis (69), estrogen receptor $\alpha$ -dependent cell growth/tumorigenesis (78), parathyroid hormone related protein activation (79) | oncogene         |
|           |        | prostate         | Upregulation of MDR1 & MMP-9 (70)                                                                                                                                                 | oncogene         |
|           |        | lung (NSCLC)     | Twist-1 activation (72), induction of tumorigenesis through endothelial-like differentiation (80)                                                                                 | oncogene         |
|           |        | melanoma         | Non-nuclear TERT expression (71), activation of Mcl-1 via XBP-1 (ER stress) (81)                                                                                                  | oncogene         |
|           |        | bladder          | increase in cell migration and invasion (82)                                                                                                                                      | oncogene         |
|           |        | T cell           | Upregulation of CCR7 (68)                                                                                                                                                         | oncogene         |
|           |        | thymocytes       | Cell cycle deregulation and induction of thymocyte differentiation tumor invasion through binding of Rb1 E2F to the ETS-1 promoter (83)                                           | oncogene         |
|           |        | colon            | Apoptosis induced by caspase-1 (73), reduced tumorigenic phenotypes (74)                                                                                                          | tumor suppressor |

|       |                   |                      |                                                                                                    |                  |
|-------|-------------------|----------------------|----------------------------------------------------------------------------------------------------|------------------|
| ETS-2 |                   | prostate             | p63 pathway (84)                                                                                   | oncogene         |
|       |                   | hypopharygeal        | MAPK/p38/Erk/JNK (85)                                                                              | oncogene         |
|       |                   | renal                | EMT via PI3K/Akt (86)                                                                              | oncogene         |
| TEL   | TEL               | lung (NSCLC)         | Correlation with poor prognosis (87)                                                               | oncogene         |
|       |                   | NIH 3T3              | Induction of apoptosis by downregulation of Bcl-xL (88), repression of stromelsin transcripts (89) | tumor suppressor |
|       | TEL2              | nasopharyngeal       | Downregulation of SERPIN1 promoter (90)                                                            | tumor suppressor |
| ESX   | ESX (ESE-1, Elf3) |                      | (described in section 1.5.4.)                                                                      |                  |
|       | ESE-3/EHF         | pancreas             | Upregulation of E-cadherin (91)                                                                    | tumor suppressor |
|       |                   | prostate             | Induction of apoptosis by procaspase-3 (92)                                                        |                  |
|       |                   | ovary                | Regulation of ERK/Akt signaling (93)                                                               | oncogene         |
|       | ESE-2/Elf5        | breast               | Inhibition of EMT by repressing snail (94)                                                         | tumor suppressor |
|       |                   | renal carcinoma      | Only phenotypes affected (95)                                                                      | tumor suppressor |
|       |                   | myeloid leukemia     | Inhibition of p21/p53(96)                                                                          | oncogene         |
|       |                   | ovary                | Inhibition of invasion and migration by EMT, PI3k/Akt (97)                                         | tumor suppressor |
| ETS-4 | PDEF              | gastric              | Only phenotypes affected (98)                                                                      | tumor suppressor |
|       |                   | bresat (ER negative) | Upregulation of Myc genes via MAD1 degradation (99), association with poor prognosis (100), p21    | oncogene         |

|     |               |                   |                                                                                                                                                  |                  |
|-----|---------------|-------------------|--------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
|     |               |                   | inhibition through DNA methylation (101)                                                                                                         |                  |
|     |               | prostate          | Integrin $\beta$ 3 pathway (upstream of talin and paxillin) (102), downregulation of stathmin (103)                                              | tumor suppressor |
|     |               | bladder           | Inhibition of EMT via down-regulation of N-cadherin, snail, slug and vimentin (104)                                                              | tumor suppressor |
|     |               | colon             | Target gene regulation (p21 & uPA) (105)                                                                                                         | tumor suppressor |
| ELG | GABP $\alpha$ | prostate          | Androgen receptor related target gene activation (106)                                                                                           | oncogene         |
| ERG | Fli1          | breast            | TET1 & DNMT1 regulation (107), anti-apoptosis via Bcl-2 (108)                                                                                    | oncogene         |
|     |               | endothelial cells | EndMT inhibition (109)                                                                                                                           | tumor suppressor |
|     |               | leukemia          | Notch 1 pathway signaling (110)                                                                                                                  | oncogene         |
|     |               | sarcoma           | DKK-1 inhibition and $\beta$ -catenin/TCF transcript activation (111), inhibition of thrombospondins (112), repression of TGF- $\beta$ RII (113) | oncogene         |
| TGF | Elk-1         | thyroid           | Early grow response-1 and PTEN (114)                                                                                                             | oncogene         |
|     |               | lung              | Wnt/ $\beta$ -catenin activation (115)                                                                                                           |                  |
|     |               | Renal             | NADH dehydrogenase 1 $\alpha$ subcomplex 4-like 2 (116)                                                                                          |                  |
| ELF | Elf1          | breast/ovary      | Activation human pygopus 2 promoter (117)                                                                                                        | oncogene         |



|      |          |                                        |                                                                                                   |                  |
|------|----------|----------------------------------------|---------------------------------------------------------------------------------------------------|------------------|
|      |          | nasopharyngeal carcinoma               | CCL2/CCR2 signaling (118)                                                                         | oncogene         |
|      | MEF/Elf4 | (Various)                              | Prevention of DNA damage (119)                                                                    | oncogene         |
| PEA3 | E1AF     | breast                                 | Progression of cell cycle via induction of cyclin D3 (120)                                        | oncogene         |
|      |          | NSCLC                                  | Activation Rho/Rho associated kinase (121)                                                        | oncogene         |
|      |          | CRC                                    | Overexpression of MMP-1 and matrilysin (122)                                                      | oncogene         |
|      | ETV1     | prostate                               | TGF- $\beta$ regulated smad signaling (123), $\beta$ -catenin signaling (123)                     | oncogene         |
|      |          | gastric/gastrointestinal stroma tumors | EMT via upregulation of snail (124)/ KIT regulation (125)                                         | oncogene         |
| SPI  | PU.1.    | leukemia                               | Increase in cell proliferation and inhibition apoptosis (126), Cooperation with IRF4 & IRF8 (127) | tumor suppressor |
| ER71 | ETV2     | glioblastoma                           | Development of endothelial transdifferentiation (128)                                             | oncogene         |

### 1.5.3. Biological function of ESE-1

ESE-1 is an epithelial-specific ETS-1 and is thus named epithelial-specific ETS-1 (63). ESE-1 has been given different names according to the characteristics used for its identification. The name Jen resulted from screening of a cDNA library in human foreskin keratinocytes and identification of a conserved 3' region of the ETS domain (129). The name ESX was based on identification of sequence tags with an 8 amino acid motif in the C-terminal region of the ETS domain for epithelial restricted with serine box (130). The name ERT was reported on the basis of a yeast two-hybrid system used to screen a human placenta cDNA library for ETS-related transcription factor (131). Finally, the name Elf3 was reported on the basis of its belonging to the ELF subfamily in the ETS transcription factor family (132).

ESE-1 is highly expressed in epithelia rich tissue, such as the gastrointestinal tract, whereas it is not expressed in monocytes, lymphocytes, endothelial cells or fibroblasts (63). ESE-1 controls late stages of differentiation in epidermal cells (133). As a transcription factor, ESE-1 regulates the expression of target genes involved in diverse biological processes including cell division, differentiation, apoptosis and tissue development (134). ESE-1 has a multidomain structure, including an N-terminus pointed domain (PNT) implicated in protein-protein interactions and a C-terminal ETS domain. In addition, three other domains (TAD, SAR and AT hook) are located between the PNT and ETS domain in tandem (63). The presence of these motifs indicates that ESE-1 may possess biological activities through interaction with other proteins rather than its nuclear transcriptional activities. The identification of such

interacting partners may enhance understanding of the mechanism(s) through which ESE-1 affects biological functions.

#### 1.5.4. ESE-1 and cancer

The biological function of ESE-1 in cancer remains unclear, because most studies have shown conflicting results. For example, ESE-1 expression is correlated with poor prognosis in breast cancer (135) and colorectal cancer (52), whereas the downregulation of ESE-1 expression is associated with reduced survival in ovarian cancer (136). In *in vitro* conditions, ESE-1 expression induces transformation of MCF-12A human mammary epithelial cells and anchorage-independent growth (132,137,138). ESE-1 is highly expressed in human primary and metastatic tumors (132,139), and it transforms phenotypes *in vitro* and *in vivo* in mammary epithelial cells (140,141). Colon cancer cell proliferation was inhibited by knockdown of ESE-1 (52), whereas ESE-1 facilitates cell growth arrest and apoptosis in colon cancer cells (54), and inhibits the invasion of oral squamous cell carcinoma (53). ESE-1 expression has also been described to lead to two conflicting findings in prostate cancer cells: ESE-1 expression mediates the development of phenotypes transformation (139), whereas ESE-1 represses androgen-receptor action and growth/migration of prostate tumors (142). These contradictory results strongly support multiple and dichotomous roles of ESE-1 in cancer development and progression (143).

Recently, two independent analyses of exome sequence have reported that ESE-1 is a substantially mutated driver gene in ampullary carcinoma. ESE-1 mutations have been observed in 9.5% of bile duct carcinoma cases (144): in patients with ampullary

carcinoma, 13.3% have somatic mutations in ESE-1, including frameshift, nonsense and missense mutations; moreover, knockdown of ESE-1 is associated with aggressive invasion and EMT (145). In addition, duodenal and distal bile duct adenocarcinoma is identified by a high frequency of inactivating mutations in ESE-1 (146). Recent RNA-Seq analysis has indicated that ESE-1 antagonizes the Wnt and Ras oncogenic signaling pathways, and The Cancer Genome Atlas (TCGA) and protein localization in colorectal cancer tumor panels have shown that ESE-1 is negatively associated with EMT markers and is correlated with clinical prognosis in patients with colon cancer (147). ESE-1 downregulation is also associated with decreased survival of patients with ovarian cancer, and it negatively regulates EMT (136). ESE-1 has also been shown to inhibit invasion of oral squamous cell carcinoma via controlling the transcriptional activity of matrix metalloproteinase 9 (MMP-9) (53).

Even within the same type of cancer cells, ESE-1 expression has been found to produce conflicting results. For example, in prostate cancer, ESE-1 expression regulates transformation of phenotypes (139) but also suppresses tumorigenesis via the inhibition of androgen receptor transcriptional activity (142). In the context of breast cancer, ESE-1 knockdown attenuates the growth of HER2<sup>+</sup> cells (148), but overexpression of ESE-1 decreases the growth of MCF7 cells by repressing estrogen receptor- $\alpha$  (ER- $\alpha$ ) (149). In studies of non-small cell lung cancer (NSCLC), overexpression of ESE-1 has been found to facilitate cell growth and metastasis in A549 cells (75), but to suppress growth and invasion/migration in ESE-1 deficient H1299 and H1703 cells (150). In colon cancer, ESE-1 is associated with decreased cell growth and tumorigenicity (52), and it also mediates anti-cancer drug-induced

apoptosis (54). These contradictory results strongly support multiple and dichotomous roles of ESE-1 in cancer development and progression (143). A detailed literature review on ESE-1 and cancer is provided in Table 2.

#### 1.5.5. ESE-1 and colon cancer

ESE-1 is highly expressed in epithelial cells (63) and it is involved in terminal epithelial cell differentiation (133). The first report on ESE-1 as an oncogene in colon cancer proposes a mechanism of  $\beta$ -catenin signaling (52). According to that study, ESE-1 binds to  $\beta$ -catenin and transactivates promoter of  $\beta$ -catenin. Consequently, c-myc, VEGF, CCND1, MMP-7, and c-jun are regulated.

However, conflicting results have been found for ESE-1 in other studies. Despite its original function in normal epithelial cell differentiation, whether ESE-1 is pro-tumorigenic or a tumor suppressor gene remains unclear.

Table 1.2. Role of ESE-1 in cancer

| Role             | Tissue/Cell lines                | Mechanisms                                                                                                                                          |
|------------------|----------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|
| Oncogene         | breast                           | Akt & cyclin D1 regulation (148), increased EMT (138), activation of malignancy-associated promoter (151), activation of mTOR & cyclin D1 (135)     |
|                  | lung (NSCLC)                     | PI3K/Akt & Erk signaling pathway (75)                                                                                                               |
|                  | prostate                         | NFκB activation (139)                                                                                                                               |
|                  | CRC                              | β-catenin transactivation (52)                                                                                                                      |
|                  | Epithelial in ulcerative colitis | NFκB activation (152)                                                                                                                               |
|                  | thyroid                          | MAPK/Erk & PI3K/Akt signaling pathways (153)                                                                                                        |
|                  | liver                            | EMT by protecting ZEB1(154)                                                                                                                         |
| Tumor suppressor | breast                           | Apoptosis induced by nuclear localization (141), induction of TGF-β-II receptor (155), decrease in ERα DNA binding affinity by binding to ERα (149) |
|                  | lung (NSCLC)                     | Inhibits cell growth and invasion (76)                                                                                                              |
|                  | prostate                         | Repression of androgen receptor (142)                                                                                                               |
|                  | CRC                              | Negative regulator of ZEB1 (156)                                                                                                                    |
|                  | bladder                          | EMT suppressor (157)                                                                                                                                |
|                  | ampullary cancer                 | Negative correlation with Wnt (146)                                                                                                                 |
|                  | oral squamous cell carcinoma     | Suppression of MMP-9 (53)                                                                                                                           |

## 1.6. Anti-cancer compounds as ESE-1 inducer

### 1.6.1. Chemoprevention and phytochemicals

High consumption of fruits and vegetables is negatively correlated with cancer prognosis. Fruits and vegetables contain phytochemicals, which have diverse beneficial health effects, such as anti-cancer, anti-inflammatory and/or anti-obesity effects. Notably, their applications in chemoprevention have developed in recent decades.

Studies have examined carotenoids such as  $\beta$ -carotene, lycopene, lutein and zeaxanthin in red, orange and green fruits and vegetables. A variety of studies have indicated anti-cancer activities of carotenoids in different types of cancer cells/models. For example, the anti-cancer activity of  $\beta$ -carotene in colon cancer has been described (158). Lycopene also showed anti-tumorigenic activity in prostate cancer (159). Lutein also inhibited tumorigenesis in breast cancer (160), and zeaxanthin has been reported as an anti-cancer carotenoid in human uveal melanoma *in vivo* (161). Although this section describes representative studies on each carotenoid, many studies have shown anti-cancer activities with diverse mechanisms in different cancer areas.

Flavonoids such as anthocyanins and quercetin also possess anti-cancer activities. Those compounds are commonly found in berries and grapes (anthocyanins), and onions (quercetin). Anthocyanin treated cells show less cell proliferation and migration in lung cancer (162). Many studies have also reported anti-cancer activities of quercetin, including in breast and prostate cancer (163,164).

Indole-3-carbinol is abundant in cruciferous vegetables such as cabbages and broccoli. Many research groups have reported its anti-cancer activity in diverse types

of cancer, including breast cancer (165). One of its metabolites, 3,3'-diindolylmethane, has been found to have anti-tumorigenic effects in several studies (166,167).

Phytic acid is a six-fold dihydrogenphosphate ester of inositol (specifically the myo isomer), also known as inositol hexakisphosphate (IP6) or inositol polyphosphate. The major phytate anion stores phosphorus in many plant tissues, especially bran and seeds. It is also found in many legumes, cereals and grains (168). One beneficial health effect of phytic acid is its anti-cancer activity in colorectal cancer (169).

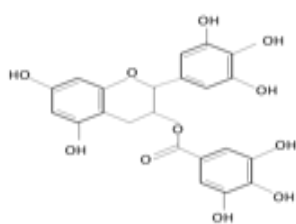
Isoflavones are polyphenolic compounds that include daidzein and genistein. In cancer studies, daidzein has been found to inhibit tumorigenesis by reducing FGFR3 pathways in bladder cancer (170), and genistein results in G2/M arrest in colorectal cancer and apoptosis in hepatocytes (171,172). Isothiocyanates are naturally synthesized through enzymatic conversion of glucosinolate, which is also known as mustard oil. Isothiocyanate has been found to decrease cancer cell proliferation *in vitro* and *in vivo* (173). Resveratrol, a phytochemical found in grape skins or red wine, has been reported by several research groups to have anti-cancer activity through diverse mechanisms (174,175).

Unlike anti-cancer drugs, dietary anti-cancer compounds and natural phytochemicals usually have less toxicity and fewer side effects. In addition, synergistic effects of combinations of dietary compounds have also been reported (176); therefore, the format of consumption, including combination, should be considered.



### 1.6.2. Green tea compound, EGCG

Green tea has been widely consumed by large populations of people worldwide for several thousand years, and it is considered a healthful beverage. Green tea extract contains many polyphenolic compounds, epigallocatechin gallates, epicatechin and flavonols (177). Epigallocatechin gallate, also known as epigallocatechin-3-gallate



**Fig. 1.2. Chemical structure of EGCG**

(EGCG), is made through esterification of epigallocatechin and gallic acid. EGCG is the most abundant catechin in tea.

The beneficial health effects of EGCG include protection against obesity (178) and inflammation (179). Because of its beneficial effects, EGCG has been used in the food

supplement industry.

EGCG is also a well-known anti-cancer compound in several human cancer cell types (180-188). In particular, many research groups have reported an anti-cancer effect of EGCG in colon cancer and have elucidated the anti-cancer mechanisms in human colon cancer cells and/or *in vivo* animal models. EGCG induces apoptosis via the inhibition of Akt and induction of the ERK pathway (189), and the effects of EGCG on colon tumorigenesis involve AMPK (190). EGCG inactivates IGF-1 (191), HER & COX-2 expression (192). In addition, EGCG upregulates the Nrf2-UGT1A signaling pathway (193) and inhibits EGFR at Ser 1046/1047 via activation of p38 MAPK (194). EGCG treatment inhibits Met signaling (195) and induces p53-mediated apoptosis associated with VEGF and AMPK (196). Other mechanisms of the anti-cancer effect of EGCG include down-regulation of Wnt signaling (197) and induction of growth arrest/apoptosis after EGCG treatment via p53-p21-PUMA growth arrest/apoptosis

(198). Several studies have used *in vivo* animal rodent models: EGCG has been found to be effective against colon tumorigenesis in AOM-DSS combination ICR mice (199). Because of its strong anti-cancer activities and relatively lower toxicity, EGCG has been commonly used for prevention of cancer.

### 1.6.3. Essential oils and their health beneficial activities

Essential oils were used as ancient medicine. Because of their unique aromatic characteristics, however, the usage of essential oils has primarily focused on fragrance/perfume applications. An essential oil is a concentrated hydrophobic liquid containing volatile chemical compounds from plants. “Essential” indicates that an essential oil contains the specific plant species’ characteristic fragrance. Essential oils have become increasingly popular in aromatherapy, an alternative medicine using essential oils. In the aromatherapy industry, essential oils are used as massage oils, in diffusers or candles, and as skin treatments.

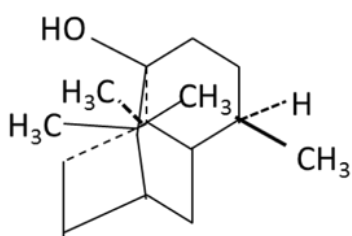
Because essential oils are used in aromatherapy on the basis of their fragrance characteristics, the effects of inhalation of essential oils have been reported (200,201). Essential oils are also helpful for pain relief (202), and inhalation of essential oils might also assist cancer patients under chemotherapy (203). According to the traditional usage of essential oils, anti-microbial activity against bacteria (204) and repellent effects against insects (especially mosquitos) (205) have also been studied and reported.

Beneficial health effects of either inhalation or oral consumption of essential oils have also been reported, and anti-oxidative (206) and anti-inflammatory (207) activities of essential oils have been investigated. Many groups of essential oils exist,

owing to the diverse plant sources and/or their extraction methods. Therefore, the spectrum of essential oil studies can be widely ranging and exploring novel findings regarding essential oils and their active chemical components holds promise.

Terpenes are an essential oil component used by plants as a repellent to protect against predators. The anti-cancer activities of terpenes such as perillyl alcohol (208), limonene (209) and carnosol (210) have been reported, and some of these compounds are chemically synthesized for industrial purposes. Diverse studies involving encapsulation of those hydrophobic compounds have been reported (211,212).

#### 1.6.4. General characteristics of patchouli alcohol



**Fig. 1.3. Chemical structure of patchouli alcohol**

Patchouli (*Pogostemon cablin*) is a plant species from the genus *Pogostemon*. The plant originates from tropical Asia. The intense fragrance of patchouli has long been used in perfume industry.

Patchouli is currently used in alternative medicines, cosmetics, incense and insect repellents. The major chemical compound of patchouli oil is patchouli alcohol (patchoulol), a sesquiterpene alcohol. Patchouli alcohol ( $C_{15}H_{26}O$ ), a naturally occurring tricyclic sesquiterpene, is the critical biological active constituent in the patchouli oil extracted from *Pogostemon cablin*. However, although patchouli alcohol has been demonstrated to possess multiple beneficial pharmacological properties, such as anti-inflammatory (213), antioxidative

(214), antitumor (215,216), antimicrobial (217), insecticidal (218), and antiatherogenic effects (219), practices using patchouli alcohol are novel in the medical field.

#### 1.6.5. Health beneficial effect of patchouli alcohol

Patchouli alcohol is a relatively novel compound in chronic disease studies. There are no reports on metabolism related chronic diseases of patchouli alcohol, and its effects in other chronic diseases have not been fully investigated.

According to previous studies, the effects of patchouli alcohol in human health can be categorized into those on inflammation and cancer, especially in the colon and lung (which have epithelial tissue origins). The anti-inflammatory activity of patchouli alcohol has been reported *in vitro* (213,219,220) *in vivo* animal models (221,222). We have recently identified patchouli alcohol as an anti-cancer compound in colon cancer (223). Patchouli alcohol decreases cell growth by activating p21 and inhibiting cyclin-dependent kinase 4 (CDK4) and cyclin D1 (215). In addition, Lu et al. (216) have proposed an anti-cancer effect of patchouli alcohol in lung cancer via induction of cell apoptosis through regulation of the EGFR-MAPK pathway.

Interestingly, in contrast to the findings of these two studies, patchouli alcohol has been found to be protective against *Helicobacter pylori* urease-induced apoptosis, oxidative stress and inflammatory responses in human gastric epithelial cells (224). In addition, according to pharmacokinetic studies and acute toxicity studies, patchouli alcohol does not show toxicity at low (20 mg/kg body weight) and high (80 mg/kg body weight) doses (225,226). Therefore, this indirect evidence suggests that patchouli alcohol may be an anti-cancer compound that selectively affects cancer.

## Chapter 2: Identification of ESE-1 as a tumor suppressor in colon cancer

### 2.1. Abstract

Colon cancer is one of the third leading factor of cancer related to death in the United States and identifying promising molecular target is essential for effective chemoprevention. Epithelium specific ETS-1 (ESE-1) belongs to the E26 transformation specific superfamily and its role in any types of cancer still remains contradictory and unclear. The biological function of ESE-1 in colon cancer was investigated in this chapter. ESE-1 knockout transgenic mice model with azoxymethane (AOM) & dextran sulfate sodium (DSS) combination model and orthotopic implantation mice model were used to elucidate the role of ESE-1 in colon cancer. ESE-1 knockout mice showed higher aberrant crypt foci (ACF) formation in the colon and implantation of ESE-1 overexpressing colon cancer cells showed lower metastasis rate in lung and liver *in vivo*. SW480 ESE-1 overexpressing stable cell lines were adopted for *in vitro* study. Anchorage independent cell growth, cell migration and invasion were reduced in the presence of ESE-1 in SW480 human colorectal cells. Apoptosis and G1 arrest were induced in ESE-1 overexpressing stable cells and DNA damage related gene were also induced in the presence of ESE-1. Tumor suppressor effect of ESE-1 was also confirmed under loss of function condition. HCT116 ESE-1 knockdown stable cells showed higher anchorage independent cell growth ( $p=0.09$ ), migration and invasion. Taken together, ESE-1 was identified as a tumor suppressor gene in colon cancer.

## 2.2. Introduction

New emerging trend of cancer research is focused on targeted therapy that prevents cancer by modulation of the molecules responsible for cancer progression. Targeted therapy requires understanding mechanisms of target molecules and the actual role in specific cancer. Therefore, understanding biological function of gene in specific cancer is significant.

Colon cancer is the third leading cancer in terms of mortality and prevalence in the US. In addition, once colon cancer is progressed and under the late stage of tumorigenesis, the possibility of death due to colon cancer might be significantly higher. Therefore, prevention of cancer ahead of colon cancer progression is critical for colon cancer treatment/prevention.

In this dissertation, we focused on ESE-1, one of the E26 transformation-specific (ETS) family of transcription factors. ETS family proteins include the ETS DNA binding domain, a highly conserved 85-amino acid motif. More than thirty ETS proteins have been identified and they are involved in the regulation of pathological processes including inflammation and neoplasia. ESE-1 is also one of the ETS family factors which is specifically expressed in epithelial cells, including small and large intestines. The ESE-1 protein contains multiple domains, including an ETS domain, a Pointed domain, an A/T hook domain, a Transactivation (TAD) domain and a Serine- and Aspartic acid-rich (SAR) domain, as described in detail in Chapter 1 & 3.

As indicated in Chapter 1 (section 1.5.4), there are conflicting evidence of ESE-1 as an either tumor suppressor or an oncogene. However, in spite of its clinical importance, little is known about the mechanisms by which the effects of ESE-1 on

oncogenesis are decided in any type of cancer. Moreover, there has been no *in vivo* study providing direct evidence for the role of ESE-1 in cancer. The purpose of this chapter study is to examine the biological function of ESE-1 in the initiation and progression of colon cancer focusing on its phenotypes.

## 2.3. Materials and Methods

### 2.2.1. Materials

The V5 antibody (R96025) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies for ESE-1 (sc-376055), tubulin (sc-5286), U1snRNP70 (sc-9571), and actin (sc-1615) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ESE-1 shRNA glycerol bacterial stock (TRCN0000013865, Clone ID: NM\_004433.3-4184s1c1, Sequence: CCGGGCCATGAGGTACTACTACAAACTCGAGTTTGTAGTAGTACCTCATG GCTTTTT) was purchased from Sigma Aldrich (St. Louis, MO, USA). Cell culture media were purchased from Invitrogen (Carlsbad, CA, USA). All chemicals were purchased from Fisher Scientific (Hampton, NH, USA) or VWR (Radnor, PA), unless otherwise specified.

### 2.2.2. Cell culture and established stable cell lines

Human colon carcinoma cell lines (HCT116, SW480, HCT15, HT29, LoVo, and Caco2) and human embryonic kidney cells (HEK293) were purchased from the

American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM/F-12 media supplemented with 10% fetal bovine serum (FBS) and a mixture of penicillin (100 U/mL) and streptomycin (100 µg/mL) under a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. Stable cell lines expressing LacZ (control) or full length were established by transfecting expression vectors and subsequently selecting using G418 (800 µg/mL for selection and 400 µg/mL for maintenance) as described previously (76). Knockdown stable cell lines were established using HCT116 cell line and control and ESE-1 shRNA and subsequently selecting using puromycin (2 µg/mL for selection and maintenance).

#### 2.2.3. Transient transfection and luciferase assay

The expression vectors (V5-tagged ESE-1) and luciferase reporters (ESE-1 promoter) were transiently transfected using Lipofectamine 2000 (Thermo Fisher Scientific) or the PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturer's instruction. For luciferase assays, cells were lysed in 1x Passive Lysis Buffer for 15 minutes, and the enzymatic activity of luciferase was measured using a luminometer (Promega, Durham, NC, USA). Enzyme activity was normalized to that of pRL-null using a dual luciferase assay system (Promega) as described previously (227).

#### 2.2.4. Western blot

The cells were washed two times with cold PBS and incubated with radio-immunoprecipitation assay (RIPA) buffer containing a protease/phosphatase inhibitor cocktail (Sigma) for 30 minutes before being centrifuged at 14,000 g for 10 minutes at



4 °C. Protein concentration was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific). SDS-PAGE was performed to separate proteins based on size. After transfer to nitrocellulose membranes, a specific primary antibody was added in TBS containing 0.1% Tween 20 (TBS-T) and 3% BSA, and incubated overnight at 4 °C. Subsequently, the membranes were incubated with horse radish peroxidase (HRP)-conjugated IgG for one hour at room temperature, washed three times with 1X TBS-T (0.1% Tween-20), and visualized using enhanced chemiluminescence (ECL) (Amersham Biosciences, Piscataway, NJ, USA). The image was taken using a ChemiDoc Imaging system (BioRad Laboratories, Hercules, CA, USA).

#### 2.2.5. Soft agar assay

SW480 ( $2 \times 10^3$  cells) and HCT 116 ( $5 \times 10^2$  cells) were plated with 0.6% agarose gel in complete media onto a 0.6% agarose gel bottom layer. Cells were incubated at 37 °C and 5% CO<sub>2</sub> with complete media added onto the plate every other day. Afterwards, cells were stained overnight at 37 °C with 200 µL of 1 mg nitroblue tetrazolium chloride/mL PBS. The image was taken using a ChemiDoc Imaging system (BioRad Laboratories).

#### 2.2.6. Cell migration and invasion assay

Cell migration and invasion assays were performed as previously described (139). For the migration assay, stable cell lines ( $6 \times 10^5$ ) were plated onto a six-well plate. After reaching confluency, cells were scratched with pipette tips and washed

three times with phosphate buffered saline before taking the first image. Microscope images were taken every 24 hours to observe cell migration. The *in vitro* invasion assay was performed using Matrigel. Briefly,  $1 \times 10^6$  cells were plated onto 24 well invasion chamber in serum free media, and the chambers were inserted onto DMEM containing 10% FBS (chemoattractant). After 48 hours, non-invading cells on the upper membrane surface were removed using cotton swab, invading cells on the bottom surface were fixed using 100% methanol for 2 minutes and stained using 1% toluidine blue in 1% borax for 2 minutes. After washing the chambers, the membranes were cut and mounted on the immersion oil. The images were taken under microscope.

#### 2.2.7. *In vivo* tumorigenicity

The *in vivo* study using azoxymethane (AOM)-treated mice was approved by the Animal Care and Use Committee (University of Maryland) and performed as described previously (228). Briefly, ESE-1 transgenic mice (*ESE-I*<sup>+/-</sup>) (133) were bred to produce wild type (*ESE-I*<sup>+/+</sup>) and heterozygous knockout mice (*ESE-I*<sup>+/-</sup>). We excluded homozygous knockout mice (*ESE-I*<sup>-/-</sup>) due to low breeding and survival rates. Mice were fed a standard chow diet throughout the experimental period. At six weeks of age, azoxymethane (10 mg/kg body weight) was injected once intraperitoneally, and then 2% dextran sulfate sodium (DSS) was given in the drinking water for one week. At 26 weeks of age, the mice were euthanized by CO<sub>2</sub> asphyxiation; their colons were removed, flushed with 0.9% saline, and slit open longitudinally and fixed in 10% formaldehyde solution. Fixed samples were then stained with 0.2% methylene blue,

and the numbers of aberrant crypt foci (ACF) were counted under a dissecting microscope.

#### 2.2.8. Orthotopic implantation of cancer cells: *in vivo* metastasis

The *in vivo* study using surgery method for implantation of human colon cancer cells was approved by the Animal Care and Use Committee (University of Maryland). Four week-old athymic immunosuppressive male nude mice (B6.Cg-*Foxn1*<sup>nu/J</sup>; Cat#: 000819) were purchased from Jackson lab (West Grove, PA, USA). At 6 week old age, the mice were anesthetized with isoflurane and SW480 control (LacZ) or ESE-1 stable cell lines ( $1 \times 10^6$ ) in 50  $\mu$ L of PBS were injected in to the spleen using 30-gauge needle. After injection, the abdominal cavity and skin were closed with surgical sutures. At the age of 12-week, all animals were sacrificed. The number and size of tumors in the spleen, liver and lung were counted and measured.

#### 2.2.9. Cell cycle distribution

SW480 stable cells ( $6 \times 10^5$ ) were plated onto 6 well plate and incubated for 48 hours. All cells were collected using trypsin EDTA and centrifuged at 1000 rpm for 5 min. Precipitated cells were washed using PBS, then 70% ethanol/PBS was slowly added. Cells were incubated on ice for 30 min, washed with PBS twice, and mixture of propidium iodide (70  $\mu$ M) and RNase (1 mg/mL) was added. Cell cycle distribution was analyzed using the cell cycle distribution was analyzed using FACS flow

cytometry at the University of Maryland MPRI Flow Cytometry and Cell Sorting Facility.

#### 2.2.10. Cell death detection/Apoptosis

The amount of cleaved DNA/histone complexes (nucleosomes) was measured using Cell Death Detection ELISA Kit (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer's instruction and previous study (229). The extract was obtained from SW480 stable cell lines mixed with immune reagent for 2 h. After washing, the extract was incubated with 100  $\mu$ L of ABTS solution for 10 min. The absorbance was recorded at 405 and 490 nm in the same microplate reader described above.

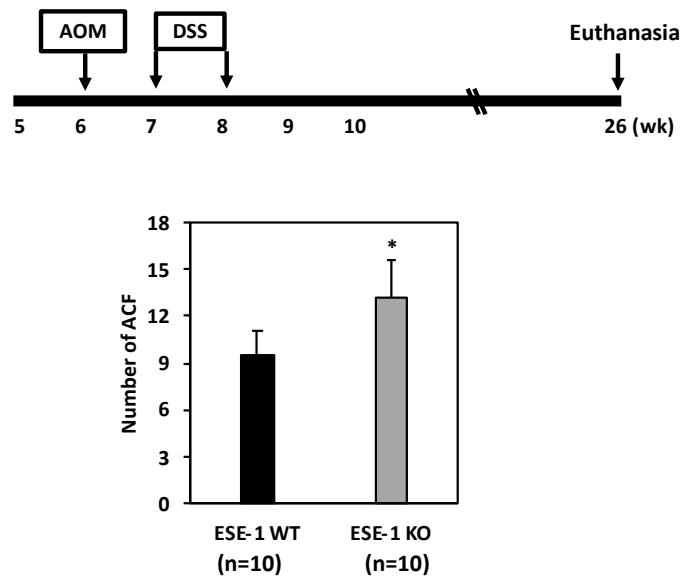
#### 2.2.11. Statistical analysis

Statistical analyses were conducted using Student's unpaired t-test with statistical significance set at  $*P < 0.05$ .

## 2.4. Results

### 2.3.1. ESE-1 Knockdown increases formation of aberrant crypt foci (ACF) in an azoxymethane (AOM)-induced dextran sulfate sodium (DSS)-promoted mouse model

Despite the importance of the biological role of ESE-1 in cancer, no studies have investigated the effect of ESE-1 on tumorigenesis using a transgenic mouse model. To test whether ESE-1 knockout influences chemically-induced tumorigenicity of colon tissues, an AOM-induced and DSS-promoted colon cancer mouse model was employed. As shown in Fig. 2.1, a significant increase in the number of ACF was observed for ESE-1 knockout mice (*ESE-1*<sup>+/-</sup>) compared to wild type (*ESE-1*<sup>+/+</sup>).



**Fig. 2.1. ESE-1 knockdown increases formation of aberrant crypt foci (ACF) in an azoxymethane (AOM)-induced and dextran sulfate sodium (DSS)-promoted mouse model.** Ten wild-type (*ESE-1*<sup>+/+</sup>) and ten knockout mice (*ESE-1*<sup>-/-</sup>) were injected with AOM (10 mg/kg body weight) and then exposed to 2% DSS for seven days in drinking water. ACF numbers were counted from colon and rectum.

\* Significant compared to control at  $p < 0.05$ .

### 2.3.2. ESE-1 overexpression reduced metastasis *in vivo*

Colorectal cancer is metastatic and rapidly spreads to adjacent tissues including lung and liver. To investigate the effect of ESE-1 on metastasis *in vivo*, ESE-1 overexpressing SW480 stable cells were implanted into spleen by surgery. Those implanted cells are supposed to spread to the liver and lung. Metastasis rate (%) was calculated by:

$$[\text{the number of tumors in the group} / \text{total number of tumors in organ}] \times 100 (\%)$$

As indicated Table 2.1, mice implanted with ESE-1 overexpressing cells showed lower metastasis rate compared to mice implanted with control cells in both liver and lung.

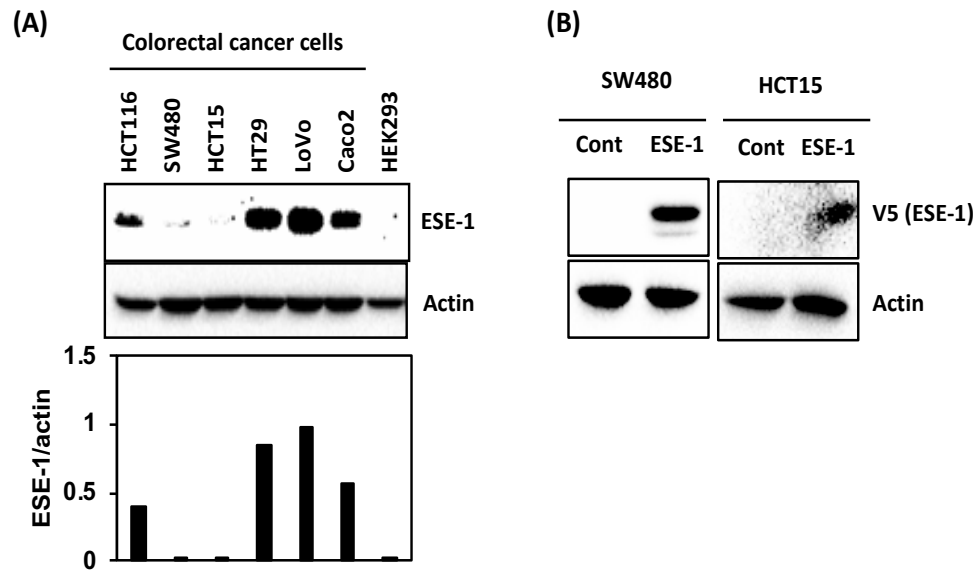
**Table 2.1. Metastasis rate of liver and lung in mice implanted with ESE-1 overexpressing SW480 stable cells.**

| Group (n = 10) | metastasis rate, % |       |
|----------------|--------------------|-------|
|                | Cont               | ESE-1 |
| Liver          | 60                 | 10    |
| Lung           | 30                 | 20    |

SW480 stable cells ( $1 \times 10^6$ ) were orthotopically implanted into 6 week-old nude mice spleen. At age of 12 weeks, all animals were sacrificed and tumors in liver and lung were analyzed.

### 2.3.3. Basal expression of ESE-1 in different human colon cancer cells and establishment of ESE-1 overexpressing stable cell lines.

Next, six human colon cancer cell lines were selected (HEK293 was selected as a negative control) and their basal expression of ESE-1 was compared. ESE-1 was highly expressed in HCT116, HT29, LoVo, and Caco2 cells, while SW480 and HCT15 cells expressed minimal levels (Fig. 2.2A). To investigate the effect of ESE-1 on cancer phenotypes, SW480 and HCT15 stable cell lines overexpressing LacZ (control) and ESE-1 (full-length) were established and validated using V5 antibody (Fig. 2.2B).

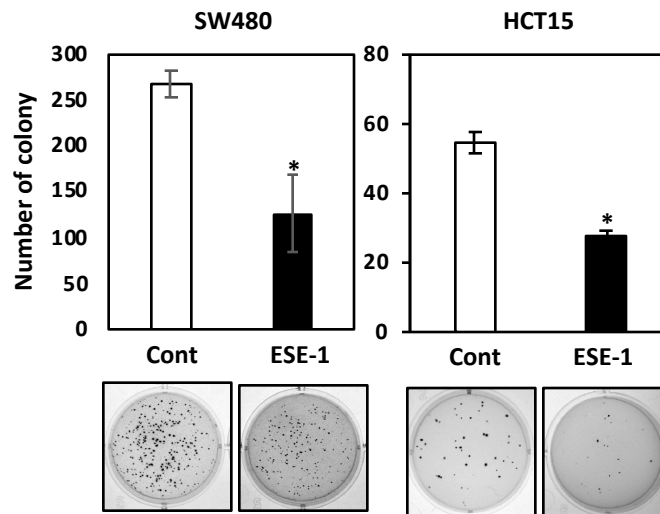


**Fig. 2.2. Expression of ESE-1 in human colon cancer cells and establishment of ESE-1 overexpressing stable cell lines. (A)** Expression of ESE-1 in different human colon cancer cells. **(B)** Validation of SW480 and HCT15 stable cell lines expressing V5-tagged LacZ and ESE-1.



#### 2.3.4. Overexpression of ESE-1 reduced anchorage independent cell growth

To investigate the effect of ESE-1 on cancer phenotypes, we conducted soft agar assay using SW480 and HCT15 stable cell lines overexpressing LacZ (control) and ESE-1 (full-length). Soft agar assay result indicates that the number of colonies for SW480 cells expressing ESE-1 was lower than control cells (66% inhibition compared to control). A similar result (50% reduction by ESE-1 expression) was observed in HCT15 stable cell lines, indicating that ESE-1 suppressed the anchorage-independent growth of these human colon cancer cells (Fig 2.3).

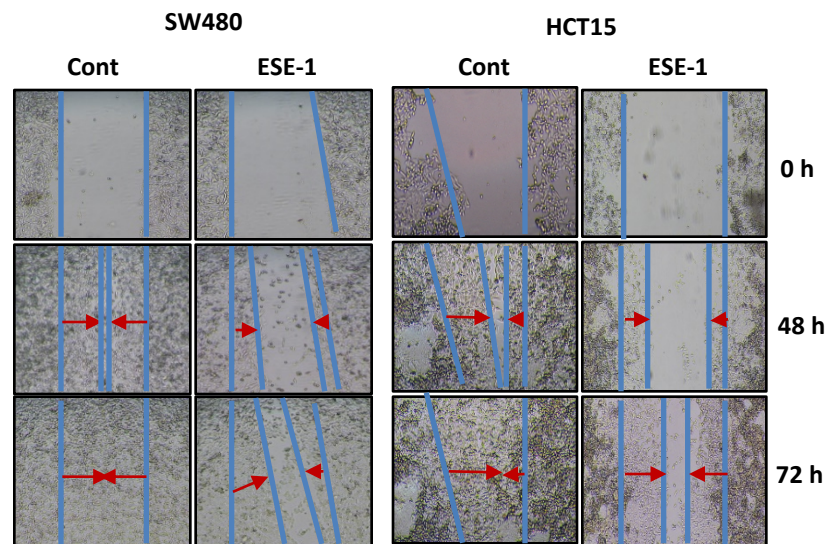


**Fig. 2.3. ESE-1 overexpression represses anchorage independent cell growth.** Soft agar assays were performed using stable cell lines. The image was taken two weeks after inoculation. Data represent the mean  $\pm$  SD from three independent experiments

\* Significant compared to control at  $p < 0.05$ .

### 2.3.5. ESE-1 represses migration of human colon cancer cells

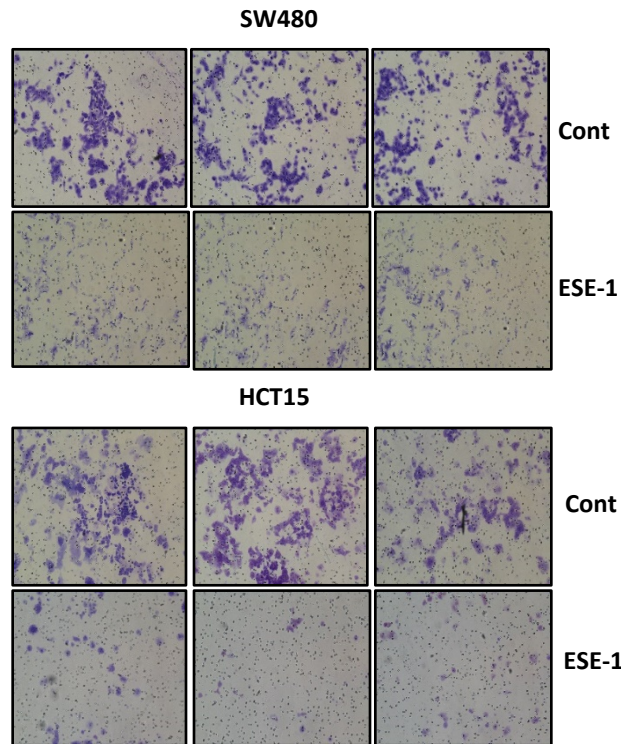
Cell migration was also measured in the same SW480 and HCT15 stable cell lines. In wound healing assays, ESE-1 overexpressing stable cells showed less migration than control cells (Fig. 2.4). Interesting finding in this result is that control cells completely migrates toward the center, showing there was no space found between scratched lines after 72 hours. However, ESE-1 overexpressing stable cells still remains space even 72 hours after the cells were scratched on both SW480 and HCT15 cell lines.



**Fig. 2.4. ESE-1 suppressed migration in SW480 and HCT15 human colon cancer stable cell lines.** Wound healing assays were performed after 48 and 72 hours of incubation.

### 2.3.6. ESE-1 inhibits invasion of human colon cancer cells

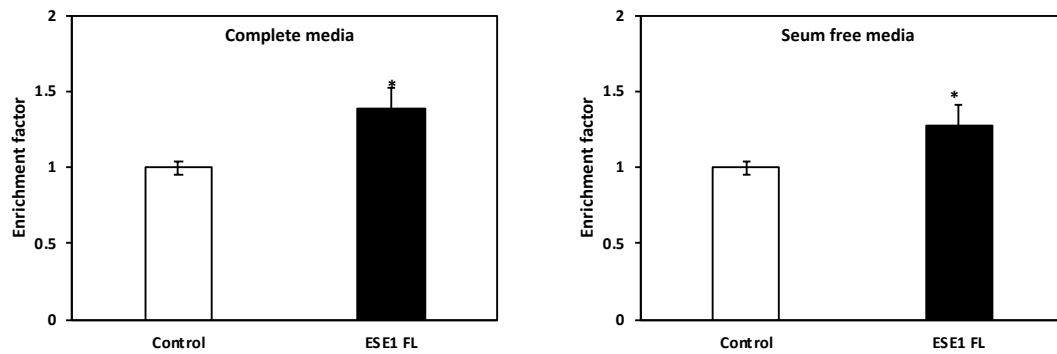
To investigate the effect of ESE-1 on metastasis, *in vitro* invasion assay was conducted using Corning® BioCoat™ Matrigel® Invasion Chambers (Corning, Corning, NY, USA). Both ESE-1 overexpressing SW480 and HCT15 stable cells showed much less invasive activity (Fig. 2.5), which is similar pattern of the migration result. Taken together, ESE-1 reduced metastasis in both SW480 and HCT15 human colon cancer cell lines.



**Fig. 2.5. ESE-1 suppressed invasion in SW480 and HCT15 human colon cancer stable cell lines.** Cells were plated on trans-well and incubated for 48 hours, then stained and imaged using a microscope.

### 2.3.7. ESE-1 overexpression induced apoptosis/cell death in SW480 human colon cancer stable cell line

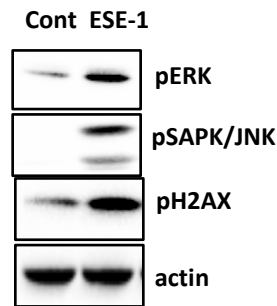
To test whether ESE-1 is involved apoptosis in human colon cancer or not, apoptosis was also measured using cell death ELISA kit in both complete media and serum free media. In the presence of serum and absence of serum (serum free media), ESE-1 overexpressing stable cells showed significant higher apoptosis/cell death compared to control cells (Fig. 2.6). This result indicates that tumor suppressor effect of ESE-1 might result from apoptosis.



**Fig. 2.6. ESE-1 overexpressing SW480 stable cells showed higher apoptosis.** Cells were plated and media was changed after incubation overnight. 24 hours after media change, Apoptosis was measured using Cell Death Detection ELISA kit as described in Materials and Methods. Data represent the mean  $\pm$  SD from three independent experiments. \* Significant compared to control at  $p < 0.05$ .

#### 2.3.8. ESE-1 overexpression induced DNA damage in human colon cancer cells

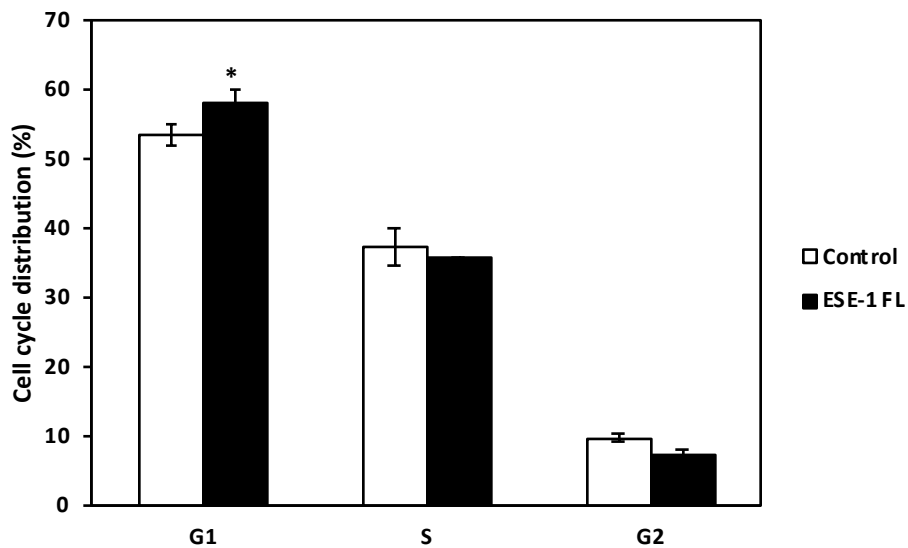
DNA damage was proposed as one of possible mechanisms of tumor suppressor effect of ESE-1. Molecular marker of DNA damage (phosphorylation of H2AX) related genes were measured in control cells and ESE-1 overexpressing stable cells using western blot. As indicated Fig. 2.7, pERK, pSAPK/JNK, DNA damage marker pH2AX were induced by ESE-1 overexpression. This data indicates that tumor suppressive effect of ESE-1 might be mediated from DNA damage via ER stress.



**Fig. 2.7. ESE-1 overexpression induced DNA damage related genes.** The cell lysates were subjected to western blot analysis for pERK, pSAPK/JNK, pH2AX and actin.

### 2.3.9. ESE-1 overexpression induced G1 arrest in SW480 human colon cancer stable cell line

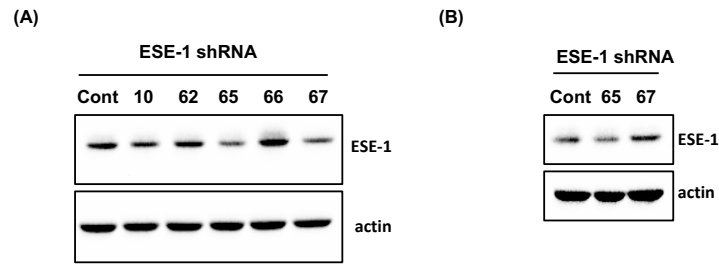
To examine whether retarded growth by ESE-1 overexpression is associated with cell growth arrest, cell cycle distribution was compared between control and ESE-1 expressing SW480 stable cells. Cell cycle analysis data showed that ESE-1 overexpression resulted in a significant increased percentage of cells in the G1 phase (LacZ,  $53.4 \pm 1.65$  vs. ESE-1,  $58.17 \pm 1.6\%$ ) and no change in the S phase (LacZ,  $37.2 \pm 2.6$  vs. ESE-1,  $35.5 \pm 0.26\%$ ), and decreased in the G2 phase (LacZ,  $9.64 \pm 0.45$  vs. ESE-1,  $7.02 \pm 0.97\%$ ) (Fig. 2.8).



**Fig. 2.8. ESE-1 overexpression in SW480 cells induced G1 arrest.** Cell cycle distribution was performed by staining the cells with propidium iodide and subsequent FACS analysis. Data represent the mean  $\pm$  SD from three independent experiments (right). \* $P < 0.05$  vs. the control.

### 2.3.10. Establishment of ESE-1 knockdown stable cell line

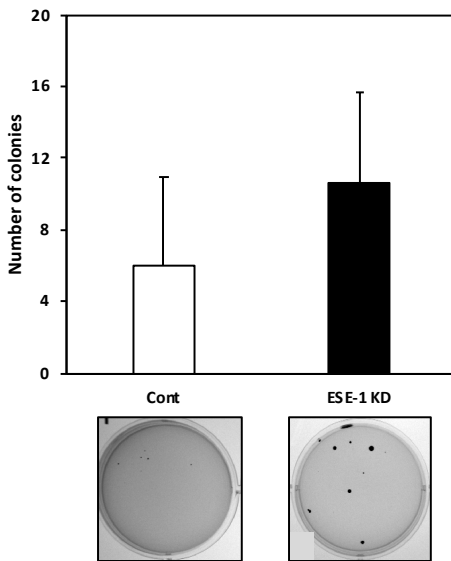
To confirm whether ESE-1 plays a role as a tumor suppressor gene in ESE-1 expressing human colorectal cancer cell line (loss of function study), we screened several lines of commercially available shRNAs. As a result, approximately 40% inhibition of ESE-1 protein expression was observed in #65 shRNA compared to control (Fig. 2.9A). Therefore, HCT116 control ShRNA and ESE-1 ShRNA stable cell lines were established using this #65 shRNA (Fig. 2.9B).



**Fig. 2.9. Validation of HCT116 ESE-1 Knockdown stable cell lines.** (A) cell lysates were analyzed 48 hours after transient transfection. (B) 48 hours after transient transfection of control or ESE-1 ShRNA, cells were stably selected using puromycin 2  $\mu$ g/mL. After completion of selection, cell lysate was analyzed using western blot.

### 2.3.11. Effect of ESE-1 knockdown on anchorage-independent growth of human colorectal cancer cells

To examine whether ESE-1 knockdown affect tumorigenesis in colon cancer, anchorage independent cell growth of ESE-1 knockdown stable cells was measured using soft agar assay. The number of colonies were counted three weeks after plating the cells. The average number of colonies of ESE-1 knockdown stable cells tend to be higher than control cells, although significant difference was not found ( $P=0.09$ ) (Fig. 2.10). However, the size of colonies in HCT116 knockdown cells is much bigger than control cells.

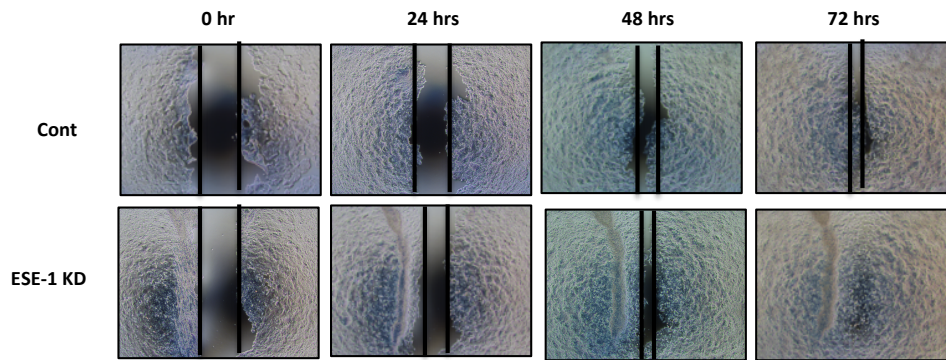


**Fig. 2.10. Effect of ESE-1 knockdown on anchorage independent cell growth in HCT116 human colon cancer cells.** Soft agar assay was performed using established HCT116 stable cell line. Data represent the mean  $\pm$  SD from three independent experiments.



### 2.3.12. ESE-1 knockdown increased migration of human colorectal cancer cells

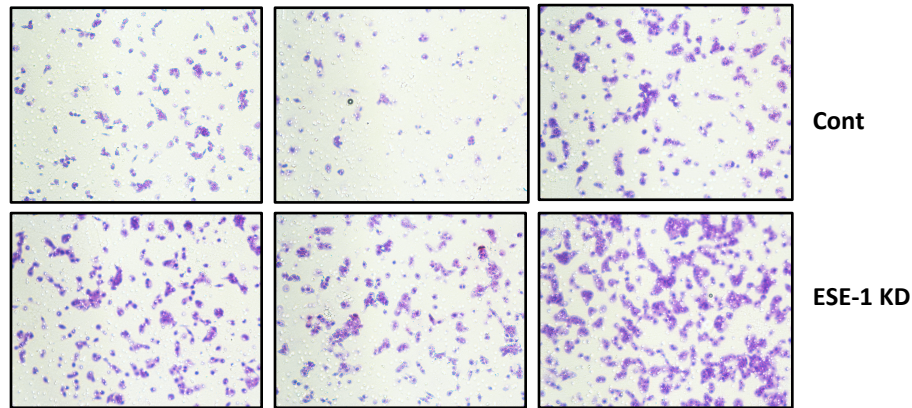
To test if ESE-1 knockdown increases migration in HCT116 stable cells, migration assay was conducted using the established ESE-1 knockdown stable cell line. 48 hours after the scratch was created, the space between two lines in control cells showed higher compared to ESE-1 knockdown stable cells. Finally, there was no space found in ESE-1 knockdown cells 72 hours after the scratch was performed (Fig. 2.11). This result supports gain of function result indicating that ESE-1 is tumor suppressor gene in colon cancer.



**Fig. 2.11. Effect of ESE-1 knockdown on cell migration in HCT116 stable cell lines.** Images were taken under microscope every 24 hours after the scratch was performed.

### 2.3.13. ESE-1 knockdown increased invasion of human colorectal cancer cells

To investigate the effect of ESE-1 knockdown on metastasis of colon cancer cells, *in vitro* Matrigel invasion assays (Corning) were performed using HCT116 stable cell lines. ESE-1 knockdown cells showed more invasive cells compared to control cells (Fig. 2.12). This result indicates similar pattern with result of migration and it is consistent as gain of function studies, which represents anti-metastatic role of ESE-1 in human colorectal cancer cells.



**Fig. 2.12. ESE-1 knockdown induces cell invasion *in vitro*.** Control and ESE-1 knockdown HCT116 stable cell lines were plated on trans-well and incubated for 24 hours, then stained and imaged using a microscope.

## 2.5.Discussion

ESE-1 belongs to the ETS family of transcription factors and has been associated with cancer progression. In addition, as described in literature reviews, ESE-1 is highly expressed epithelial cells and plays a significant role in terminal differentiation of epithelial cells. That might be the direct evidence that ESE-1 possesses biological function in colorectal cancer progression/tumorigenesis.

However, observation of the effects of ESE-1 on cancer still remains unclear, whether ESE-1 is oncogenic or tumor suppressive in colon cancer or in any other cancer types. There are conflicting results from colon (52,156) and lung cancer studies (75,76); In particularly colon cancer, Wang et al (52) reported that ESE-1 activated  $\beta$ -catenin signaling and they proposed the result as the mechanism of the oncogenic role of ESE-1. On the contrary, recent study characterized ESE-1 as a tumor suppressor gene, suggesting that ESE-1 inhibits EMT transcriptional factor ZEB1 (156). However, none of those studies has identified biological function of ESE-1 using *in vivo* mouse model. Therefore, this chapter only focused on cancer phenotypes in colon cancer using *in vitro* and *in vivo* model. In addition, both gain of function and loss of function studies were conducted to determine and confirm the role of ESE-1 in tumorigenesis in colon cancer.

Since carcinogenesis resulted from inflammatory bowel disease (IBD) is a common pathological and clinical aspect of colon cancer, we adopted an AOM-induced and DSS-promoted colon cancer model using ESE-1 knockout mice, which may potentially be more applicable to inflammation and neoplasia in colon. In this study,

we observed tumor suppressive effect for ESE-1. We tested metastasis *in vivo* using orthotopic implantation animal model. The ESE-1 overexpressing cell injected mice showed lower metastasis rate. Because most of the observed tumors in the liver and lung seemed like micro-metastasis tumors, the image could not be taken, and tumor area calculation software was not available as well. Therefore, we alternatively calculated based on the ratio of the number of tumors in each gene expressing cells to the total number of tumors. However, this data represents that ESE-1 suppresses (micro) metastasis *in vivo*.

Cancer cells grow abnormally without control and their survival rate is higher than normal cells. Therefore, anchorage independent cell growth and cell cycle distribution could be important to parameter to represent growth rate and activity of cancer cells. Our data indicate that ESE-1 expression is negatively associated with anchorage independent growth of human colorectal cancer cells as observed in soft agar assay using ESE-1-expressing SW480 and ESE-1 knockdown-HCT116 cells (Fig. 2.3 and 2.10). Due to equipment and software limitation, only number of colonies were counted manually. Since the size of colonies in HCT116 knockdown cells is overall bigger than control cells, standard deviation of the result might become smaller so that significant difference might be changed under colony area scan. Moreover, only  $5 \times 10^2$  cells were plated because HCT116 cells are more invasive and grow faster compared to SW480 cell line. The number of cells might be too low to obtain normal distribution of data. However, knockdown cells also showed similar trend that ESE-1 inhibits cancer cell growth. Additionally, apoptosis and cell cycle results from SW480 cells also support that ESE-1 is tumor suppressor gene in colon cancer. DNA damage related

genes are also upregulated by the presence of ESE-1. This might be one of possible mechanisms of ESE-1 as a tumor suppressor gene.

Migration and invasion assays results indicate that ESE-1 represses metastasis *in vitro*. Overexpression of ESE-1 in SW480 stable cell lines showed less migrative cells compared to control cells. Control cells completely recovered 72 hours after the scratch, whereas the wider space between the scratch lines were found in ESE-1 overexpressing cells. HCT116 knockdown results also showed similar trend; ESE-1 knockdown cells seemed to migrate more compared to control cells. Moreover, the invasion results are clear and fully explain that ESE-1 acts as a tumor suppressor gene under knockdown condition in ESE-1 abundant cells.

In this chapter, we investigated the biological function of ESE-1 in colon cancer only focusing on its phenotypes, including cell growth, apoptosis, cell cycle, migration and invasion. Those are widely known hallmarks of cancer representing not only tumorigenic activity in the primary tumor site but also metastatic activity. Our conclusion is that ESE-1 possesses tumor suppressive activity in human colorectal cancer. Based on results in this chapter, further mechanisms of ESE-1 as a tumor suppressor gene in colon cancer will be investigated in next Chapters.

## Chapter 3: Mechanistic study of ESE-1 in colon cancer

### 3.1. Abstract

To elucidate the hypothesis that tumor suppressive activity of ESE-1 rely on its nuclear localization, subcellular localization of ESE-1 using internally deleted ESE-1 expression vectors ( $\Delta$ NES1,  $\Delta$ NLS1,  $\Delta$ NES2,  $\Delta$ NLS2) was investigated. Internal deletion of nuclear localization sequence 2 (NLS2) decreased ESE-1 in the nucleus while full length of ESE-1 mainly localized in the nucleus. Three lysine residues ( $^{318}\text{KKK}^{320}$ ) in the NLS2 were responsible for its nuclear localization. To further investigate the biological function of these three lysine residues in colon cancer, SW480 stable cell lines expressing point-mutated at three lysine residue ( $^{318}\text{KKK}^{320}$ ) was established. The suppression of anchorage independent cell growth, cell proliferation, migration and invasion were reversed by point mutation of three triple lysine residues( $^{318}\text{KKK}^{320}$ ). Reduced  $\beta$ -catenin transcriptional activity in ESE-1 full length overexpressing stable cells was fully recovered by triple lysine mutation. Lastly, acetylation and methylation were tested using immunoprecipitation immunoblotted to acetylation and methylation antibodies. Acetylated ESE-1 did not show significant difference, but methylation seemed to occur more significantly in ESE-1 full length cells, while NLS2 deleted ESE-1 cells reduced down back to the similar level as control cells. In summary, nuclear localization of ESE-1 and subsequent tumor suppressive activity was determined by methylation at three lysine residues of NLS2. Therefore, inhibition of tumorigenesis in colon cancer by ESE-1 might be regulated by triple lysine residue in NLS2 and methylation through NLS2 residues. These results suggest that

localization of ESE-1 might be mediated specifically by methylation via the three lysine residues of NLS2 and acetylation might not be involved in nuclear localization of ESE-1 by NLS2.

### 3.2. Introduction

In previous chapter, we identified ESE-1 tumor suppressor gene in colon cancer *in vitro* and *in vivo*. The main purpose of this chapter is to investigate responsible mechanism of ESE-1 as a tumor suppressor in colon cancer.

As described in Chapter 1, every ETS family protein is characterized based on its own different structure. The ESE-1 protein includes multiple domains, which are an ETS domain, a Pointed domain, an A/T hook domain, a Transactivation (TAD) domain, and a Serine- and Aspartic acid-rich (SAR) domain. Each domain possesses different function; the ETS domain regulates binding to DNA sites with a core consensus sequence of GGA(A/T), found within the promoter regions of target gene (230,231). The Pointed domain has been appeared to enable dimerization, whereas the A/T hook domain is involved in binding to the minor groove of AT-rich sequences (63). The TAD domain is considered to play a role in protein stabilization (132), and the SAR domain was reported that it mediates cellular transformation in breast cancer via a cytoplasmic mechanism (140).

One of the unique characteristics of ESE-1 is its cytosolic localization. Unlike other ETS transcriptional family proteins, localization of ESE-1 is diverse; other ETS family members are detected only in the nucleus, but a significant amount of ESE-1 localizes to the cytoplasm of colon (54) and breast cancer cells (141), suggesting that a unique biological role of ESE-1 might rely on subcellular localization. First study indicated that cytoplasmic ESE-1 induces transformation of normal mammary epithelial cells, whereas its nuclear localization causes apoptosis (141). However, none



of ESE-1 related studies in colon cancer has been studied, focusing on mechanisms related to either nuclear localization or the function of ESE-1 domain. In addition, our previous study showed that ESE-1 is  $\beta$ -catenin binding protein (232). Thus, I assume that interaction of ESE-1 and  $\beta$ -catenin might be one of possible mechanisms of ESE-1 biological function in colon cancer.

Therefore, this chapter hypothesizes that mechanisms of ESE-1 as a tumor suppressor gene in colon cancer is mediated by ESE-1-domain-dependent subcellular localization and interaction with  $\beta$ -catenin.

### 3.3. Materials and methods

#### 3.3.1. Materials

The V5 antibody (R96025) was purchased from Thermo Fisher Scientific. Antibodies for tubulin (sc-5286), U1snRNP70 (sc-9571), and actin (sc-1615) were purchased from Santa Cruz Biotechnology. Cell culture media were purchased from Invitrogen. All chemicals were purchased from Fisher Scientific or VWR unless otherwise specified.

#### 3.3.2. Cloning of ESE-1 deletion and point mutation vectors

Internal deletion and point mutation of the ESE-1 expression vector was performed as described previously (233). Briefly, the full-length ESE-1 plasmid cloned into pcDNA3.1-V5-His (54) was amplified with the indicated primers (Table 3) using

the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Savage, MD, USA) according to the manufacturer's instruction.

### 3.3.3. Isolation of cytosol and nuclear fractions

Cytoplasmic and nuclear fractions were prepared with the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, cells were washed two times with ice-cold PBS containing phosphatase inhibitors and transferred to an ice-cold tube. Collected cells were centrifuged at 500 rpm for five minutes at 4 °C. After discarding the supernatant, cell pellets were resuspended in 250 µl 1X Hypotonic Buffer and incubated for 20 minutes on ice. The cytoplasmic fraction was obtained by collecting the supernatant after vortexing with 12 µl detergent for ten seconds and centrifuging at 14,000 g for 30 seconds at 4 °C. The nuclear fraction was obtained by resuspending and incubating pellets with 50 µl of Complete Lysis Buffer and 0.25 µl of protease inhibitor and 5 uL of 10 mM DTT on a rocking platform for 30 minutes at 4 °C and centrifuging for ten minutes at 14,000 g.

### 3.3.4. Cell culture and established stable cell lines

Human colon carcinoma cell line (SW480, HCT15 and HCT116) and human embryonic kidney cells (HEK293) was purchased from the American Type Culture Collection and maintained in DMEM/F-12 media supplemented with 10% fetal bovine serum (FBS) and a mixture of penicillin (100 U/mL) and streptomycin (100 µg/mL) under a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. Stable cell lines expressing LacZ

(control), full length (ESE-1 FL), or point-mutated ESE-1 (ESE-1 PM) were established by transfecting expression vectors and subsequently selecting using G418 (800 µg/mL for selection and 400 µg/mL for maintenance) as described previously (76).

#### 3.3.5. Transient transfection and luciferase assay

The expression vectors (V5-tagged ESE-1) and luciferase reporters (ESE-1 promoter) were transiently transfected using Lipofectamine 2000 (Thermo Fisher Scientific) or the PolyJet DNA transfection reagent (SignaGen Laboratories) according to the manufacturer's instruction. For luciferase assays, cells were lysed in 1x Passive Lysis Buffer for 15 minutes, and the enzymatic activity of luciferase was measured using a luminometer (Promega). Enzyme activity was normalized to that of pRL-null using a dual luciferase assay system (Promega) as described previously (227).

#### 3.3.6. Western blot

The cells were washed two times with cold PBS and incubated with RIPA buffer containing a protease/phosphatase inhibitor cocktail (Sigma) for 30 minutes before being centrifuged at 14,000 g for 15 minutes at 4 °C. Protein concentration was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific). SDS-PAGE was performed to separate proteins based on size. After transfer to nitrocellulose membranes, a specific primary antibody was added in TBS containing 0.1% Tween 20 (TBS-T) and 3% BSA, and incubated overnight at 4 °C. Subsequently, the membranes were incubated with horse radish peroxidase (HRP)-conjugated IgG for one hour at room temperature, washed three times with 1X TBS-T (0.1% Tween-20), and

visualized using enhanced chemiluminescence (ECL) (Amersham Biosciences). The image was taken using a ChemiDoc Imaging system (BioRad Laboratories).

#### 3.3.7. Soft agar assay

SW480 stable cell lines ( $2 \times 10^3$  cells) were plated with 0.6% agarose gel in complete media onto a 0.6% agarose gel bottom layer. Cells were incubated at 37 °C and 5% CO<sub>2</sub> with complete media added onto the plate every other day. Afterwards, cells were stained overnight at 37 °C with 200 µL of 1 mg nitroblue tetrazolium chloride/mL PBS. The image was taken using a ChemiDoc Imaging system (BioRad Laboratories).

#### 3.3.8. Cell proliferation

Cell proliferation was measured as we described previously (229). Briefly, the SW480 stable cells ( $2 \times 10^3$  cells) were grown onto 96-well culture plate. MTT assay was performed every 24 hours. After removal of media, 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added and incubated for 3 h at 37 °C in a CO<sub>2</sub> incubator. Then, MTT dye was extracted using 200 µL of dimethyl sulfoxide, and the absorbance was measured at 540 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA).

### 3.3.9. Cell migration and invasion assay

Cell migration and invasion assays were performed as previously described (139). For the migration assay, stable cell lines ( $6 \times 10^5$ ) were plated onto a six-well plate. After reaching confluency, cells were scratched with pipette tips and washed three times with phosphate buffered saline before taking the first image. Microscope images were taken every 24 hours to observe cell migration. The *in vitro* invasion assay was performed using Matrigel kit (Corning). Briefly,  $1 \times 10^6$  cells were plated onto 24 well invasion chamber in serum free media, and the chambers were inserted onto DMEM containing 10% FBS (chemoattractant). After 48 hours, non-invading cells on the upper membrane surface were removed using cotton swab, invading cells on the bottom surface were fixed using 100% methanol for 2 minutes and stained using 1% toluidine blue in 1% borax for 2 minutes. After washing the chambers, the membranes were cut and mounted on the immersion oil. The images were taken under microscope.

### 3.3.10. Luciferase assay

Transient transfection was carried out using PolyJet reagent (SignaGen Laboratories). The cells ( $6 \times 10^5$  cells/well) were plated in 24-well culture dishes. The next day, master mixtures of plasmid (0.5  $\mu$ g of TOP Flash or FOP Flash plasmids and 0.05  $\mu$ g of pRL-null plasmids) were prepared per each well and incubated with cells for 48 h. The cellular extracts were harvested using lysis buffer. Luciferase activity (a ratio of firefly/renilla) was measured according to protocol of a dual-luciferase assay kit (Promega, Madison, WI, USA).

### 3.3.11. Immunoprecipitation

SW480 cells were plated and transfected with control (LacZ), full length of ESE-1, and NLS2 deleted ESE-1 ( $\Delta$ NLS2) using Polyjet reagent (SignaGen Laboratories). Deacetylase inhibitor was treated next day for 24 hours. Cells were harvested in RIPA buffer or NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40) supplemented with protease inhibitor cocktail (Sigma-Aldrich) and cleared by centrifugation at  $12,000 \times g$  for 20 min at 4°C. Pre-cleared cell lysates (1,000  $\mu$ g) were incubated with 1  $\mu$ g of anti-V5 antibody (Invitrogen), anti-methylated lysine (Santa Cruz) or anti-pan acetyl antibody (Santa Cruz) at 4°C overnight. Immunocomplex was rotated with 30  $\mu$ l of 50% of agarose-conjugated protein A/G beads (Santa Cruz) at 4°C for 4 h. The pellets were washed four times with either RIPA or NP-40 lysis buffer, boiled in a protein loading buffer and then subjected to immunoblotting analyses using an anti-V5 (Invitrogen) or anti-pan acetyl antibody (Santa Cruz).

### 3.3.12. Statistical analysis

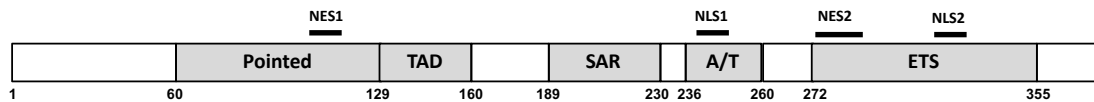
Statistical analyses were conducted using Student's unpaired t-test with statistical significance set at  $*P < 0.05$ .

### 3.4. Results

#### 3.4.1. Schematic representation of human ESE-1 protein and its functional domains

Two putative nuclear export sequences (NES) and two nuclear localization sequences (NLS) were found in ESE-1 (234). The first NES sequence (NES1: <sup>102</sup>LCNCALEELRL<sup>112</sup>) is in the Pointed domain and the other (NES2: <sup>275</sup>LWEFIRDILI<sup>284</sup>) is in the ETS domain, while the first NLS sequence (NLS1: <sup>242</sup>HGKRRR<sup>247</sup>) is in the AT-hook domain and the other (NLS2: <sup>314</sup>LWGQKKKNSN<sup>323</sup>) is located in the ETS domain (Fig. 3.1).

Since the anti-cancer activity of ESE-1 might be determined through cellular localization, the effects of each NLS and NES on its subcellular localization and anti-cancer activity were investigated. Four internally deleted ESE-1 expression vectors ( $\Delta$ NES1,  $\Delta$ NLS1,  $\Delta$ NES2,  $\Delta$ NLS2) were cloned for further study.



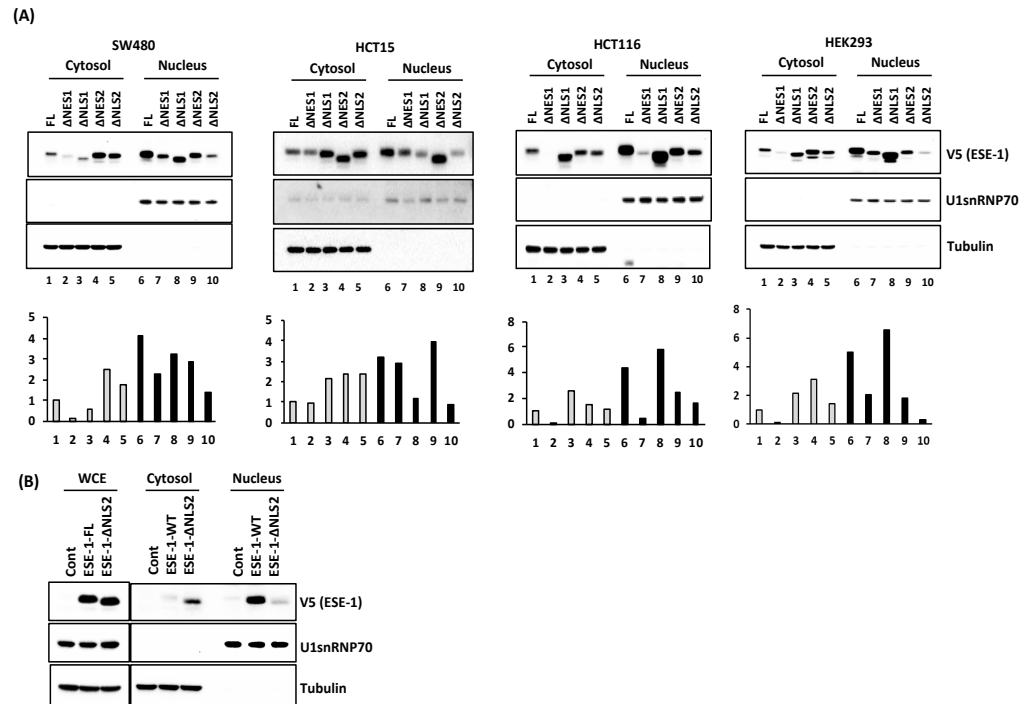
**Fig. 3.1. Schematic representation of human ESE-1 protein and its functional domains.** Schematic representation of human ESE-1 protein and its functional domains: The Pointed domain, Transactivation domain (TAD), Serine- and Aspartic acid-rich domain (SAR), AT hook domain, and ETS domain are shown. Short black lines indicate the positions of NES1, NES2, NLS1, and NLS2.

### 3.4.2. Internal deletion clones of ESE-1 and localizations

Unlike other ETS transcription factors, ESE-1 localizes to both the cytosol and nucleus. Therefore, the deleted ESE-1 expressing vectors (Fig. 3.1) were transfected in three different human colon cancer cell lines (SW480, HCT15, HCT116) and in HEK293 cells. After isolation of the cytosolic and nuclear fractions, subcellular fractionations were analyzed to determine which sequence was functionally responsible for ESE-1 localization. As shown in Fig. 3.2A, abundant amount of full-length ESE-1 is present in the nucleus, while a small amount was detected in the cytoplasm (Lane 1 and lane 6) (Fig. 3.2A). Interestingly, deletion of NLS2 ( $\Delta$ NLS2) increased cytoplasmic ESE-1, while nuclear ESE-1 was dramatically decreased (Lane 10). This similar pattern was observed in all other tested cell lines under the experiment condition (Fig. 3.2A).

Fig. 3.2B compares the nuclear localization of ESE-1 between ESE-1 full-length and ESE-1  $\Delta$ NLS2 lines. The results imply that NLS2 is functionally significant for the localization of ESE-1 protein in nucleus of human colon cancer cells. Interesting finding in this result is, deletion of NES1 decreased the ESE-1 amount in both cytosol and nucleus (lane 2 & 7) in SW480, HCT116 and HEK293 cells, indicating that NES1 might be involved in the stabilization of ESE-1 protein. Deletion of NLS1 (HCT15) and NES2 (SW480, HCT116, HEK293) also moderately repositioned cellular localization.





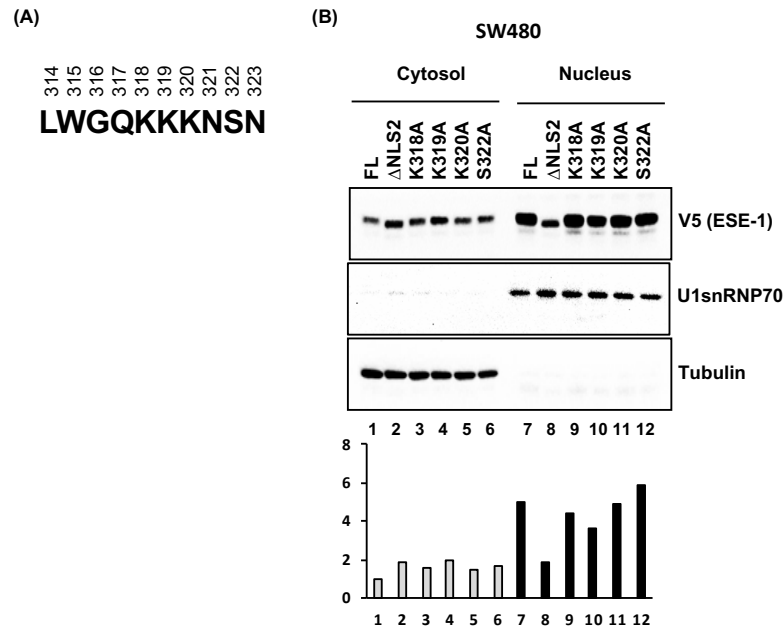
**Fig. 3.2. Effect of internal deletion of NESs and NLSs on nuclear localization of ESE-1 in human colon cancer cells. (A)** Internal deletion clones lacking NES ( $\Delta$ NES1 and  $\Delta$ NES2) and NLS ( $\Delta$ NLS1 and  $\Delta$ NLS2) sequences were constructed. Each deletion clone was transfected into SW480, HCT15, HEK293 and HCT116 for 48 hours. Nuclear and cytosolic fractions were isolated and Western blots were performed to detect V5 (ESE-1). **(B)** Distribution of ESE-1 FL and ESE-1  $\Delta$ NLS2 between cytosol and nucleus in HEK293 cells. Cytoplasmic and nuclear fractions were separated and Western blot was performed using antibodies for V5, U1snRNP70 and tubulin. Densitometry analysis of each fraction (V5/tubulin and V5/U1snRNP70) is expressed as a bar graph.

#### 3.4.3. The effect of single point mutation of ESE-1 on its nuclear localization

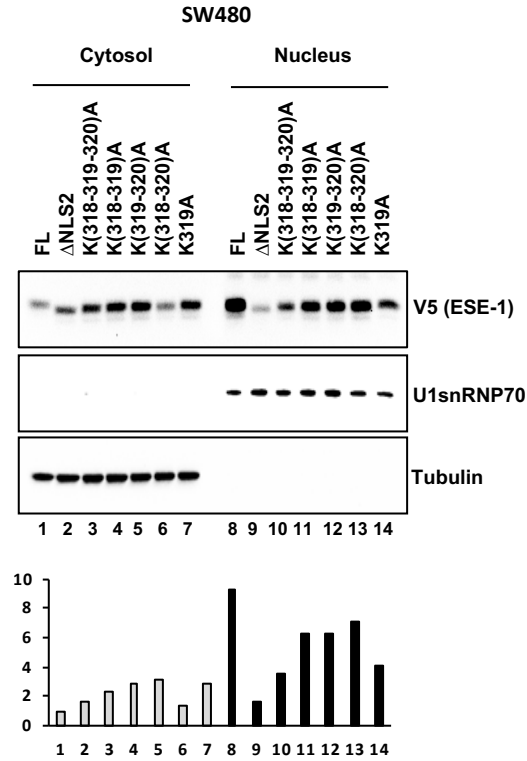
Next, amino acids in NLS2 which are significant for the nuclear localization of ESE-1 were identified. The NLS2 sequence (<sup>314</sup>LWGQKKKNSN<sup>323</sup>) includes three lysine residues and one serine residue at which protein modifications occur (Fig. 3.3A). The contribution of each residue to nuclear localization of ESE-1 was examined by generating single point mutation clones that replacing each of these four residues to alanine. No single point mutation was found to reverse the localization of full-length ESE-1 (Fig. 3.3B).

#### 3.4.4. The effect of double/triple mutations on ESE-1 subcellular localization

Double and triple point mutations were also produced for further experiment. While the double point mutation of lysine residues reduced nuclear localization of ESE-1, substitution of all three lysine residues (<sup>318</sup>KKK<sup>320</sup>→<sup>318</sup>AAA<sup>320</sup>) to alanine recovered the localization of full-length ESE-1. This result indicates that the three lysine residues are cooperatively responsible for the nuclear localization of ESE-1 protein (Fig. 3.4).



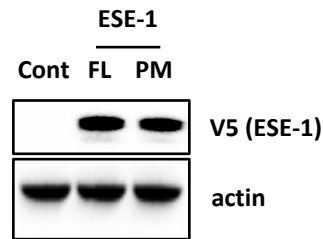
**Fig. 3.3. Effect of single amino acid substitution on nuclear localization of ESE-1 in human colon cancer cells.** (A) Amino acid sequence of NLS2. (B) SW480 cells were transfected with V5-tagged ESE-1 FL (full length), ESE-1 ΔNLS2, and ESE-1 point mutant plasmids (K318A, K319A, K320A, and S322A single point mutants) for 48 hours. Cytoplasmic and nuclear fractions were separated and Western blot was performed using antibodies for V5, U1snRNP70 and tubulin. Densitometry analysis of each fraction (V5/tubulin and V5/U1snRNP70) is expressed as a bar graph.



**Fig. 3.4. Effect of double and triple lysine substitution on nuclear localization of ESE-1 in human colon cancer cells.** SW480 cells were transfected with V5-tagged ESE-1 FL, ΔNLS2, and double or triple point mutants (K318A-K319A-K320A triple mutant, K318A-K319A double mutant, K318A-K320A double mutant, K319A-K320A double mutant, and K319A single point mutant) for 48 hours. Cytoplasmic and nuclear fractions were separated and Western blot was performed using antibodies for V5, U1snRNP70 and tubulin. Densitometry analysis of each fraction (V5/tubulin and V5/U1snRNP70) is expressed as a bar graph.

### 3.4.5. Establishment of ESE-1 triple lysine deleted stable cell lines

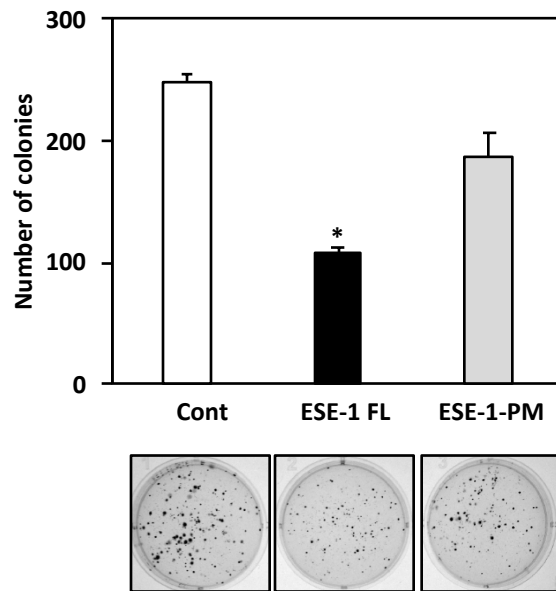
To test the effect of three lysine residues within NLS2 on cancer phenotypes, SW480 and stable cell lines overexpressing LacZ (control) ESE-1 with full-length (ESE-1 FL) and ESE-1 with substitution at triple lysine residue (ESE-1 PM) were established and established stable cell lines was validated through Western blot using V5 antibody (Fig. 3.5).



**Fig. 3.5. Establishment of SW480 stable cell lines expressing full length (FL) and triple lysine substitution (PM) of ESE-1.** Validation of SW480 stable cell lines expressing V5-tagged LacZ (cont), full length ESE-1 (ESE-1 FL) and triple lysine substitution (ESE-1 PM).

#### 3.4.6. Lysine residues of NLS2 are responsible for tumor suppressive effect of ESE-1 in colon cancer

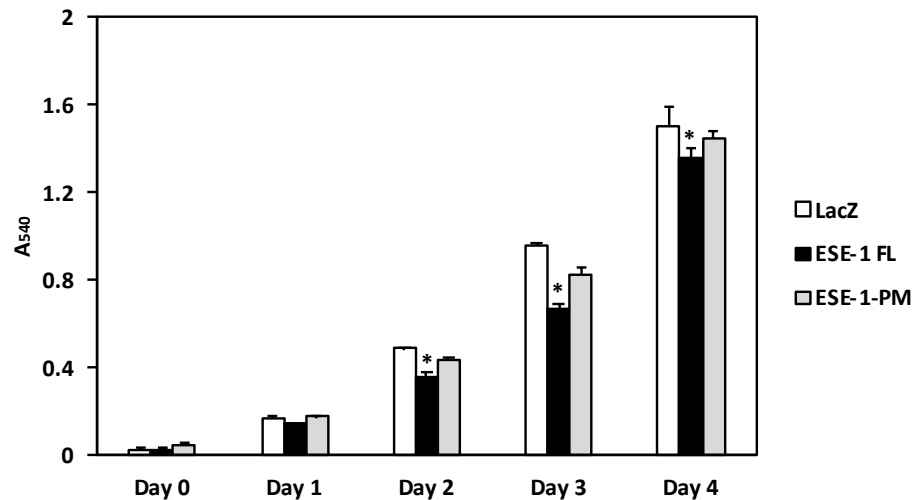
To examine whether lysine residues of NLS2 are responsible for inhibition of anchorage independent cell growth by ESE-1 in colon cancer, soft agar assay was conducted using SW480 stable cell lines expressing LacZ (control), full length of ESE-1 (ESE-1 FL) or triple lysine mutated ESE-1 (ESE-1-PM). As shown in previous chapter, ESE-1 overexpression reduced anchorage independent cell growth in SW480 stable cells significantly (56.63% inhibition). However, the growth of triple lysine deleted ESE-1 stable cells was partially recovered compared to full length of ESE-1 overexpressing stable cells. The triple lysine mutation recovered up to 75.5% of total number of colonies of control cells (Fig. 3.6).



**Fig. 3.6. Effect of triple lysine substitution on anchorage independent growth of human colorectal cancer cells.** Soft agar assays were performed using stable cell lines. The image was taken two weeks after inoculation. Data represent the mean  $\pm$  SD from three independent experiments. \* Significant compared to control at  $p < 0.05$ .

### 3.4.7. Triple lysine residues in NLS2 are responsible for suppressed cell proliferation by ESE-1

To confirm that ESE-1 inhibits cell growth in colon cancer and triple lysine residues in NLS2 are responsible for reduced cell growth by ESE-1, cell proliferation was tested using MTT assay. The result showed similar pattern as soft agar assay result, indicating that triple lysine residues partially contributed cell growth inhibition in SW480 stable cells [Day 2: ESE-1 FL: 27.57% inhibition, ESE-1-PM up to 89% recovered, Day 3: ESE-1 FL: 30.25% inhibition, ESE-1-PM 86.03% recovered, Day 4 ESE-1 FL: 9.16% inhibition, ESE-1-PM 96.49% recovered, compared to control cells].

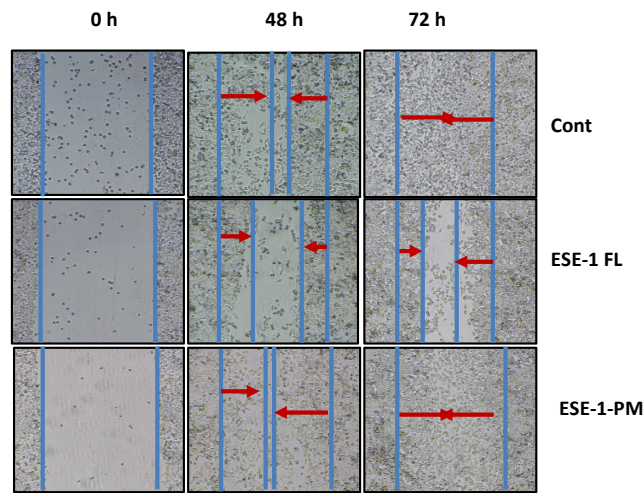


**Fig. 3.7. Effect of triple lysine substitution of ESE-1 on proliferation of human colon cancer cells.** SW480 stable cells were grown in 10% fetal bovine serum containing complete media for 1, 2, 3, and 4 days. MTT assay was performed as described under Materials and Methods. Values are the mean  $\pm$  SD,  $n = 3$ . (\*)  $p < 0.05$  versus control cells and triple lysine deleted ESE-1 FL and ESE-1 PM.



#### 3.4.8. Triple lysine residues in NLS2 are responsible for suppressed cell migration by ESE-1

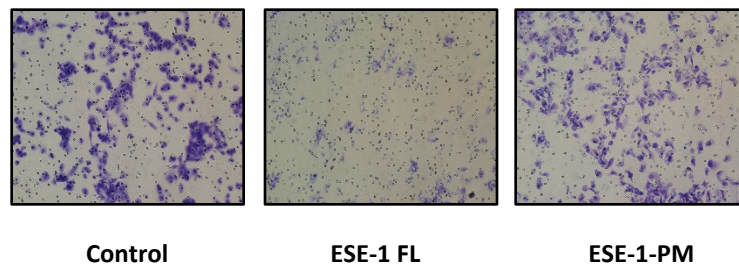
To investigate whether triple lysine residues in NLS2 are critical for inhibited migration by ESE-1 in colon cancer cells, wound healing assay was performed including triple lysine mutated ESE-1 overexpressing SW480 stable cell line. As shown in Chapter 2, stable cells expressing full length of ESE-1 migrated less than control cells. However, triple lysine residues mutated ESE-1 overexpressing stable cells recovered their migrative activity (Fig. 3.8).



**Fig. 3.8. Effect of triple lysine substitution of ESE-1 on migration in human colon cancer cells.** Wound healing assays were performed after 48 and 72 hours of incubation. SW480 stable cells were plated onto 6-well plate. After confluency, cross-scratch was drawn onto the layer of the cells. The distance between scratch lines was observed every 24 hours and the images were taken under microscope.

#### 3.4.9. Triple lysine residues in NLS2 are responsible for reduced invasiveness of colon cancer cells by ESE-1

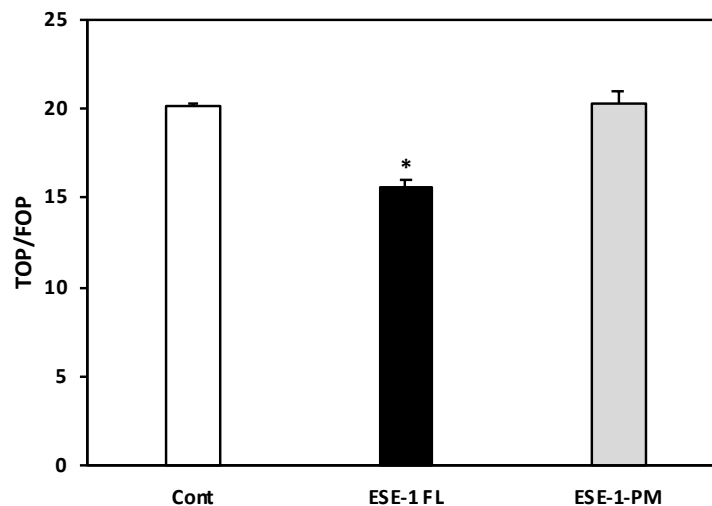
To confirm if the triple lysine residues in NLS2 is critical for cell invasion, Matrigel *in vitro* cell invasion assay was performed and invasive cells were observed under microscope. As a result, triple lysine residue deletion in NLS2 recovered reduction of invasive cells by ESE-1 (Fig. 3.9). This result is consistent to migration result, which means that triple lysine residues in NLS2 play a key role in metastasis in colon cancer.



**Fig. 3.9. Effect of triple lysine substitution of ESE-1 on invasion of human colon cancer cells.** Cells were plated on trans-well and incubated for 48 hours, then stained and imaged using a microscope.

#### 3.4.10. Triple lysine residues in NLS2 is responsible for downregulation of $\beta$ -catenin transactivation by ESE-1

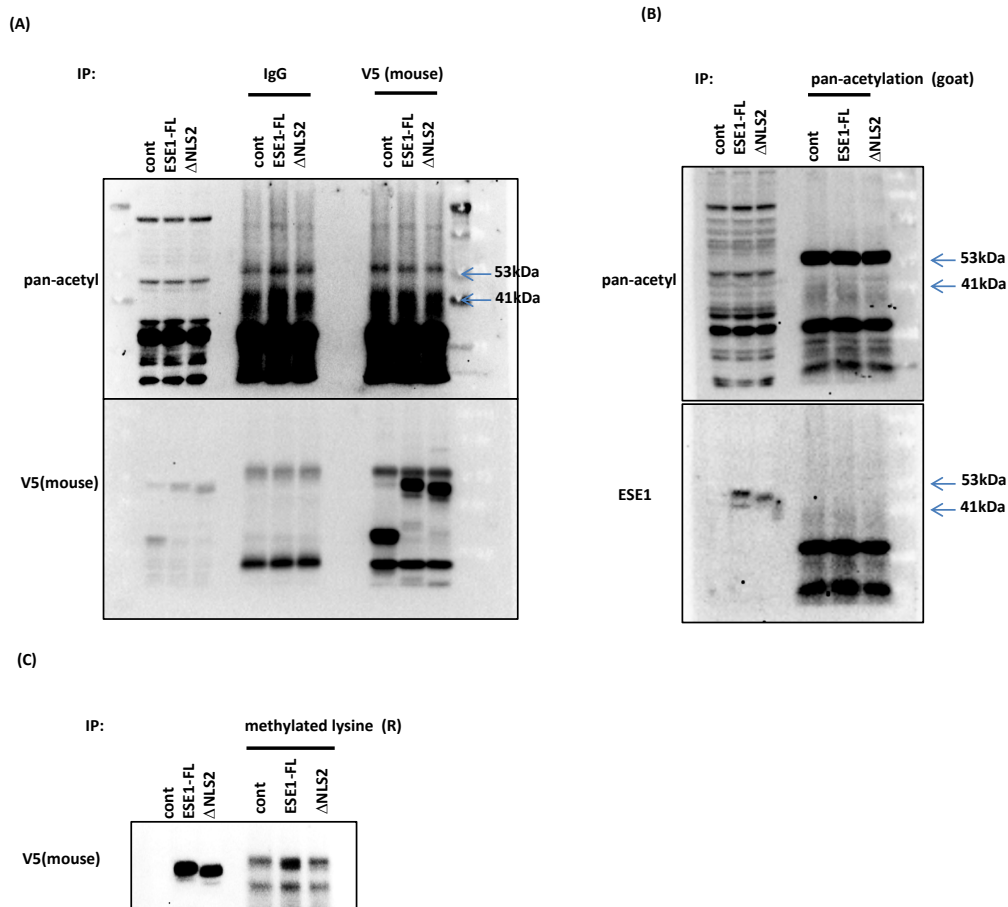
To propose possible mechanism of ESE-1 as a tumor suppressor gene,  $\beta$ -catenin transcriptional activity was tested using SW480 established stable cell lines. As a result, full length of ESE-1 overexpressing cells showed significantly lower TOP/FOP  $\beta$ -catenin transcriptional activity compared to control cells. In addition, triple lysine residues mutated ESE-1 (ESE-1 PM) almost recovered  $\beta$ -catenin transcriptional activity as originally control cells showed (Fig. 3.10). Therefore,  $\beta$ -catenin is involved in tumor suppressive effect of ESE-1 and triple lysine residues in NLS2 are significant in ESE-1.



**Fig. 3.10. Effect of triple lysine substitution of ESE-1 on transactivation of  $\beta$ -catenin in human colon cancer cells.** TOP flash or FOP flash plasmids were transfected into SW480 stable cells for 48 h. The assay of reporter gene (luciferase) was performed as described under Materials and Methods.

#### 3.4.11. Post-translational modification at the amino acid residue NLS2

Acetylation and methylation are common amino acid modifications occurring within lysine residues. We performed immunoprecipitation using acetylation and methylation-specific antibodies. ESE-1 was pulled down with V5 and acetylated ESE-1 was immunoblotted with pan-acetyl antibody. For methylation, the cell lysates were pulled down with anti-methylated-lysine and immunoblotted with V5 antibody. As a result, neither ESE-1 FL nor ESE-1  $\Delta$ NLS2 influences acetylation of ESE-1 compared to control cells (Fig 3.11A & B). However, ESE-1 overexpression showed change of the level of methylation. In addition, deletion of NLS2 reduced methylated lysine. (Fig. 3.11C), indicating that NLS2 residues might be important for methylation of ESE-1.



**Fig. 3.11. Post-translational modification at the amino acid residue NLS2.** Cell lysates extracted from SW480 cells were transfected each ESE-1 clones and immunoprecipitated using anti-v5 antibody (A) pan-acetyl (B) or IgG and immunoblotted with pan-acetyl antibody (upper blot) or anti-V5 antibody (bottom). (C) Cell lysates were immunoprecipitated using methylated lysine or IgG and immunoblotted with V5 antibody. Whole-cell lysates (first three lanes of each blot) were used as control.

### 3.5. Discussion

Because ESE-1 belongs to ETS transcriptional factor family and nuclear localization is significant for target gene transactivation, the significance of NLS and NES sequence within the ESE-1 protein was tested. As shown in Fig. 3.1, ESE-1 includes four putative NLS and NES sequences. The NLS1 sequence (NLS1: <sup>242</sup>HGKRRR<sup>247</sup>) is located in the AT-hook domain, NLS2 (NLS2: <sup>314</sup>LWGQKKKNSN<sup>323</sup>) and NES2 (NES2: <sup>275</sup>LWEFIRDILI<sup>284</sup>) are within the ETS domain, and NES1 (NES1: <sup>102</sup>LCNCALEELRL<sup>112</sup>) is in the Pointed domain. To explore the role of each sequence, four internal deletion clones ( $\Delta$ NLS1,  $\Delta$ NLS2,  $\Delta$ NES1,  $\Delta$ NES2) were generated using an ESE-1 FL expression vector and found that NLS2 is functionally important in the nuclear localization of ESE-1 protein (Fig. 3.2). Within NLS2, point mutation clones were produced and further investigated to identify which amino acid sequence is responsible for nuclear localization of ESE-1. As a result, three lysine residues (<sup>318</sup>KKK<sup>320</sup>) in NLS2 were functionally critical for the nuclear localization of ESE-1 (Fig. 3.4). The importance of lysine residues in nuclear localization has been reported (234). Prescott et al. (140) reported this sequence is lack of nuclear localization activity in breast cancer cells. Protein modification including acetylation, methylation and ubiquitination commonly occur within lysine residues. In this study, a specific protein modification that might regulates the nuclear localization of ESE-1 was identified. Any significant result in acetylation was observed. However, methylation could be one of the mechanisms of both nuclear localization and the role of tumor suppressor gene of ESE-1. It might be possible that experiment condition

might show the more significance since the overall data quality is not high enough. Another interesting finding in this study is that NES1 sequence in the Pointed domain might be significant for stability of ESE-1 protein; deletion of NES1 reduced the amount of ESE-1 in both cytosol and nucleus (Fig. 3.2B).

Overall results from cancer phenotypes indicate that ESE-1 is tumor suppressor gene as investigated in Chapter 2. More interesting finding in this chapter is that triple lysine residues are involved in anti-tumorigenesis as well as nuclear localization of ESE-1. ESE-1 is primarily existed in nucleus and Chapter 2 already proved that the presence of ESE-1 is essential for inhibition of tumorigenesis in colon cancer. Therefore, triple lysine mediated nuclear localization of ESE-1 is responsible for its tumor suppressive effect in colon cancer.

Previously, we reported that ETS domain in ESE-1 binds to N-terminal of  $\beta$ -catenin (232). One of our hypothetical and possible mechanisms of ESE-1 as a tumor suppressor might result from reduced  $\beta$ -catenin transcriptional activity. Rather than regulation of target genes of ESE-1, ESE-1 might mediate the formation of complex of  $\beta$ -catenin and TCF/LEF binding sites, which target genes are mostly oncogenes, such as MMP-9 (235), cyclin D1 (236), c-myc (237) etc. Moreover, overexpression of ESE-1 reduced  $\beta$ -catenin transcriptional activity and triple lysine substitution almost completely recovered original activity as control. This is still incomplete conclusion since direct interaction of ESE-1 and  $\beta$ -catenin in different cellular area has not been investigated yet. Other possible mechanisms related to other signaling pathways also could be explored in follow-up study.

## Chapter 4: Identification of anti-cancer compounds as ESE-1 inducers in colon cancer

### 4.1. Abstract

This chapter designed to identify bioactive dietary compounds to stimulate expression of ESE-1. Various phytochemicals were tested in human colorectal cancer cells and it was found that EGCG and patchouli alcohol increased expression of ESE-1 in protein level. EGCG treatment increased ESE-1 protein expression in a dose- and time-dependent manner. Nuclear ESE-1 was induced by EGCG treatment. mRNA expression of ESE-1 was increased and promoter assay showed induced two versions of ESE-1 promoter. Dose-dependent increase of ESE-1 expression was also observed in human colorectal cancer cells treated with patchouli alcohol, and nuclear ESE-1 protein was also increase by 100  $\mu$ M of patchouli alcohol. Interaction between ESE-1 and  $\beta$ -catenin was also investigated. Patchouli alcohol treatment did not change binding affinity of ESE-1 and  $\beta$ -catenin protein. In addition, a significant reduction of tumor number and tumor load was observed in *Apc<sup>min/+</sup>* mice treated with patchouli alcohol (25 mg/kg and 50 mg/kg body weight). However, no induced ESE-1 protein was observed in patchouli alcohol treated tumor tissue in the colon, although patchouli alcohol reduced  $\beta$ -catenin protein expression level significantly (patchouli alcohol 50 mg/kg body weight). Taken together, anti-cancer dietary compound EGCG and patchouli alcohol were identified as ESE-1 inducers through transcriptional regulation. In addition, anti-cancer activity of patchouli alcohol was confirmed using colon cancer animal model *Apc<sup>min/+</sup>*-DSS combination model for the first time.



## 4.2. Introduction

In recent decades, a large number of epidemiology studies have focused on uncovering the causes of chronic diseases including cancer. One such discovery was that dietary intake is very important in the development of these chronic diseases. This has led many food industries to developing diverse supplements called ‘nutraceuticals’ that integrate the benefits of nutrition and pharmaceuticals. Such supplements contain compounds from certain foods with desirable health benefits. Many phytochemicals have been shown to provide anti-obesity, anti-diabetes or anti-inflammatory benefits. Therefore, researching natural products with potential health benefits is promising.

An emerging research area seeks to identify the molecular target genes responsible for the initiation and progression of chronic diseases in various cellular and tissue contexts. In many epidemiology studies, an increased consumption of fruit and vegetables is highly correlated with a lower prevalence of chronic diseases, including cancer. Therefore, a nutritional approach and changes in specific dietary habits prevent cancer; anti-cancer compounds have been reported to control these chronic diseases with specific targets. Diverse pharmaceutical products and chemically synthesized drugs have also been developed based on molecular target genes. Therefore, this nutritional approach depends on the accurate elucidation of molecular targets.

In previous chapters, we focused on the biological function of ESE-1 in colon cancer and its mechanism. Briefly, we identified ESE-1 as a tumor suppressor in colon cancer and explored mechanisms related to ESE-1 nuclear localization and triple lysine residues in NLS2. The objective of this chapter is to explore the potential use of ESE-1 in the treatment of colon cancer with the mechanism by which ESE-1 determine anti-

oncogenic activity in previous chapter, to identify related bioactive compounds based on their expressions and the mechanism(s) of their actions. Here, based on the hypothesis that ESE-1 could be a molecular target for anti-cancer compounds, a well-known anti-cancer dietary compound EGCG and a novel phytochemical were explored.

### 4.3. Materials and methods

#### 4.3.1. Materials

Antibodies for ESE-1 (sc-376055), tubulin (sc-5286), U1snRNP70 (sc-9571), and actin (sc-1615) were purchased from Santa Cruz Biotechnology. Cell culture media were purchased from Invitrogen. Epigallocatechin-3-gallate (EGCG) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Genistein (#345834) and resveratrol (#554325) were purchased from EMD Millipore (Burlington, MA, USA). Capsaicin (E2028), isorhamnetin (#17794), hispidin (H5257), amygdaline (A6005), protocatechualdehyde (D108405), and 2-methoxy-4-vinylphenol (W267500) were purchased from Calbiochem (Burlington, MA, USA). Actigenin (#1777) was purchased from Tocris Bioscience (Minneapolis, MN, USA), and 3,3'-diindolylmethane (ALX-350-370) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Patchouli alcohol for *in vitro* and *in vivo* study was purchased from Santa Cruz (SC-281124) and Carbosynth USA (FP09677) (San Diego, CA, USA), respectively. All chemicals were purchased from Fisher Scientific or VWR, unless otherwise specified.

#### 4.3.2. Cell culture and treatment

HCT116 human colon carcinoma cell line was purchased from the American Type Culture Collection and maintained in DMEM/F-12 media supplemented with 10% fetal bovine serum (FBS) and a mixture of penicillin (100 U/mL) and streptomycin (100 µg/mL) under a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C.

#### 4.3.3. Cell cycle distribution

HCT116, SW480, Caco-2 and HT-29 cells ( $6 \times 10^5$ ) were plated onto 6 well plate and incubated with vehicle or 100  $\mu$ M of patchouli alcohol for 24 hours. All cells were collected using trypsin EDTA and centrifuged at 1000 rpm for 5 min. Precipitated cells were washed using PBS, then gradient washing was conducted using 70% ethanol/30% PBS, 50% ethanol/50% PBS and 20% ethanol/80% PBS. Finally, mixture of propidium iodide (70  $\mu$ M) and RNase (1 mg/mL) was added. Cell cycle distribution was analyzed using the cell cycle distribution was analyzed using FACS flow cytometry at the University of Maryland MPRI Flow Cytometry and Cell Sorting Facility.

#### 4.3.4. Transient transfection and lucifersase assay

The luciferase reporters (ESE-1 promoter), TOP or FOP were transiently transfected using PolyJet DNA transfection reagent (SignaGen Laboratories) according to the manufacturer's instruction. For luciferase assays, cells were lysed in 1x Passive Lysis Buffer for 15 minutes, and the enzymatic activity of luciferase was measured using a luminometer (Promega). Enzyme activity was normalized to that of pRL-null using a dual luciferase assay system (Promega) as described previously (227).

#### 4.3.5. Western blot and RT-PCR

The cells were washed two times with cold PBS and incubated with RIPA buffer containing a protease/phosphatase inhibitor cocktail (Sigma) for 30 minutes before being centrifuged at 14,000 g for 10 minutes at 4 °C. The tissue weight was

measured and RIPA buffer added 1 mL per 3 g of tissue weight. Then, tissues were ground using tissue homogenizer on ice. Protein concentration was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific). SDS-PAGE was performed to separate proteins based on size. After transfer to nitrocellulose membranes, a specific primary antibody was added in TBS containing 0.1% Tween 20 (TBS-T) and 3% BSA, and incubated overnight at 4 °C. Subsequently, the membranes were incubated with horse radish peroxidase (HRP)-conjugated IgG for one hour at room temperature, washed three times with 1X TBS-T (0.1% Tween-20), and visualized using enhanced chemiluminescence (ECL) (Amersham Biosciences). The image was taken using a ChemiDoc Imaging system (BioRad Laboratories). ESE-1 mRNA was measured using RT-PCR. Total RNA was extracted from HCT116 cells treated with different dose of EGCG for 24 hours. cDNA was synthesized and PCR was performed with following primers: ESE-1: forward 5'-TTAGCAACTACTTCAGTGCGATGT-3' and reverse 5'-GTTCTTCTCCACTTGGTAGCTGAT-3' , glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward 5' -ACCCAGAAGACTGTGGATGG-3' and reverse 5' - TTCTAGACGGCAGGTCAGGT-3'.

#### 4.3.6. *In vivo* study (*Apc*<sup>Min/+</sup>)

We purchased *Apc*<sup>Min+</sup> mice which have mutation of tumor suppressor (*Apc*) from Jackson's laboratories (Stock Number: 002020) and bred with B6 mice. Twenty-seven *Apc*<sup>Min</sup> mice at 4 weeks of age were assigned to one of following three groups;

1) vehicle (0.5% methylcellulose), 2) low dose of patchouli alcohol (25 mg/kg body weight), and 3) high dose of patchouli alcohol (50 mg/kg body weight) respectively (nice mice/each group). Then 2% (w/v) DSS was given to mice through drinking water for one week. Patchouli alcohol were orally administrated three times a week for 6 weeks from week 4 to week 10. At age 10 week, all animals were sacrificed and the entire small and large intestinal tract were removed, flushed with cold saline and opened flat longitudinally. The tumors were determined and counted under a dissecting microscope and tumor load was calculated in both small and large intestines.

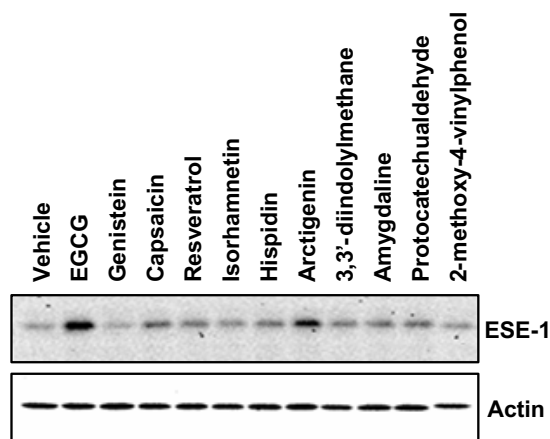
#### 4.3.7. Statistical analysis

Statistical analyses were conducted using Student's t-test with statistical significance set at  $*P < 0.05$ .

## 4.4. Results

### 4.4.1. Evaluation of natural phytochemicals as an ESE-1 inducer

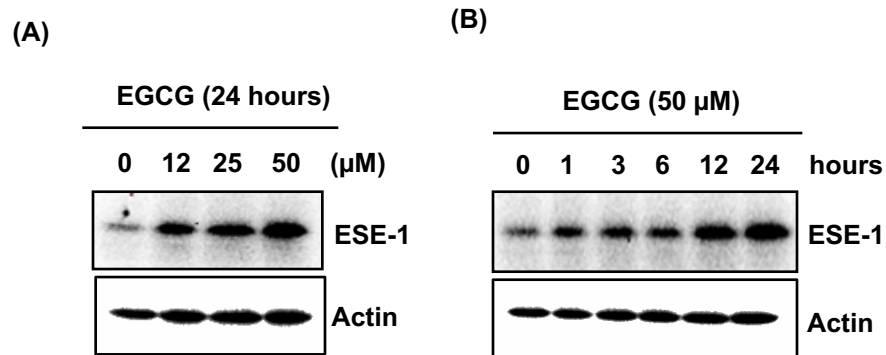
Many studies support that diverse dietary phytochemicals possess anti-cancer activities in humans. To evaluate potential natural dietary compounds which facilitate ESE-1 expression, HCT116 cells were treated with commonly used phytochemicals (EGCG, genistein, capsaicin, resveratrol, isorhamnetin, hispidin, arctigenin, 3,3'-diindolylmethane, amygdaline, protocatechualdehyde and 2-methoxy-4-vinylphenol) at doses of 50  $\mu$ M. As shown in Fig. 4.1, EGCG and arctigenin induced ESE-1 protein expression. Since the effect of EGCG was more significant, it was used for further studies.



**Fig. 4.1. ESE-1 expression in human colorectal cancer cells treated with different phytochemicals.** HCT116 human colon cancer cells were exposed to 11 different phytochemicals at a dose of 50  $\mu$ M for 24 hours. Cells were harvested with RIPA buffer and subjected to Western blot to detect ESE-1 and actin.

#### 4.4.2. Induction of ESE-1 by treatment of EGCG

Next, protein expression level of ESE-1 was investigated after treatment of EGCG with different doses and different time points (50  $\mu$ M). As shown in Fig. 4.2A, EGCG treatment increased ESE-1 expression in a dose dependent manner. ESE-1 expression level was increased 12 hours after treatment of EGCG, (Fig. 4.2B).

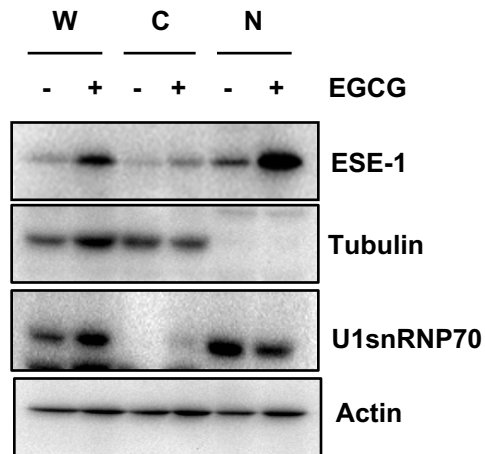


**Fig. 4.2. EGCG induced ESE-1 expression in a dose- and time-dependent manner in human colon cancer cells. (A)** HCT116 cells were exposed to different doses of EGCG for 24 hours. **(B)** HCT116 cells were exposed to 50  $\mu$ M of EGCG for different durations (1, 3, 6, 12, 24 hours) and Western blots were performed using antibodies for ESE-1 and actin.



#### 4.4.3. Nuclear localization of ESE-1 by treatment of EGCG

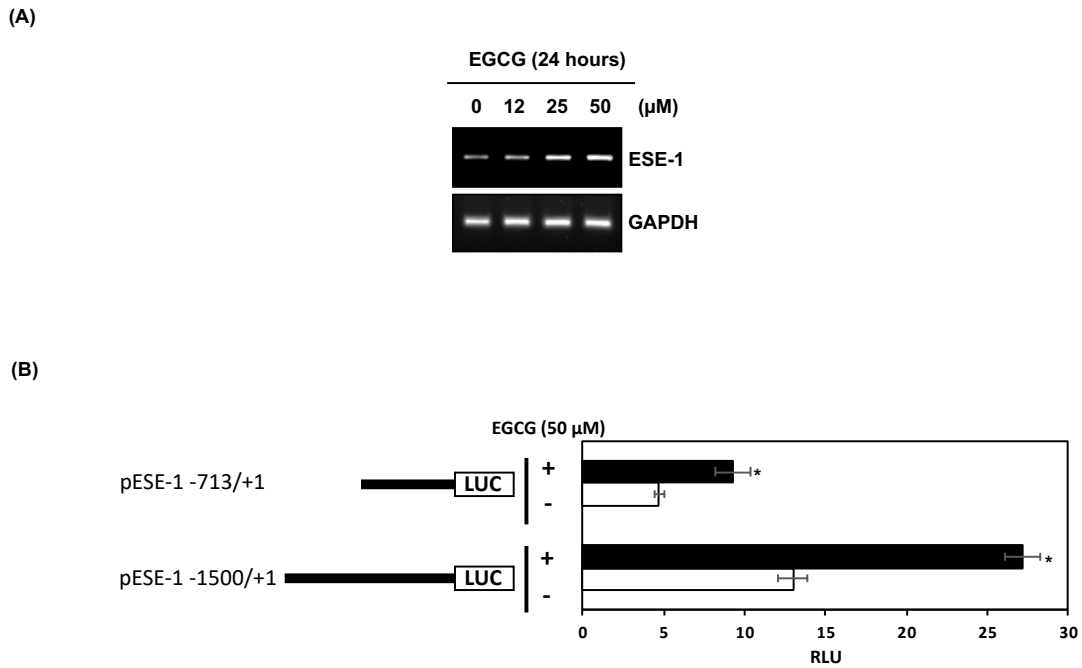
Since increased accumulation of ESE-1 in nucleus is significant in tumor suppressive activity, whether treatment of EGCG affects amount of nuclear ESE-1 was tested. Tubulin and U1snRNP70 was used as positive controls for cytosol and nuclear fraction. A huge increase of ESE-1 is observed in nucleus whereas minimal increased is observed in cytosol of cells treated with EGCG. (Fig. 4.3).



**Fig. 4.3. EGCG increases the amount of ESE-1 protein in nucleus.** Cytoplasmic and nuclear fractions were prepared from HCT116 cells after 24 hours of EGCG treatment. Tubulin and U1snRNP70 were detected as specific markers for the cytosol and nucleus.

#### 4.4.4. Effect of EGCG on transcriptional regulation

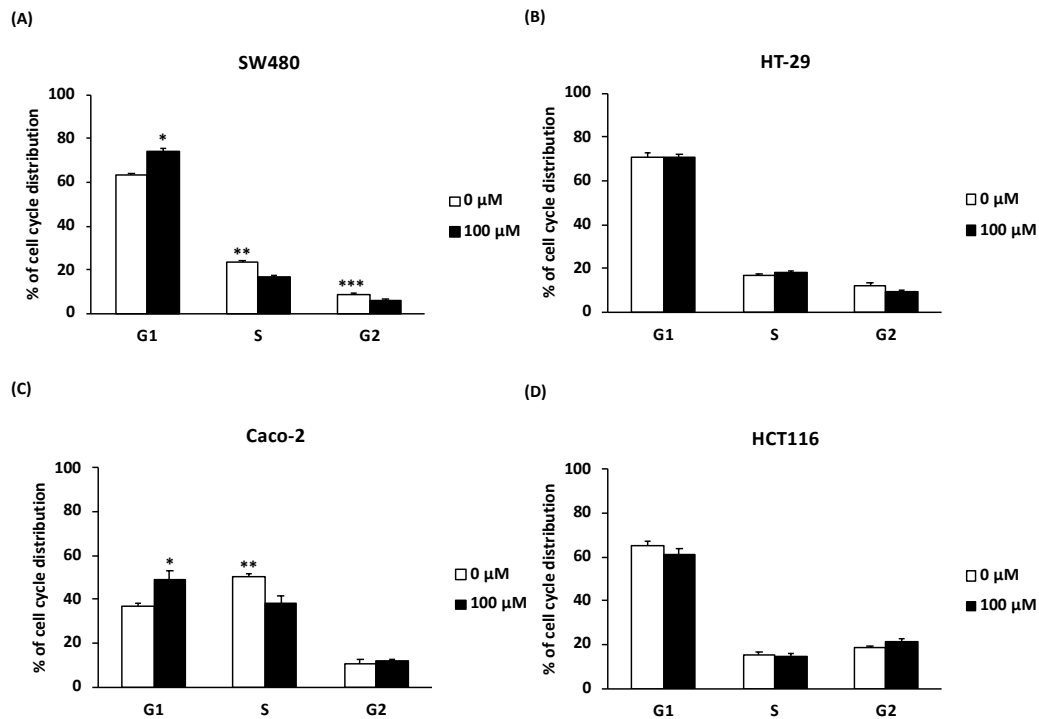
For further mechanism, whether increased ESE-1 is associated with transcriptional upregulation was examined. As shown in Fig. 4.4A and B, EGCG increased ESE-1 mRNA expression levels and the activity of ESE-1 promoters.



**Fig. 4.4. ESE-1 increased ESE-1 mRNA and transcriptional activity of ESE-1 promoters.** (A) HCT116 cells were exposed to different doses of EGCG, and RT-PCR was performed to detect ESE-1 and GAPDH mRNA levels. (B) HCT116 cells were transfected with ESE-1 promoters, and then treated with 50  $\mu$ M of EGCG and luciferase activity measured.

#### 4.4.5. Patchouli alcohol induces G1 arrest in certain types of human colon cancer cell lines

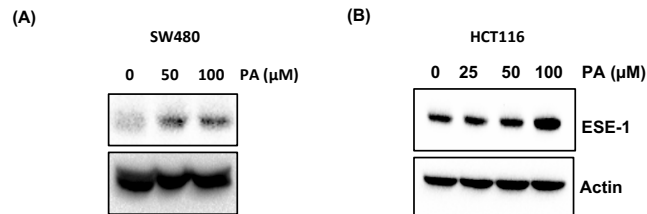
Previously, we reported anti-proliferative activities of patchouli alcohol in human colon cancer cells (223). As a follow-up study, we performed FACS analysis to see patchouli alcohol affects cell cycle distribution using the different type of human colorectal cancer cells treated with 100  $\mu$ M of patchouli alcohol. As a result, patchouli alcohol induced G1 arrest in SW480 and Caco-2 cell lines (Fig. 4.5).



**Fig. 4.5. Effect of patchouli alcohol on cell cycle distribution of colon cancer cell lines.** Cell cycle distribution was performed by staining the cells with propidium iodide and subsequent FACS analysis. Data represent the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$  vs. the control.

#### 4.4.6. Patchouli alcohol induces ESE-1 protein expression in different human colon cancer cell lines

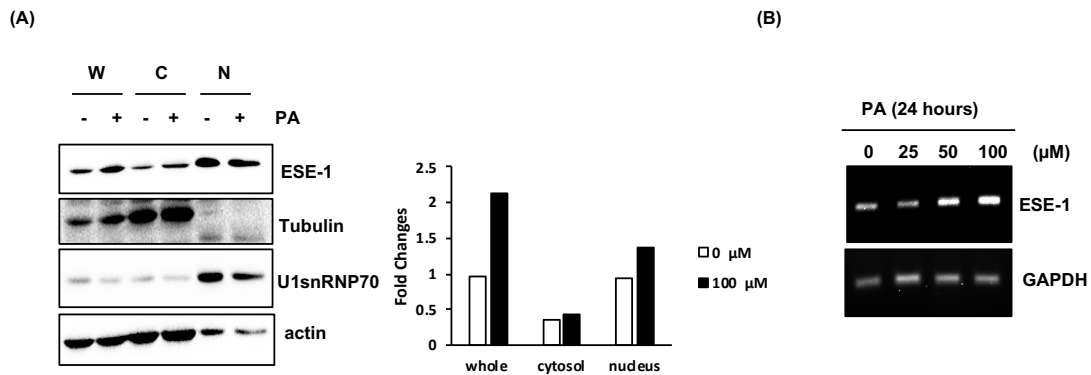
To test whether patchouli alcohol induces ESE-1 expression, patchouli alcohol was treated and ESE-1 expression level was observed using western blot. Patchouli alcohol treatment induces ESE-1 expression in both SW480 and HCT116 cells (Fig. 4.6).



**Fig. 4.6. Patchouli alcohol induces ESE-1 expression in human colon cancer cell lines.** Patchouli alcohol was treated to SW480 (A) and HCT116 (B) human colon cancer cell lines for 24 hours in serum free media and western blots were performed using antibodies for ESE-1 and actin.

#### 4.4.7. Patchouli alcohol induces ESE-1 via its transcriptional upregulation

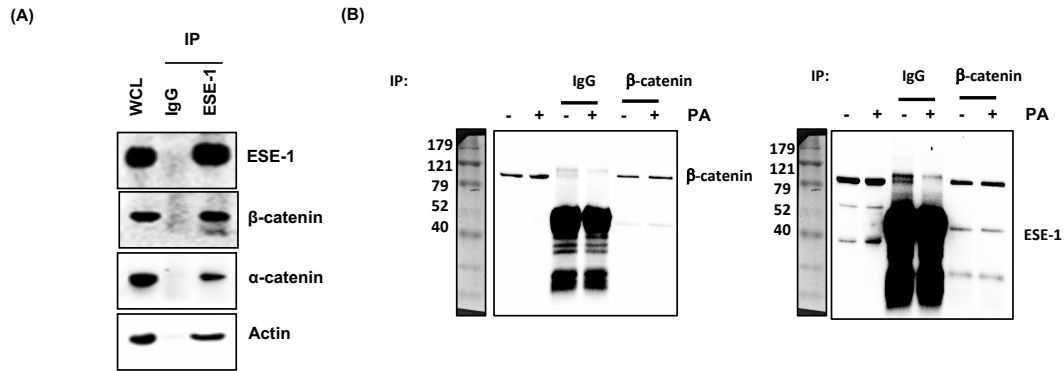
The effect of treatment of patchouli alcohol on accumulation of ESE-1 protein in nucleus was tested. Patchouli alcohol slightly induced ESE-1 protein expression in nucleus. Unlike dramatic increase of nuclear ESE-1 induced by EGCG (Fig. 4.3), patchouli alcohol did not increase nuclear ESE-1 dramatically (Fig. 4.7). To confirm whether increased ESE-1 mediated by treatment patchouli alcohol results from transcriptional upregulation, ESE-1 mRNA expression and promoter assay were performed. Patchouli alcohol increased ESE-1 mRNA expression levels, but patchouli alcohol did not increase the activity of either any type of the ESE-1 promoter (data not shown).



**Fig. 4.7. Effect of patchouli alcohol on transcriptional regulation of ESE-1.** (A) HCT116 cells were exposed to different doses of patchouli alcohol, and RT-PCR was performed to detect ESE-1 and GAPDH mRNA levels. (B) HCT116 cells were transfected with ESE-1 promoters, and then treated with 100  $\mu$ M of patchouli alcohol and luciferase activity measured. Cytoplasmic and nuclear fractions were prepared from HCT116 cells after 24 hours of patchouli alcohol treatment. Tubulin and U1snRNP70 were detected as specific markers for the cytosol and nucleus.

#### 4.4.8. Effect of patchouli alcohol on interaction between ESE-1 and $\beta$ -catenin

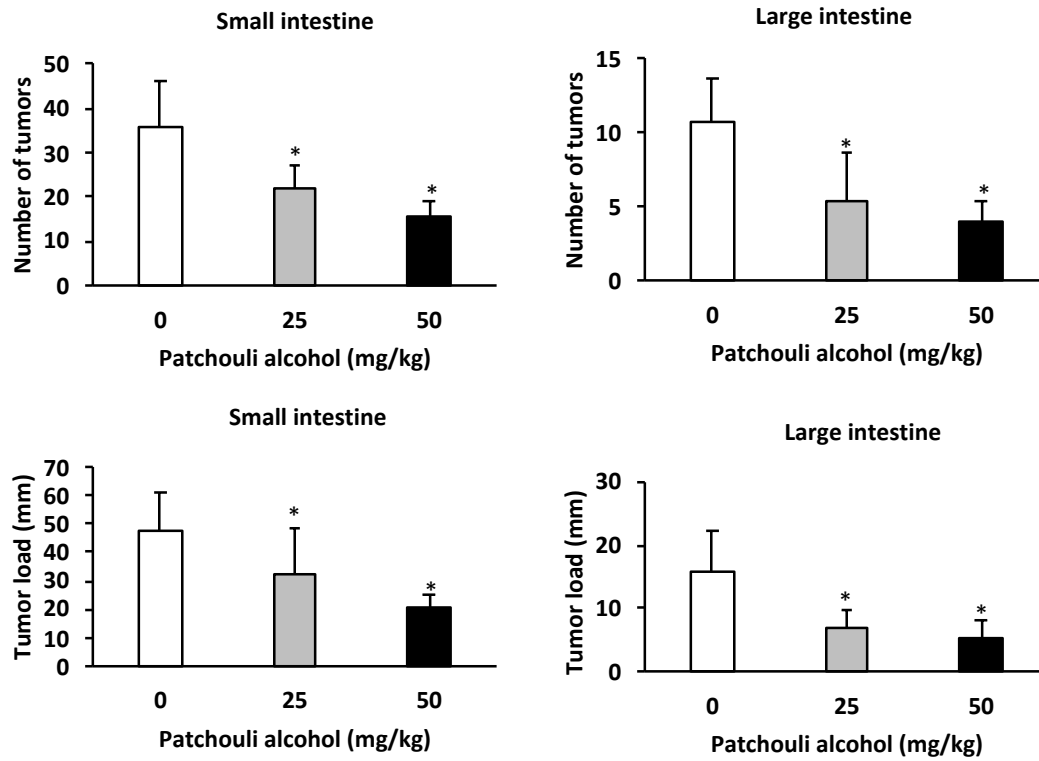
Interaction between ESE-1 and  $\beta$ -catenin was proposed as another potential mechanism by which ESE-1 suppresses cancer as shown in Fig. 4.8A. Therefore, immunoprecipitation was performed to see if patchouli alcohol affects interaction between ESE-1 and  $\beta$ -catenin. Protein was pulled down with  $\beta$ -catenin and was immunoblotted with ESE-1 antibody. Patchouli alcohol did not affect protein-protein interaction between ESE-1 and  $\beta$ -catenin (Fig. 4.8B).



**Fig. 4.8. Effect of patchouli alcohol on protein-protein interaction between ESE-1 and  $\beta$ -catenin.** (A) Cell lysates were pulled down using IgG or ESE-1 antibody and immunoblotted with  $\beta$ -catenin,  $\alpha$ -catenin, or actin. (B) Cell lysates extracted from HCT116 cells that were treated with vehicle or patchouli alcohol 100  $\mu$ M and immunoprecipitated using anti- $\beta$ -catenin antibody or IgG and immunoblotted with  $\beta$ -catenin antibody (left blot) or ESE-1 antibody (right). Whole-cell lysates (first two lanes of each blot) were used as control.

#### 4.4.9. Patchouli alcohol reduced colon cancer tumorigenesis *in vivo*

We investigate whether patchouli alcohol shows anti-cancer activity in colon cancer *in vivo*. The *Apc*<sup>Min/+</sup> mice exposed to DSS for 1 week via drinking water produced average 35.78 and 10.67 tumors in small and large intestine, respectively. Administration of patchouli alcohol via gavage significantly reduced both number of tumors and tumor load in both small intestine and large intestines. The 32.67% and 57.45% of reduction in tumor load was observed in small intestine of *Apc*<sup>Min/+</sup> mice fed with 25 mg/kg and 50 mg/kg patchouli alcohol, respectively. And 55.15% and 67.15% of reduction in tumor load was observed in large intestine (colon and rectum) of *Apc*<sup>Min/+</sup> mice fed with 25 mg/kg and 50 mg/kg patchouli alcohol, respectively. These data indicate anti-tumorigenic activity of patchouli alcohol in colon cancer model.

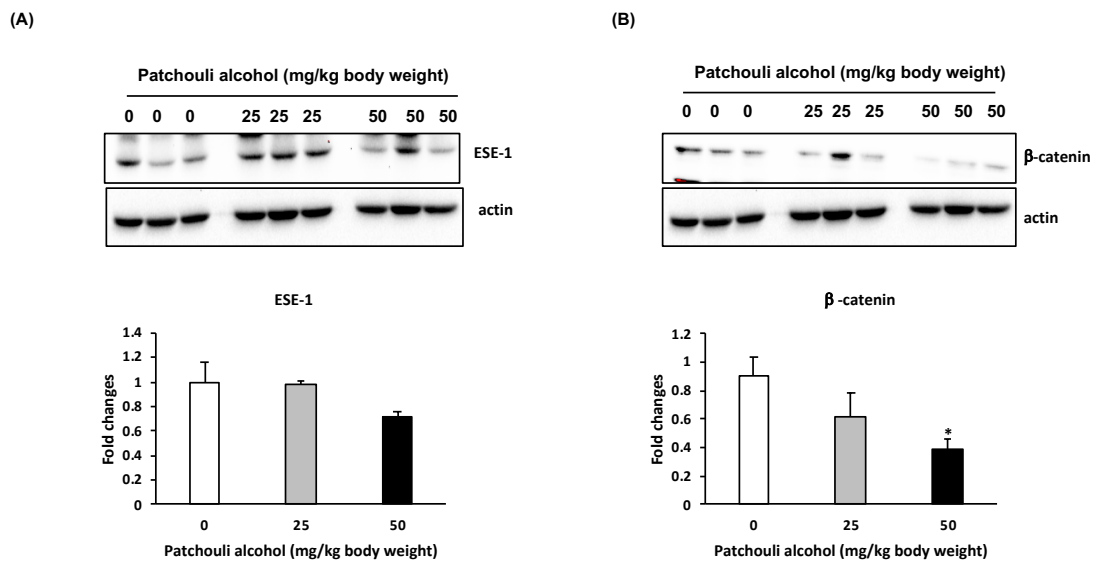


**Fig. 4.9. Patchouli alcohol inhibits tumorigenesis *in vivo*.** Patchouli alcohol was orally administered three times a week (0, 25, 50 mg/kg body weight) for 6 weeks. *Apc*<sup>Min/+</sup> mice were exposed to DSS for 1 week via drinking water. The number of tumors in colon and small intestine was counted and tumor size in colon and small intestine was measured. Tumor load was calculated by multiplying tumor size (mm) and the number of each size of tumor (bottom). \* Significant compared to control at  $p < 0.05$ .



#### 4.4.10. ESE-1 expression in tumor from colon (*in vivo*)

To elucidate the mechanisms of anti-cancer activity of patchouli alcohol *in vivo* and to investigate whether ESE-1 is involved in tumor suppressor effect of patchouli alcohol *in vivo*, protein expression of ESE-1 and  $\beta$ -catenin was observed. No trend of ESE-1 change was found in colon tumor tissues but decrease of  $\beta$ -catenin by patchouli alcohol treatment was observed at patchouli alcohol 50 mg/kg body weight (Fig. 4.13).



**Fig. 4.10. Effect of patchouli alcohol treatment on gene expression *in vivo*.** Tumors from the  $Apc^{Min/+}$  mice colon was lysed and western blots were performed using antibodies for ESE-1,  $\beta$ -catenin and actin. \* indicates significance difference compared to control at  $p < 0.05$ .

#### 4.5. Discussion

Natural phytochemical products were tested for induced ESE-1 expression mediated by those products and identified EGCG and patchouli alcohol as ESE-1 inducers in human colon cancer cells. Some of those tested compounds include other effective tumor suppressors such as genistein, capsaicin, resveratrol, isorhamnetin, and 3,3'-diindolylmethane. However, ESE-1 expression was activated only by EGCG and patchouli alcohol, implying that they might be specific and selective in ESE-1 based cancer treatment.

EGCG is a polyphenol compound and originated from green tea. EGCG is widely known and studied anti-cancer compound, which a lot of studies reported apoptotic and cell cycle arrest effect in the *Apc<sup>Min/+</sup>* colon cancer animal model (238) and in colon cancer cells (239). EGCG could be a positive control as an anti-cancer compound in colon cancer to study whether ESE-1 could be a molecular target of chemopreventive agent.

On the other hand, patchouli alcohol is a relatively novel anti-cancer compound in colon cancer. Several health benefits of patchouli alcohol have been reported in lung cancer (216), colon cancer (223) and gastric cancer (217,240). However, patchouli alcohol has not been discovered much in cancer area. Moreover, there was no report about anti-cancer activity in colon cancer *in vivo*. *Apc<sup>Min/+</sup>* mice also represent the human familial adenomatous polyposis (FAP) syndrome. The weakness of *Apc<sup>Min/+</sup>* mice model is that mostly tumors are formed in the small intestine rather than colon. However, *Apc<sup>Min/+</sup>* mice model with combination of DSS developed more multiple

colonic neoplasms (241) which means that this combination model is more ideal to examine how patchouli alcohol affects colorectal tumorigenesis.

Based on results in previous chapters, main focus of this chapter was on accumulation of nuclear ESE-1 mediated by either EGCG or patchouli alcohol in this chapter. As a result, both EGCG and patchouli alcohol increased the amount of ESE-1 in the nucleus, and both EGCG and patchouli alcohol increased mRNA expression, supporting that induced ESE-1 expression might result from transcriptional upregulation. In addition, two versions of ESE-1 promoter activity was also increased by treatment of EGCG. As shown in Chapter 2, the presence of ESE-1 was already critical for its biological function as a tumor suppressor gene and its accumulation in nucleus is important for its tumor suppressor effect. Therefore, induction of nuclear ESE-1 accumulation mediated by natural compounds could be promising for anti-cancer drugs/chemopreventive agents. Another interesting finding of this chapter is that patchouli alcohol treatment induced ESE-1 even in SW480 which is considered as an ESE-1 null cell line. This effect might be the similar effect as overexpression of ESE-1 in ESE-1 null cells although no relevance study was conducted in this chapter. Therefore, this approach also will later be applied to other potentially effective compounds.

Since anti-cancer activities of patchouli alcohol using a colon cancer animal model was not reported, *Apc*<sup>Min/+</sup> mice were used to investigate tumor suppressive effect of patchouli alcohol in colon cancer. Both the number of tumors and tumor load were decreased and significance difference was observed in patchouli alcohol 50 mg/kg treatment group.  $\beta$ -catenin protein level was measured in colon tumor tissues, because

*Apc*<sup>Min/+</sup> mice model is ideal to study  $\beta$ -catenin signaling pathway and  $\beta$ -catenin is one of typical oncogenes easily regulated and changed by treatment. In addition,  $\beta$ -catenin transactivates a lot of target genes which are mostly oncogenes. As expected, reduced  $\beta$ -catenin protein expression was observed in patchouli alcohol treatment group. However, there was no change in ESE-1 protein expression by treatment of patchouli alcohol in animal tissue analysis.

Lastly, immunoprecipitation was performed to test whether patchouli alcohol changes binding between ESE-1 and  $\beta$ -catenin protein. Although interaction between ESE-1 and  $\beta$ -catenin was already reported (232), binding of ESE-1 to  $\beta$ -catenin was confirmed (Fig. 4.8A). However, there was no change of interaction between those two proteins by treatment of patchouli alcohol. This result indicates that anti-cancer activity of patchouli alcohol is not associated with ESE-1 binding to  $\beta$ -catenin protein.

## Chapter 5: CONCLUSION AND PROSPECTS

### 5.1. Integrative Discussion

Colon cancer is one of leading causes of cancer-related deaths in the United States and its clinical importance in targeted treatment has been emerging for decades. In targeted therapy for chemoprevention, understanding underlying molecular mechanisms is significant.

ESE-1 belongs to the ETS transcription factors family and has been considered as significant gene in cancer progression. ESE-1, epithelial specific ETS-1, could be one of the molecular targets for chemoprevention especially in colon cancer because ESE-1 is involved in terminal cell differentiation of epithelial cells (133). However, ESE-1 research studies are still contradictory and whether ESE-1 is tumor suppressor or oncogene still has not been completely verified yet, either in colon cancer or in other cancer types.

In this study ESE-1 knockout mouse model was adopted and treated with AOM and DSS. This is an ideal colon cancer animal model in terms of pathological and clinical relevance because inflammatory bowel disease (IBD) is primary precondition for carcinogenesis. ESE-1 knockout transgenic mice showed significant higher ACF formation, indicating that ESE-1 is tumor suppressor gene in colon cancer.

The results suggest that *in vitro* gain of function and loss of function study using human colorectal cancer stable cell lines. As a result, in the presence of ESE-1 gene, anchorage independent cell growth was decreased in both SW480 and HCT15 cells. G1 cycle arrest and apoptosis were induced by ESE-1 expression in SW480 and HCT15

cells. In addition, cell migration and invasion were inhibited in both SW480 and HCT15 stable cell lines expressing ESE-1. Another interesting finding is that ESE-1 overexpression might induce ER stress related DNA damage. This indicates that ER stress might be one of the mechanisms of ESE-1 as a tumor suppressor gene.

Tumor suppressive effect of ESE-1 was also verified by loss of function study. HCT116 was selected to establish ESE-1 knockdown stable cell lines because of its abundant ESE-1 expression level. HCT116 ESE-1 knockdown cells showed higher anchorage independent cell growth, cell migration and invasion. Overall, all results were consistent to each other and they showed that ESE-1 plays a key role as a tumor suppressor gene in colon cancer.

As a follow-up mechanistic study in Chapter 3, the effect of sublocalization of ESE-1 on determination of tumorigenicity was investigated. Internally deleted ESE-1 expression vector lacking two NLS and NES within the ESE-1 protein was used. As a result, NLS2 (NLS2: <sup>314</sup>LWGQKKKNSN<sup>323</sup>) is functionally important in terms of nuclear localization of ESE-1. Within NLS2, any single or double amino acids was not significant for nuclear localization of ESE-1. In a further study, three lysine residues (<sup>318</sup>KKK<sup>320</sup>) in NLS2 were critical for the nuclear localization of ESE-1.

Therefore, triple lysine mutated ESE-1 overexpressing cell line was established to test whether those residues are responsible for colon cancer tumorigenesis. The inhibited anchorage independent cell growth, migration and invasion were recovered in triple lysine mutated ESE-1 overexpressing stable cells. In addition, similar pattern was also observed in the result of cell proliferation, representing that ESE-1 and its triple lysine residues might also affect cancer cell growth in a short-term period.

Since ESE-1 is  $\beta$ -catenin binding protein and their interaction was identified (232),  $\beta$ -catenin transcriptional activity was also measured;  $\beta$ -catenin transcriptional activity was decreased in the presence of ESE-1. However, triple lysine mutated ESE-1 stable cells showed recovered  $\beta$ -catenin transcriptional activity as high as control cells. This result is promising because Wnt/ $\beta$ -catenin is significant in tumorigenesis and most of  $\beta$ -catenin target genes are oncogenic. Lysine residues sites are common locations for protein modification (acetylation, methylation and ubiquitination). Therefore, whether NLS2 residues could be a target of acetylation and/or methylation was investigated. As a result, neither ESE-1 nor NLS2 residues affect acetylation. However, methylation was induced by ESE-1 and reduced down to the level as control cells in NLS2 transfected cells. This data indicates that methylation might regulate nuclear localization of ESE-1 via NLS2 residues. Although triple lysine residues in NLS2 was identified that they are responsible for nuclear localization of ESE-1 and  $\beta$ -catenin was proposed as a possible mechanism, the mechanisms of how those triple lysine residues modify the interaction between  $\beta$ -catenin and ESE-1 still remains unclear. This question would be future study topics/objectives.

Moonlighting proteins are a family of genes sharing multiple functions without gene fusions, multiple RNA splice variants, or pleiotropic effects. Several moonlighting transcriptional factors have been determined, such as STAT3, ATF2 and ERK2. Like other regular transcriptional factors, moonlighting proteins bind to DNA to regulate transcription of gene, but also plays a role as structural proteins in other subcellular components (242). As other moonlighting proteins, ESE-1 might possesses dual function based on subcellular localization of ESE-1 (243). According to results

from this dissertation, it was assumed that nuclear ESE-1 might mainly function as a tumor suppressor gene, whereas ESE-1 in cytoplasm might regulate cellular transformation through regulation in non-nuclear and non-transcriptional ways. This speculation is supported by other's finding that nuclear localization of ESE-1 facilitate apoptosis while ESE-1 in cytoplasm results in transformation of normal mammary epithelial cell through SAR domain-dependent mechanism (140).

Diet is highly associated to colon cancer, and high consumption of plant-based foods is low risk factor of colon cancer (244). There probably might be other reasons, but probably main reason might be based on phytochemicals in plant-based products. Diverse phytochemicals were identified as anti-cancer compounds.

Therefore, to test whether ESE-1 could be molecular target for potential or well-known anti-cancer drugs, patchouli alcohol and EGCG were treated and ESE-1 was tested in diverse ways. As known as an effective anti-cancer dietary compound, overall EGCG treatment highly induced ESE-1 expression. The amount of nuclear ESE-1 was highly induced and its transcriptional activity was induced by EGCG treatment. Patchouli alcohol also showed similar pattern, but the level of induction was not effective as EGCG. However, patchouli alcohol also showed strong anti-cancer activities in colon cancer (223) and our *in vivo* data also supports its tumor suppressive effect. Anti-cancer activities are complex and ESE-1 might not be only gene affected by treatment of phytochemicals; and the amount of ESE-1 affected by certain compound also might determine results.



## 5.2. Concluding Remarks

It is important to screen and identify effective compounds and to study their working mechanisms for the treatment and management of chronic diseases including cancer. Identifying the biological function of ESE-1 would be a significant contribution to the field. The mechanisms in this dissertation could suggest a basis for development of novel nutraceuticals which prevents colon cancer. The results will be basis to expand our knowledge and further create a novel and potential molecular target to repress colon cancer. This mechanistic study with practical application using dietary compounds/anti-cancer drugs possesses clinical relevance for transition to chemopreventive or therapeutic strategies.

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