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Study on gene regulation and post-translational modification of Ets transcription factors

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This study focuses on a group of functional factors named as Ets family of transcription factors, whose gene products control various cellular functions and biological processes, such as hematopoiesis, differentiation, apoptosis, immunity and oncogenesis, through regulating transcriptions. The research contents present here are mainly divided into two parts: one part concentrates on the identification of Ets target genes and investigation of the related regulatory mechanisms; the other attempts to elucidate the influence of post-translational protein-protein interaction and modification on one Ets factor, MEF.

The diverse functions of Ets family are finally fulfilled by a series of downstream genes that are targeted and controlled by Ets factors, and discovering new Ets target genes involved in physiological or pathological processes will facilitate towards understanding the vital functions of Ets family. The functions of Ets factors in epithelial cells have been a subject of active investigations. In the first part of this study, the transcriptional control mechanisms of two Ets target genes are investigated in epithelial cells respectively.

The results of Chapter 1 demonstrate that ETS2 is a crucial transcription factor and a target of PKC (protein kinase C), which regulates GM-CSF (granulocyte-macrophage colony-stimulating factor) expression through transferring the activating signals from upstream PKC to GM-CSF promoter. ETS2 and a PKC activator, PMA (phorbol 12-myristate 13-acetate), can synergistically up-regulate the transcription of GM-CSF.

The study in Chapter 2 indicates that another Ets factor, MEF, acts as a novel transactivator of HBD2 (human β-defensin 2) in epithelial cells, which functions through binding to the EBS in the promoter of HBD2. Thus MEF may have the potential to enhance basic HBD2 expression under non-irritated physiological conditions.

The transcriptional action is not a simple process that turns on or off depending only on the binding or releasing of transcription factors to their consensus DNA, but is a
subtle, cellular and environmental context-dependent and responsive regulatory course. Post-translational protein-protein interaction and modification are effective ways to influence the transactivating potential of Ets factors, their mutual interplays and the more subtle and comprehensive mechanisms, for instance, where and when the transcriptional affairs occur. The main concerns of the second part of this study are to evaluate the post-translational protein-protein interaction and modification of MEF, and the consequent impacts on its functions in epithelial cells.

First, the data present in Chapter 3 show that the transactivity of MEF on HBD2 is enhanced by PML (promyelocytic leukemia protein), which alone did not show activating effect. Furthermore, exogenous heat shock stress promoted the co-localization between endogenous MEF and PML nuclear bodies and consequently up-regulated both HBD2 promoter activity and its endogenous expression. Taken together, the results above suggest that the transcriptional activity of MEF on HBD2 may be regulated by PML especially under stress conditions.

Next, the SUMO modification of MEF is investigated in Chapter 4. The in vitro experiments using the E. coli sumoylation system demonstrated that MEF is modified by SUMO with the lysine 657 within the SUMO motif in its C-terminus. The SUMO modification of MEF was also verified in mammalian cells. The functional investigations indicate that SUMO modification of lysine 657 does not correlate with MEF localization in PML NB, but probably contributes to the modulation of its transcriptional activity.
Research Background

1. General information about Ets family of transcription factors.

Protein is one of the basic substances that support the vitalities of all creatures. The initial step for synthesizing a protein is called transcription, which transfers the genetic information from DNA to the messenger RNA (mRNA), then finally to the protein by the step named translation. Transcription begins from the binding of transcription complex to the promoter region of a gene. For the smooth processing of transcription, for example in eukaryotes, besides the basal enzymes like RNA polymerase II and the basal or general transcriptional factors like TFIID, the special regulatory transcription factors to activate or suppress transcription are also required and contained in the transcription complex. The present study is focusing on a group of functional factors named as Ets (E26 transformation-specific) family of transcription factors, whose gene products control various cellular functions through regulating transcriptions.

The Ets family of transcription factors is characterized by an evolutionarily conserved ETS domain, which is a fragment of about 85 amino acid residues. The ETS domain, through its winged helix-turn-helix (wHTH) structure [1], mediates the binding between the Ets proteins and the purine-rich DNA sequence with a central GGAA/T core consensus in target gene promoters [2, 3]. Consensus DNA sequences for Ets factors are called ETS-binding site (EBS). Alteration of one or two nucleotides in the core consensus can abolish the DNA binding of Ets factors. Different members of the Ets family proteins display distinct DNA-binding specificities, which is supposed to be influenced by the ETS domains and the flanking amino acid sequences [2]. Furthermore, the adjacent DNA sequences of the core consensus also affect the preferential binding affinity of individual Ets family proteins [4].

Since v-ets, the first found Ets family gene, was identified as a gag-myb-ets fusion oncogene of the avian transforming retrovirus E26 which induces both erythroblastic and myeloblastic leukemia [5], many cellular homologs were isolated thereafter from Caenorhabditis elegans or Drosophila melanogaster to humans [6, 7]. So far, approximately 30 members of this family have been identified in mammals which are shown to encode transcription factors to regulate gene expression.

The Ets family proteins can be divided into several subfamilies on the basis of their structural compositions and their similarities in the DNA-binding ETS domains (Fig. B-1). Most of them have the ETS domain in the C-terminal regions. However, several
Ets proteins like the ternary complex factor (TCF) subfamily have the ETS domain in their N-terminal regions. In addition, besides the conserved ETS domain, a subset of Ets family proteins has another evolutionarily conserved domain called the Pointed (PNT) domain at their N-terminal region, which forms a helix-loop-helix (HLH) structure for protein-protein interactions [8].

As for the expression pattern, some Ets factors are expressed ubiquitously, some in a tissue-specific manner. Expression patterns of representative Ets factors are shown in Table B-1.

<table>
<thead>
<tr>
<th>Subfamily member</th>
<th>structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETS</td>
<td>ETS1, ETS2</td>
</tr>
<tr>
<td>ERG</td>
<td>Erg, Fli1/ ErgB, FEV</td>
</tr>
<tr>
<td>TEL</td>
<td>TEL/ETV6, TEL</td>
</tr>
<tr>
<td>PFA3</td>
<td>PFA3/F1AF, FR71, FR81/ETV1, FRM</td>
</tr>
<tr>
<td>ELF</td>
<td>ELF1-3/ERT/ESX/ESE1, MEF</td>
</tr>
<tr>
<td>ERF</td>
<td>PE1/METS, PE2/ERF</td>
</tr>
<tr>
<td>TCF</td>
<td>ELK1, Sap1, NETNetb</td>
</tr>
</tbody>
</table>

Fig. B-1. Schematics of the structures of the ETS family factors. ETS: DNA-binding domain; HLH: helix-loop-helix domain (pointed domain); AD: activation domain; ID: auto-inhibitory domain; RD, repression domain.

Table B-1. Tissue distribution of major ETS family factors

<table>
<thead>
<tr>
<th>Factor</th>
<th>Tissue distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETS1</td>
<td>Lymphoid organs, brain, vascular endothelial cell</td>
</tr>
<tr>
<td>ETS2</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>Erg</td>
<td>vascular endothelial cells, hematopoietic cells, kidney, etc</td>
</tr>
<tr>
<td>TEL</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>PFA3</td>
<td>Epidermis, mammary gland, brain, etc</td>
</tr>
<tr>
<td>ELF1</td>
<td>Hematopoietic cells, liver, kidney, intestine, lung, etc</td>
</tr>
<tr>
<td>ESE1/ESX</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>TCFs</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>PU.1</td>
<td>B cells, macrophages, neutrophils</td>
</tr>
<tr>
<td>MEF</td>
<td>Hematopoietic cells, ovary, placenta, colon, lung, etc</td>
</tr>
</tbody>
</table>
The Ets family of transcription factors is known to play important biological roles in a wide range of processes, such as hematopoiesis, differentiation, apoptosis, immunity and oncogenesis [9, 10]. The remarkable roles of Ets factors in diverse biological processes are illustrated by the observations from individual Ets factor-deficient mice (Table B-2). These diverse functions are finally fulfilled by a series of downstream genes that are targeted and controlled by Ets family of transcription factors. So discovering new Ets target genes involved in physiological or pathological processes will facilitate towards understanding the vital functions of Ets family.

Table B-2. Phenotypes in mice deficient for the Ets family genes.

<table>
<thead>
<tr>
<th>ETS1</th>
<th>Increase in T cell apoptosis, defects in B cell function</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETS2</td>
<td>Embryonic lethal, defects in extraembryonic tissue (placenta), deficient expression of MMP-9</td>
</tr>
<tr>
<td>TEL/ETV6</td>
<td>Embryonic lethal, defects in yolk sac angiogenesis, defects in bone marrow hematopoiesis</td>
</tr>
<tr>
<td>PEA3/E1AF</td>
<td>Male infertility</td>
</tr>
<tr>
<td>ER81/ETV1</td>
<td>Defects in synaptic connection to motor neuron</td>
</tr>
<tr>
<td>PU.1</td>
<td>Death soon after birth, defects in B cells and macrophages</td>
</tr>
<tr>
<td>NET</td>
<td>Death soon after birth, respiratory failure due to dilated lymphatic vessels</td>
</tr>
<tr>
<td>MEF</td>
<td>Impairment in NK and NK-T cell development and lack of NK cell function</td>
</tr>
</tbody>
</table>

The transcriptional action is not a simple process that turns on or off depending only on the binding or releasing of transcription factors to their consensus DNA, but is a subtle, cellular and environmental context-dependent and responsive regulatory course. The subtle regulation refers to the mechanisms as diverse as exogenous stimulation, signaling pathway, protein-protein interaction, post-translational modification, subcellular localization, chromatin status, developmental and differentiation background. Considering the versatile and critical functions of Ets family factors, it is necessary to explore the connections between the functions of Ets factors and the context-dependent regulatory mechanisms.

2. Signaling pathways of Ets family factors.
Phosphorylation of the proteins sometimes affects their DNA-binding affinities, transcriptional capacities, subcellular localization and association with other co-factors. Numerous Ets factors are phosphorylated in response to the signaling from growth factors and their receptors or cellular stresses. The signaling pathways involved in mediating the activities of Ets factor include MAP kinase, PI3 kinase, Jak/Stat and others.

One of the most important and indispensable phosphorylation pathways for Ets family factors is the MAP kinase pathway, of which the best known are the ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38 cascades [11-13]. TCF subfamily is implicated in regulating cell proliferation in response to MAP kinase cascades. Among this subfamily, ELK1 is one of the best studied protein, which was first shown to be regulated by ERK[14, 15]. Phosphorylation of ELK1 by ERK both enhances its recruitment to DNA and potentiates its transcriptional activity. For example, following serum or TPA stimulation, phosphorylated ELK1 shows increased association with serum response factor (SRF) and the subsequent allosteric changes in TCFs enhance the binding of the complex to the promoters of many immediate early genes including c-fos, erg-1 and nur77 [16]. Continuous studies initially demonstrated the convergence of ERK, JNK and p38 cascades on the regulation of ELK1 [17-19]. In contrast with the ERK signaling, which is activated by growth factors and mitogens [20], the JNK and p38 cascades respond to cytokines and stress stimuli (Fig. B-2) [21]. MAP kinase signaling also mediates the transcriptional activities of other members of Ets family, such as ETS1 and ETS2 [22, 23], which play important roles in regulating the expression of growth-, tumorigenesis-, apoptosis- and cell cycle-related genes.

![Phosphorylation of ETS transcription factors](image)

Fig. B-2. ETS family of transcription factors are regulated by three major phosphorylation cascades of MAP kinase pathway. Their immediate upstream kinase(s) in these cascades are shown. MEK, MAP kinase/ERK kinase; MKK, MAP kinase kinase.
Meanwhile, the other signaling cascades also contribute to the phosphorylation of Ets family factors. ETS1 can also be phosphorylated by calcium signaling pathway, leading to the reinforcement of autoinhibition in ETS1, which block its DNA-binding activity [24]. Autoinhibition of DNA binding is also observed in ETS2 and PEA3 [25, 26]. Additionally, it has been reported that PI3K (phosphoinoside 3 kinase)-Akt signaling is able to increase the transcriptional activity of PU.1, resulting in an enhancement in the immunoglobulin κ3’ enhancer activity in pre-B cells [27].

On the other hand, Ets family factors are controlling the expression of many cell type-specific growth factors, growth factor receptors and integrin families involved in cellular proliferation. Representative examples of the cytokine or growth factor receptors regulated by Ets family factors include the GM-CSF receptor, M-CSF receptor, G-CSF receptor, Toll-like receptor-4, IL-1β in myeloid cells [28]; the T cell receptors, TCRα/β and IL-2 receptor in T cells [9]. The expression of HGF (hepatocyte growth factor) receptor is up-regulated by ETS1, whose activation is actually induced by HGF via a Ras-MAP kinase pathway [29]. This indicates that Ets factors sometimes act both upstream and downstream of signaling receptors.

3. Protein-protein interactions of Ets family factors.

Ets family proteins regulate gene expression by functional interaction with other transcriptional factors or co-factors on composite DNA-binding sites. First, several lines of evidence have established that protein-protein interactions between Ets family proteins and other cellular partners contribute to activating gene expression. The most well-known example is the interaction of Ets factors with Jun family proteins on the DNA sequences called the Ras-responsive element consisting of an EBS and an AP-1 (Fos/Jun) site, which is located on the sequence in various cellular genes that are responsive to MAP kinase signaling [30]. This element is found in the promoters of metalloproteinase (MMP) genes [31], which are responsible for organ remodeling and tumor invasion. Occasionally ETS1 and AP-1 synergistically activate target promoters with the cooperation by the co-activator CBP/p300. Many other Ets family proteins including ETS2, ER81 have also been reported to interact with CBP/p300 to activate transcription [32]. The interaction with partner transcription factors also augments the transactivity of Ets factors. The autoinhibition of ETS1 can be relieved by interaction with AML/RUNX1, a runt domain transcription factor. This process occurs through the direct interaction of their autoinhibitory domains, resulting in mutual augmentation in their DNA-binding activity [33, 34]. In addition, ETS1 has been reported to interact with
Ubc9, which is later described as a SUMO-conjugating enzyme, resulting a substantial increase in ETS1 transactivity [35]. TEL, a transcriptional repressor, also interacts with Ubc9 and this interaction leads to the restoration in the promoter activity suppressed by TEL [36].

Conversely, there are several cases showing that protein-protein interactions lead to suppression of Ets family factors. The transcriptional activity of ETS2 is inhibited by the interaction with Erg [37]. Histone deacetylase (HDAC) acts as a transcriptional co-repressor utilized not only by histone proteins but also by diverse transcription factors. For instance, NET, a member of TCF subfamily, negatively regulates c-fos and erg-1 promoter activities when it is not phosphorylated by ERK2 MAP kinase. The negative regulation is mediated by recruitment of HDAC via the co-repressor CtBP (E1A C-terminal-binding protein) [38]. A recent report indicated that recruitment of an mSin3A-HDAC complex in an ELK1 complex negatively regulates the c-fos promoter activity [39], in contrast to the transactivating role of ELK1 when phosphorylated. Similarly, PU.1 can also act as an activator or repressor for transcription in association with CBP/p300 [40] or HDAC1 [41], respectively. These evidences suggest that the same Ets family proteins behave as activators or repressors depending on cellular conditions.


After proteins are synthesized, reversible post-translational modifications are widely used to dynamically regulate protein activities. The modifications, like phosphorylation, acetylation, methylation, ubiquitination and sumoylation, have critical roles in many cellular processes owing to their ability to cause rapid changes in the functions of substrate proteins, multiprotein complexes and subcellular structures. The studies about tumor suppressor p53 provide us a comprehensive and delicate model in which various modifications interplay synergistically or antagonistically to display their distinctive and correlated roles. Phosphorylation and acetylation either increase the stability of p53 or directly enhance its DNA-binding affinity [42]. In addition, sumoylation of p53 in C-terminus may consequently affect phosphorylation, PML (promyelocytic leukemia protein) nuclear body localization and transactivity of p53 [43-45].

The diverse modifications on Ets family proteins have been observed in past studies. It is well known that acetylation of histone tail can bring dynamic change of chromatin structure to facilitate transcription [46]. Simultaneously, the transcription factors themselves, such as above mentioned p53, are sometimes acetylated. Among the Ets
family, it has been reported that two lysine residues of ER81 are acetylated by p300 and 
PACA, resulting in not only enhanced transactivity but also increased DNA binding 
affinity and in vivo half-life of ER81 [47]. ETS1 shows rapid and prolonged acetylation 
upon TGF-β stimulation, which may up-regulate the transcription of several MMP genes 
in favor of matrix turnover in arthritis and cancer [48].

In terms of ubiquitination system, by far there have been rare Ets factors reported to be 
ubiquitinated. Some Ets factors, such as ETS1 and TEL, were found in the complexes 
with a member of the family of ubiquitin-conjugating enzymes, Ubc9, but this 
interaction does not lead to the degradation of the proteins [35, 36]. Ubc9 is actually the 
unique identified E2 enzyme for SUMO conjugation pathway. Till now the Ets family 
proteins, TEL and ELK1 have been demonstrated to be modified by SUMO conjugation 
pathway. TEL, a putative tumor suppressor, was revealed to be modified by SUMO 
within the highly conserved PNT domain. Mutation of the SUMO acceptor residue 
lysine of TEL results in an increase in transcriptional repression, presumably because of 
decreased nuclear export [49]. In another example, SUMO modifies a direct target of 
MAP kinase pathways, ELK1, by conjugating to the lysine residue in one of its 
repressive domains. Interestingly, ERK pathway activation leads to both phosphorylation 
of ELK1 and loss of SUMO conjugation and, hence, to the loss of the repressive activity. 
So a dynamic interplay exists between the activating ERK MAP kinase pathway and the 
repressive SUMO pathway [50]. The repressive effect of SUMO pathway is implied to 
be due to the recruitment of HDAC2 [51].

Concerning the conjugating machinery and functional significance of SUMO, more 
details will be described in Chapter 4.

5. Sub-cellular localizations of Ets family.

Ets proteins are nuclear proteins and some of them have nuclear export signals (NES) 
as well as nuclear localization signals (NLS).

Covalent modification can alter the localization of Ets factors. First, phosphorylation of 
Ets proteins changes their subcellular localization in several cases. Stress of UV 
irradiation or heat shock induces active nuclear exclusion of NET, a member of TCF 
subfamily, through phosphorylation of NES by JNK/SAPK [52]. Similar translocation 
pattern has also been observed in ERF, which is believed to be important in the control 
of cell proliferation during G0/G1 phase of the cell phase. Upon mitogenic stimulation, 
ERF is immediately phosphorylated and exported to the cytoplasm, which is modulated 
by JNK pathway [53].
In terms of the effect of SUMO modification, the localizations of the two sumoylated Ets proteins, TEL and ELK1, are influenced upon the modification. TEL is a predominately nuclear protein which represses transcription. In a subset of cell population, sumoylated TEL shows the cell cycle-specific nuclear speckles, named as TEL bodies. The leukemia-associated fusion protein TEL/AML1 is also modified by SUMO1 and found in the TEL bodies [54]. However, when TEL is expressed in fibroblast, it is found both in nucleus and cytoplasm. Experimental evidences indicate that SUMO conjugation is responsible for the nuclear export [49]. In the case of ELK1, sumoylation contributes to the nucleo-cytoplasmic shuttling of ELK1, which is observed in the central nervous system [55]. Some Ets family proteins can change their subcellular localization by physically interacting with other nuclear proteins. MEF, whose functions and modification are going to be described in detail in chapter 2, 3 and 4, moves to PML nuclear bodies under some circumstances, like heat shock stress, by interacting with PML protein.

6. Objectives of this study.

Several Ets proteins are preferentially expressed in specific cell lineages. However, the members of the Ets family are generally found in a wide range of tissues and organs. Concomitantly the members of this family play critical and indispensable regulatory roles in diverse tissues and cells, such as hematopoietic cells, vascular endothelial cells, neural cells, osteogenic cells as well as epithelial cells.

Epithelial cell sheets line all the cavities and free surfaces of the vertebrate body. Regarding the defense system to microbes and harmful stimuli, the epithelial surfaces, like those lining skin, lung and gut, provide a tight physical barrier between the inside of the body and the outside world. Meanwhile, epithelial cells also actively participate in the defense system by producing viscous liquids, cytokines or antimicrobial molecules, such as defensin and lactoferrin. These molecules synthesized by epithelial cells also actively take part in the regulation of tumorigenesis.

The functions of Ets factors in epithelial cells have been a subject of active investigations. Finding new target genes of Ets factors in epithelial cells will provide direct and basic information for understanding the functions of the Ets family in controlling epithelial activities. The first objective of this study is to identify the Ets transcription factors and clarify the related mechanisms that mediate the expression of two important molecules involved in immunity and tumor development using epithelial cell models. The second objective of this study is to investigate the functional
significance of one Ets factor, MEF, resulted from the post-translational protein-protein interaction and modification, which decide the transactivating potential of Ets factors, their mutual interplays and the more subtle and comprehensive mechanisms, for instance, where and when the transcriptional affairs occur.
Research Contents

Part I. The transcriptional regulation of Ets target genes by Ets transcription factors.

Identification of functional target gene promoters that are regulated by specific transcription factors is critical for initiating studies to increase understanding of the molecular mechanisms that control transcription. Furthermore, identification of target gene promoters provides insight into the regulation of the genes that are involved in control of cell growth, differentiation, immune response, as well as information critical to understanding cancer development.

The DNA consensus sequences of Ets factors, EBS have been identified in the promoter or enhancer regions of viral and cellular genes. Over 200 Ets target genes have been reported, and the number of genes shown to be regulated via EBS is constantly increasing [56]. A study demonstrated that EBS is among the eight most important DNA motifs in minimal responsive synthetic promoters generated using random oligonucleotides, establishing the importance of Ets factors [57]. The importance of the Ets family of transcription factors in various biological and pathological processes necessitates the identification of downstream cellular target genes containing EBS and the specific Ets proteins.

In this part, the transcriptional control mechanisms of two Ets target genes will be described respectively. One is about the regulation of granulocyte-macrophage colony-stimulating factor (GM-SCF) by Ets factor, ETS2 [58]; the other is about human beta-defensin 2 (HBD2) by MEF [59].
Chapter 1. Involvement of ETS2 in transcriptional regulation of granulocyte-macrophage colony-stimulating factor in human airway epithelial cells.

1.1 Introduction

Airway epithelial cells produce a significant amount of GM-CSF (granulocyte-macrophage colony-stimulating factor) [60-62], which is originally identified as a cytokine to stimulate the proliferation, differentiation, and function of myeloid progenitor cells [63]. Further, GM-CSF is believed to mediate many important physiological processes [64, 65]. For instance, it is known to improve the proceeding of infections under pathological conditions, such as asthma [66].

Recently, the production of GM-CSF is reported to be involved in both the in vitro invasiveness and the local progression of squamous cell carcinoma in the lung [67]. Recombinant GM-CSF stimulates the MMP activity and invasiveness of less invasive LK-2 and LC-1 cells, and this stimulation is abrogated by the neutralizing anti-GM-CSF antibody, which is also able to decrease the invasiveness of highly invasive EBC-1 and NCI-H157 cells [67, 68]. In addition, clinical evidences showed that patients with squamous cell carcinoma co-expressing GM-CSF and GM-CSF receptor displayed significantly poorer prognosis than those expressing neither GM-CSF nor GM-CSF receptor, suggesting that GM-CSF can have a stimulatory effect on some human non-small cell lung cancer (NSCLC) [69]. Several clinical trials of anticancer chemotherapy combined with recombinant human GM-CSF have been performed. GM-CSF can lead to rapid neutrophil recovery, which may promote tumor progression [70]. Moreover, GM-CSF is involved in mobilization of endothelial progenitor cells, so it is likely a target for inhibition of tumor angiogenesis [71]. Considering the significant functions GM-CSF plays in tumor, it is necessary to elucidate the transcriptional regulatory mechanism of GM-CSF in NSCLC.

Regarding the transcriptional control of GM-CSF, previous studies in T-lymphocytes have identified a number of regulatory elements in its promoter. A conserved lymphokine element 0 (CLE0) (-54 to -31), and an upstream NF-κB site (-85 to -76) have been identified on the proximal functional promoter region (-620 to +34) [72]. Mutation in either of these sites resulted in a decrease in phorbol 12-myristate 13-acetate (PMA)/Ca^{2+}-induced GM-CSF-luciferase reporter activation. It has also been confirmed that AP-1, NF-ATp, and a higher order NF-ATp/AP-1 complex all bind to the human
CLE0 element. In addition, an enhancer region located 3.3 kb upstream containing functional NF-AT/AP-1 binding sites appears to act in conjunction with the proximal promoter in response to T-cell receptor activation [73, 74]. As to the involvement of Ets family factors, ETS1 has been shown to transactivate human GM-CSF promoter stimulated with PMA and ionomycin in Jurkat T cells [75, 76].

The human ETS2 gene codes for a 56-kD nuclear protein that can be phosphorylated, is turned over rapidly, and responds to protein kinase C (PKC) [77, 78]. ETS2 having a high similarity with ETS1 is expressed in the extra-embryonic trophectoderm during embryonic development [79] and is expressed ubiquitously in adult tissues. ETS1 and ETS2 are activated by Ras-MAP kinase signaling. Mutation of a threonine residue to alanine located within the N-terminal PNT domains of ETS1 and ETS2 (Thr38 and Thr72, respectively) has been reported to abolish Ras-responsive enhancement of their transcriptional activities [23]. The PNT domains of ETS1 and ETS2 contain ERK2 MAP kinase docking sites, and mutation of these sites prevents Ras-mediated transactivation function of these proteins [80]. Mitogenic signaling by colony-stimulating factor-1 (CSF-1) and Ras, which activates c-myc expression, is suppressed by a dominant negative mutant of ETS2 and is restored by c-myc over-expression [81], suggesting that ETS2 also acts as an effector of a signal transduction pathway.

Previous studies have demonstrated that PKC activation of MAPK-ERK1/2 in epithelial cells is both associated with and required for PMA- and TNFα-induced GM-CSF production [82], but the responsible transcription factors involved in this pathway have not yet been reported. The goal of this study was to identify the transcription factor that regulates PMA-induced GM-CSF expression in NSCLC, and as a result the mechanism connecting PKC, ETS2 and transcriptional control of GM-CSF was established.

1.2 Materials and methods

1.2.1 Cell culture

A549 cells (human pulmonary adenocarcinoma cells) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and at 37 °C in a humidified 5% CO₂ and 95% air atmosphere.

1.2.2 Plasmid constructs

GM-CSF promoter construct GMP(-629) was prepared by PCR using a Genome Walker Kit (Clontech). 5’-primer, GM(-629-601) and 3’-primer, GM(+58+29) were used
for PCR. The PCR product was cloned into pCR2.1 vector using the Original TA Cloning Kit (Invitrogen). After confirming the sequence, it was cloned into the Xhol and HindIII sites of pGL2-basic vector (Promega), a promoter-less luciferase expression plasmid.

The GM-CSF promoter constructs, GMP(-120)WT and the GMP(-69)WT, were prepared by PCR using GMP(-629) in pCR2.1 vector as a template and the following primers: 5’-primer, GMP(-120) or GMP(-69), and 3’-primer, M13(reverse). The PCR product was cloned into the Xhol and HindIII sites of pGL2-basic vector.

GMP(-120)MUT and GMP(-69)MUT are GMP(-120)WT constructs with mutant EBS. They were prepared using a Transformer Site-directed Mutagenesis Kit (Clontech) according to the manufacturer’s instructions. Respective mutation primers, GMP100(5’)MZ and GMP40(5’)MZ which contain mutant EBS, and selection primer, Trans Oligo ScaI/StuI, were used.

The MMP-9 and IL-8 promoters were prepared as described previously [83]. The promoter fragments were respectively inserted into KpnI-XhoI and XhoI-HindIII sites of pGL2 basic vector.

MEF in pCB6: MEF cDNA (+1+1992) in pCR2.1 vector was treated by XbaI and HindIII. The fragment obtained from the digestion enzyme treatment was inserted into the XbaI and HindIII sites of pCB6 vector.

ETS2 in pCB6: ETS2 in pBluescript vector (a gift from Dr. D.K. Waston) was treated by BglII and HindIII. Then the 1.5 kb fragment obtained from the digestion enzyme treatment was inserted into the BglII and HindIII sites of pCB6 vector.

Antisense MEF construct is defined as opposite-direction inserted cDNA of MEF in pCB6 vector. The opposite-direction fragment was inserted into the EcoRI and BamHI sites of pCB6 vector.

Antisense ETS2 construct is defined as opposite-direction inserted cDNA of ETS2 in pCB6 vector. The opposite-direction fragment was inserted into the BglII and BamHI sites of pCB6 vector.

Full-length human ETS1 (1325 bp), ELF1 (1870 bp, cloned by us), PEA3 (a gift from Dr. J.A. Hassel), and ESE1 (a gift from Dr. T. A. Libermann) were cloned into the KpnI-XbaI sites of pCB6 downstream of the cytomegalovirus promoter.

Double-stranded WT and MUT ETS decoy were prepared by incubating the mixed sense and antisense oligonucleotides at 94 °C for 5 min, then cooling down gradually to 40 °C.

The sequences of primers used in this study are shown in Table 1-1.

All constructs were verified by DNA sequence using ABI Prism 377 DNA Sequencer
and manufacturer’s software (Applied biosystems).

1.2.3 Transfection

Transient transfections were performed with transfectam (Promega) according to the manufacturer’s recommendations. Specifically, cells were plated on 24-well plates. When the confluency of the cells reached about 70%, 1 µl transfectam reagent and 0.5 µg GM-CSF promoter in pGL2-basic plasmid were incubated for 10 min before application to one-well cells. Co-transfection of various plasmids was performed with a mixture of 3 µl transfectam and 0.5 µg of GM-CSF promoter in pGL2-basic plasmid, and 1 µg of Ets factor in pCB6 plasmid. Empty vector (pCB6) was added to ensure constant DNA input. Co-transfection with 10ng per sample of the pRL-CMV vector, which expresses Renilla luciferase (Promega), ensured that differences in firefly luciferase reporter gene expression were not due to differences in transfection efficiency. Cells were incubated for 2 h with the DNA mixture, after which additional medium was added. Forty-eight hours after transfection, the medium was removed and cells were harvested for luciferase assay.

1.2.4 Luciferase assay

Forty eight hours after transfecting cells with DNAs, medium was removed and the cells were washed with PBS(-) (Ca²⁺, Mg²⁺ free phosphate buffered saline). Cell lysates were recovered by adding 100ml 1 PLB (passive Lysis Buffer, Promega) and mounting on a mini-shaker platform for 15 min. Twenty ml of the lysate was used for measuring luciferase activity, which was measured using a dual-luciferase reporter assay system (Promega) and a luminometer (Lumat LB9507, EG&G Berthold). Absolute light emission generated from the luciferase activity was plotted and represents the fold induction of activity generated by experimental treatments with respect to the activity associated with basic vector alone. Values are shown as means ± S.E. (n=4).

1.2.5 RNA preparation and reverse transcription-PCR (RT-PCR)

Isogene (NIPPON GENE) was used for extracting total RNA from cells according to manufacturer’s instructions. The concentration of total RNA obtained above was determined by measuring the UV absorbance (260 nm). The RNA with high purity (OD_{260}/OD_{280} ≥ 1.80) was used for experiments.

Cells were grown to about 70% confluency and were transfected with ETS2 in pCB6 using Transfectam. After incubation for 24 h, cells were stimulated with PMA (50 nM, Sigma). Following another 24 h incubation, cells were harvested and total RNA was
extracted using Isogen. RT-PCR experiments were performed using a RNA PCR Kit (Takara) according to the manufacturer's instructions: 30 °C for 10 min, 42 °C for 60 min, 99 °C for 5 min, and 5 °C for 5 min for reverse transcription; 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 60 sec for 35 cycle. The following primers were used: for GM-CSF, 5'-primer, GM-CSF-up and 3'-primer, GM-CSF-down; for internal expression control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-primer, GAPDH-up and 3'-primer, GAPDH-down.

The sequences of primers used in this study are shown in Table 1-1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM(-629-601)</td>
<td>TTCTCAGAGCTGGCTGACATCTCGCTGCTG</td>
</tr>
<tr>
<td>GMP(-120)</td>
<td>CCGCTCGACGTGATAAGGGCCAGAGGATTC</td>
</tr>
<tr>
<td>GMP(-69)</td>
<td>CCGCTCGAGGCATTTTGTGGTCACCATTAA</td>
</tr>
<tr>
<td>GM(+58+29)</td>
<td>AAGAGCACGGGCTCTGCAGCCACATCTCT</td>
</tr>
<tr>
<td>GMP100(5'MZ)</td>
<td>G GCCAAGAGTATCAGACAGRRRAG</td>
</tr>
<tr>
<td>GMP40(5'MZ)</td>
<td>GGCACATTAAATCAATTATCTCTGTGTTATTAA</td>
</tr>
<tr>
<td>GM-CSF-up</td>
<td>GAGACATGTGAATGCCATC</td>
</tr>
<tr>
<td>GM-CSF-down</td>
<td>GATGACAAGCAGAAAAGTC</td>
</tr>
<tr>
<td>GAPDH-up</td>
<td>CGGGAGGCCTGTGTGATCAATGG</td>
</tr>
<tr>
<td>GAPDH-down</td>
<td>GCCAGTGATGGCATGGACTG</td>
</tr>
<tr>
<td>M13 (reverse)</td>
<td>CAGGAAACAGCTAGAC</td>
</tr>
<tr>
<td>Trans Oligo Scal/Stul</td>
<td>GTGACGTGAGGCGCCTCAACCAAGTC</td>
</tr>
</tbody>
</table>

1.2.6 Western blotting analysis

Whole cell lysates from A549 cells transfected with plasmid DNA or treated by PMA (50 nM) were subjected to SDS-PAGE using 10% gel, then the proteins were transferred to PVDF membrane. After blocking with 10% skim milk at 4°C overnight, membranes were incubated with affinity-purified rabbit antisera to ETS2 (1/1000; Santa Cruz Biotechnology) at 4°C for 2 h. The membranes were subsequently incubated with HRP-conjugated goat anti-rabbit IgG (1/10000; Seikagaku Corporation.) at room temperature for 1 h. Finally chemiluminescence was detected using the ECL Kit (Amersham Life Science).

1.2.7 Cell growth assay

A549 cells were plated onto 24-well plates at 1 × 10^4 cells/ well and cultured with DMEM containing 10% FBS for 12 h to allow adherence. After transfection of the
double-stranded ETS decoy ODN, the culture medium was replaced with DMEM containing 10% FBS and renewed every 2 days. Cell Counting Kit (Dojindo) was used to determine the proliferating rates of the untransfected and transfected cells by measuring the absorbance of the culture medium at 450 nm, at 0, 3, 6 and 9 days, respectively.

1.3 Results

1.3.1 PMA induces GM-CSF promoter activity.

In our preliminary study, it was revealed that GMP(-120)WT, containing a proximal fragment of GM-CSF promoter which spans from nucleotide -120 to +58 relative to the supposed transcription start site, possessed the strongest transactivity, compared to the GMP(-629) and the other truncated mutant promoter constructs with different lengths and Ets consensus sites (data not shown). Two Ets consensus sites, EBS, were found in this fragment, one at -40 bp and the other at -100 bp. Fig. 1-1 (left side) shows the constructs of GM-CSF promoter used in this study. In GMP(-69)MUT and GMP(-120)MUT, the EBS were mutated from 5’-TTCC-3’ to 5’-TATC-3’ for further experiments. As shown in Fig. 1-1, promoter activity of GMP(-120)WT measured by luciferase assay is about two times higher than that of GMP(-69)WT. These activities were reduced by the mutation of EBS. Especially, GMP(-69)MUT, which has no EBS, showed very low transactivity. PMA, a PKC activator, prominently enhanced promoter activities of GMP(-69)WT and GMP(-120)WT. These activities were also reduced by the mutation of EBS. Taken together, it is suggested that PMA induces GM-CSF promoter activity in an EBS-dependent manner.

To further confirm the involvement of PKC in the GM-CSF promoter activity control and investigate which subtype of PKC enzyme family is responsible for the activation, GMP(-120)WT was used for the following activator and inhibitor experiments, because it contains two EBS and showed the strongest transactivity (Fig. 1-1).

As shown in Fig. 1-2A, PDBu (50 nM), another protein PKC activator, also increased GMP(-120)WT activity to about same extent as PMA, but 4α-PDD (50 nM), an inactive PMA analogue, did not. These data further indicated that PKC is involved in the transactivation of GMP(-120)WT. Then to identify the subtype of PKC, the effects of several inhibitors were checked: 1) PKC specific inhibitor, Sphingosine; 2) different PKC isozyme inhibitors, Staurosporine (inhibitor to PKC-α, βI, βII, γ, δ, ε), Go6983 (inhibitor to PKC-α, β, γ, δ, ζ), Go6976 (inhibitor to PKC-α, βI, μ) and Ro-31-8220 (inhibitor to PKC-α, βI, βII, γ, ε). Except for Go6976, all the other inhibitors strongly
blocked PMA-induced GMP(-120)WT transactivity (Fig. 1-2B). Go6976 showed a weak inhibitory effect.

The above data strongly indicate that PKC contributes to the transactivation of GM-CSF promoter, although based on the current data about inhibitors of PKC, it is still difficult to exactly confirm which PKC isozyme(s) is responsible in this process.

Fig. 1-1. Transcriptional activity of human GM-CSF promoter in A549 cells. Structures of promoters with different EBS are indicated in the left side. Twenty-four hours after transient transfection, A549 cells were cultured with PMA (50nM) or NS (no stimulation) as indicated for about 20 h and then cells were harvested for luciferase assay.
1.3.2 ETS2 is involved in GM-CSF promoter transactivation.

Considering the EBS-dependent effect of PKC shown in the above experimental results, it is indicated that some members of the Ets family factors should be responsible for the GM-CSF promoter regulation. To determine the particular Ets factor(s) involved in this regulation, A549 cells were transfected with the following Ets family factors, ETS1, ETS2, PEA3, ELF1, ESE1 and MEF, to screen their individual transactivity on GM-CSF promoter. Interestingly, only ETS2 evidently transactivated GMP(-120)WT, whereas the other members checked here did not (Fig. 1-3). This result was also reproduced in HeLa cells (data not shown).
1.3.3 Cooperative effect of PMA and ETS2 on GM-CSF transactivity.

The current data suggest the necessity to investigate if ETS2 and PKC have synergistic activating effort on GM-CSF promoter. To do this, the ETS2 was transfected into cells, followed by PMA treatment. The luciferase activity induced by ETS2 was obviously enhanced by PMA treatment (Fig. 1-4). Meanwhile the construct expressing antisense ETS2 RNA was introduced into cells to block the endogenous expression of ETS2, followed by PMA treatment. It was observed that GMP(-120)WT activity was decreased by antisense ETS2 RNA and this suppressive effect was observed even in the presence of PMA stimulation.

These data imply that ETS2 may play a critical role in transferring the activating signal from upstream PKC to the downstream GM-CSF promoter. To further check the reasonableness of this hypothesis, the transactivity of ETS2 on various GM-CSF promoter constructs was examined in the absence and presence of PMA stimulation. The data showed that both the individual and combined effects of ETS2 and PMA were EBS-dependent. In detail, GMP(-120)WT containing two EBS was most strongly activated. The activity of the other two constructs containing only one EBS, GMP(-120)MUT and GMP(-69)WT, was also slightly enhanced. But ETS2 and PMA were unable to enhance the activity of GMP(-69)MUT lacking EBS (Fig. 1-5). These results suggest that the cooperative effects of PMA, a PKC activator, and ETS2 on
GM-CSF promoter are EBS-dependent.

To confirm the consequent change in ETS2 expression after transfecting related DNA constructs and directly investigate the effect of PKC on ETS2 in this study, the protein level of ETS2 was investigated by western blotting analysis. The results proved that transfection of ETS2 and antisense ETS2 plasmids into A549 cells indeed induced the protein expression change (Fig. 1-6). The upper protein (54 kD) is supposed to be the full-length isoform of ETS2 and the lower one (52 kD) may be a phosphorylated form of ETS2 [84]. PMA treatment enhanced the expression levels of both, which is consistent with previous report that PKC is able to stabilize ETS2 protein [85].

To further confirm the essential roles played by ETS2 and PKC on GM-CSF regulation, the endogenous transcription level of GM-CSF was measured by RT-PCR. As shown in Fig. 1-7, in response to the introduction of ETS2 into cells, GM-CSF showed increased mRNA level, which was further promoted by PMA treatment. The expression of the housekeeping gene, GAPDH, used as a control was not altered by any treatment.

Fig. 1-4. PMA and ETS2 coactivate GM-CSF promoter activity. A549 cells were transiently transfected with sense and antisense ETS2 as indicated in the figure. Twenty-four hours after transfection, cells were stimuluted with PMA (50nM) for about 20 h and then harvested for luciferase assay.
Fig. 1-5. Cooperative effects of PMA and ETS2 on GM-CSF promoter are EBS-dependent. A549 cells were transiently transfected with ETS2 and diverse GM-CSF promoters. Twenty-four hours after transfection, cells were stimulated with PMA (50nM) for about 20 h and then harvested for luciferase assay.

Fig. 1-6. Examination of ETS2 expression. Forty-eight hours after DNA transfection or 20 h after stimulation with PMA (50nM), whole cell lysates were harvested for western blotting.

Fig. 1-7. PMA and ETS2 increase endogenous mRNA expression of GM-CSF. RT-PCR for GM-CSF and GAPDH was performed as described in 1.2.5.
1.3.4 MEF may down-regulate GM-CSF through competing with ETS2.

As shown in Fig. 1-3, some Ets factors showed inhibitory effects on the transactivity of GMP(-120)WT, suggesting that some member of this family, like MEF, ESE1 or PEA3, may block the transcriptional activity of ETS2. Recently it was revealed that MEF plays a role as a tumor suppressor gene, through acting as a competitive factor of ETS2 for binding the EBS in target gene promoters. To check the inhibitory effect of MEF on GM-CSF promoter activity, the effect of antisense MEF RNA was examined in MEF-stably transfected A549 cells, which have high background of MEF expression. As shown in Fig. 1-8, the introduction of antisense MEF RNA enhanced GMP(-120)WT transactivity, but antisense ETS2 RNA inhibited it. These data are consistent with the demonstrated tumor suppressor role of MEF as a competitor of ETS2 in epithelial cells.

Fig. 1-8. Effect of antisense MEF RNA on GM-CSF promoter. MEF-stably transfected A549 cells were transiently transfected with GMP(-120)WT and pCB6 vectors encoding antisense RNA using Transfectam. Forty-eight hours after transfection, cells were harvested for luciferase assay.

Considering MEF is supposed to suppress the promoter transactivity of tumor-related genes, such as MMP-9, IL-8 and GM-CSF through competing for the EBS with ETS2, the feasibility of a new approach, named as ETS decoy, is implied, which can block the transactivating effects of the Ets family oncogenes on target promoters instead of MEF, whose endogenous expression is very low in epithelial tumor cells due to the methylation of MEF gene [83]. The double-stranded oligodeoxynucleotides of ETS decoy developed here contains three Ets cis-elements, whose sequences were designed based on the
sequences of EBS in MMP-9, IL-8 and GM-CSF promoters (Fig. 1-9A). The mutant decoy with mutated EBS was also synthesized to work as control. The ETS decoy was transfected into epithelial cells with the aim of catching the Ets family oncogenes, like ETS2, away from binding to their target promoters (Fig. 1-9B). As shown in Fig. 1-10, transfection of WT ETS decoy decreased MMP-9, IL-8 and GM-CSF promoter transactivity, but the mutant ETS decoy did not. Next, the proliferating capacity of tumor cells was examined and the result indicated that the ETS decoy-transfected cells have weaker proliferating potency compared to the control (Fig. 1-11). These results indicate the possibility to utilize ETS decoy as a new approach to suppress the vitality of tumor cells.

![Diagram](image)

Fig. 1-9. ETS decoy may suppress the vitality of tumor cells. A. The WT ETS decoy contains three individual EBS, which are mutated in MUT ETS decoy used as control. B. The mechanism for ETS decoy to affect the tumor cell proliferation and invasion.
Fig. 1-10. ETS decoy suppresses the transactivity of MMP-9, IL-8 and GM-CSF promoters. Respective promoters (0.2μg) with MUT or WT ETS decoy (final conc.: 200 nM) were transfected into A549 cells. Forty-eight hours after transfection cells were harvested for luciferase assay.

Fig. 1-11. ETS decoy suppresses the proliferation of A549 cells. Cells were transfected with WT or MUT ETS decoy (final conc.: 200 nM). The untransfected cells are indicated as control. The proliferating rates were measured using Cell Counting Kit (as described in 1.2.7) at 0, 3, 6 and 9 days after transfection.
1.4 Discussion

1.4.1 Data summary and analysis

Considering that GM-CSF is actively involved in cancer progression as described in introduction, it is important to know how the GM-CSF expression is regulated at transcription level. In this study, the transcriptional control of GM-CSF was investigated using A549 cells as a cancer cell model.

This study demonstrates that ETS2 and a PKC activator, PMA up-regulated not only the activity of transfected GM-CSF promoter, but also the transcription of the endogenous GM-CSF gene in A549 cells. It seems that ETS2 and PMA activated GM-CSF promoter through the two EBS, because not only the individual but also the combined effects of these two factors were intact EBS-dependent. Moreover, antisense experiment showed that inhibiting endogenous ETS2 in A549 cells attenuated not only the basal level activity of GMP(-120)WT but also the activity induced by PMA. These results suggest that ETS2 is a crucial transcription activator, which regulates GM-CSF expression in A549 cells through transferring the activating signals from upstream PKC to GM-CSF promoter. Additionally, activation of MAPK-ERK1/2 via PKC was revealed to be associated with and required for GM-CSF production induced by PMA and TNFα [82]. Taken together, the cell-specific regulation of GM-CSF is demonstrated, which is critical for local airway responses (Fig. 1-12).

![Fig. 1-12. ETS2 and PKC synergistically upregulate the transactivity of GM-CSF promoter in airway epithelial cells. PMA or TNFα activates ETS2 through PKC, which may subsequently stimulate ERK according to previous report, resulting in the phosphorylation and stabilization of ETS2. Finally the activated ETS2 transactivates GM-CSF by binding to the EBS in its promoter.](image)

It has also been reported that neither the upstream enhancer region nor the other regions outside the proximal promoter are involved in the activation of GM-CSF transcription in epithelial cells [86]. Compared to the proximal GM-CSF promoter construct GMP(-629), and to the other truncated promoter constructs with different lengths and Ets consensus
sites, GMP(-120)WT produced the strongest transcriptional activity. Therefore, in this study we focused on the two proximal EBS in this promoter. However, the possibility still exists that some other elements in this promoter, such as the NF-κB consensus site, can respond to PMA stimulation and account for the total observed activity of GMP(-120)WT induced by PMA in A549 cells. In T cells, both the proximal (-85 to -76) and the distal (-2002 to -1984) NF-κB binding sites have been reported to be involved in PMA induction of GM-CSF promoter [87].

The evidence was shown in this study that MEF may down-regulate GM-CSF transcription activity. This is supposed to occur through competing with ETS2, which was proved to have the potency to enhance the expression of GM-CSF. The similar tumor-related gene control pattern by ETS2 and MEF has been reported in another study about their roles in epithelial cell tumors [83]. Ets family factors, like ETS1, ETS2 and MEF, may interfere with each other because of their similar DNA-recognizing domains and may thereby function antagonistically, either activating or repressing the transcription of same target genes. The gene-specific regulatory pattern is supposed to be dependent on promoter context, cell type or stage of differentiation [88, 89]. Context-specific regulation of GM-CSF as well as other essential genes by different transcriptional factors may be an interesting subject for future studies.

1.4.2 Involvement of ETS subfamily in tumorigenesis

ETS subfamily is not only involved in the malignant transformation of cells but also in the promotion and progression of tumors by activating invasion and metastasis-related genes [31]. ETS1 and ETS2 are components of ETS subfamily with ETS domain located in their respective C-terminus (Fig. B-1). The in vivo research showed that the oncogene PyMT-positive mice having heterozygous ETS2 allele developed smaller size tumor than those having two wild type ETS2 alleles, which is correlated with the differentiated state of early hyperplastic growth [90]. ETS1 and ETS2 have mitogenic and transforming activities when over-expressed in NIH3T3 cells [91]. As to the involvement in tumor invasion, ETS1 is co-expressed with MMP-1 and uPA (urokinase-type plasminogen activator) in various types of tumors. Co-expression of ETS1, MMP-2 and MMP-9 has been reported in pancreatic cancer and high expression levels of these genes are a poor prognostic marker in these tumors [92]. Some Ets factors are also expressed in vascular endothelial cells and are thought to play central roles in angiogenesis. When vascular endothelial cells are stimulated with angiogenesis factors such as VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor) and TNFα (tumor necrosis factor α), expression of ETS1 is temporarily induced, followed by the
expression of cell cycle-related genes such as c-myc and the genes involved in the degradation of the extracellular matrix to promote the migration and invasion of endothelial cells. However, despite the importance of ETS1 in tumor induction, knockout of ETS1 causes no abnormality in blood vessels and lymphatic vessels in mice, although the mice shows a reduced number of T cells with B cell dysfunction (Table B-2). It is speculated that other Ets transcription factors compensate for the function of ETS1, since several members including ETS1, ETS2, Fli1, Erg, ELF1 have similar expression pattern.

As for the function of ETS subfamily in cell cycle control, ETS1 and ETS2 appear to contribute to activate c-myc expression by binding the Ets consensus site overlapping the E2F site of the promoter [93]. ETS2 also transactivates the cdc2 [94] and cyclin D1 promoters [95]. Cdk10, a Cdc2-related kinase regulating G2/M phase of the cell cycle, interacts with the PNT domain of ETS2 to inhibit ETS2-mediated transactivation [96], suggesting the involvement of ETS2 in cell cycle regulation.

In terms of apoptosis, ETS1 and ETS2 have been reported to be pro-apoptotic as well as anti-apoptotic in some cases. The role of ETS2 in blocking apoptosis is supported by the finding that expression of the Bcl-XL gene is regulated by ETS2 in mouse myeloid leukemia cells [97]. Oppositely, over-expression of ETS2 in prostate tumor cells increases apoptosis accompanied by increased level of p21WAF1/Cip1 [98], whereas over-expression of ETS1 in human umbilical vein endothelial cells induces apoptosis under serum-deprived conditions [99].

1.4.3 Survey about ETS decoy

Ets factors could be the candidates of molecular targets for selective cancer therapy, since they play important roles in maintenance of the transformed phenotypes of tumor cells. Recent progress in molecular biology has provided new techniques for inhibiting target gene expression. Accordingly, the following methods have been developed experimentally: 1) use of dominant negative mutants of Ets, 2) use of repressive Ets family factors, 3) use of antisense oligonucleotides, 4) use of RNA interference [31]. In addition, transfection of cis-element double-stranded oligodeoxynucleotides (ODNs), referred to as “decoy” ODNs, has been reported to be a powerful tool in a new class of anti-gene strategies for gene therapy and in the study of transcription regulation. Transfection of double-stranded ODNs corresponding to the cis sequence will result in the attenuation of authentic cis-trans interaction, leading to the removal of trans factors from the endogenous cis elements with subsequent modulation of gene expression (Fig. 1-13).
The decoy strategy may be useful for treating a broad range of human diseases because of its capacity in blocking constitutively expressed factors as well as multiple transcription factors that bind to the same *cis* element. The application of transcription factor decoy ODNs as means of gene therapy and study of gene expression has been established in treating cardiovascular diseases. For examples, NF-κB and E2F decoys are demonstrated to be useful in the treatments of atherosclerosis and neointimal hyperplasia in vein bypass grafts, respectively [100]. In concerns of tumor treatment, Sp1 decoy transfected to carcinoma cells suppresses the expression of VEGF, TGFβ1 (transforming growth factor β1) and also cell growth and invasion activities [101]. The research utilizing ETS decoy strategy for tumor treatment has not been widely carried out yet. The possible utility of the ETS decoy in tumor therapy was implied in the present research, although further studies, such as *in vivo* functional evaluation, need to be done about this decoy.

![Decoy ODNs: Cis-element double-stranded oligodeoxynucleotides (ODNs)](image)

Fig. 1-13. The strategy of decoy ODNs. Decoy ODNs target and block the binding of the specific transcription factors to their cis-elements in the gene promoters, and thereby alter the gene expression. TF, transcription factor.
Chapter 2. Involvement of MEF in transcriptional regulation of human β-defensin 2 in epithelial cells.

2.1 Introduction

Innate immunity provides us with a dynamic first-line host defense against invading microbes. Antimicrobial molecules secreted by various tissues constitute an essential component of innate immunity [102,103]. Defensins are extensively characterized antimicrobial peptides and have a broad spectrum of antimicrobial activity against bacteria, yeast and fungi. Like most antimicrobial peptides, defensins are cationic molecules with spatially separated hydrophobic and charged regions. This arrangement allows them to insert themselves into and consequently disrupt the microbial membranes [102].

In addition to the direct antimicrobial activity, it is now revealed that defensins also contribute to cross-talk between the innate and acquired immune response (Fig. 2-1). Defensins interact with CCR6 to recruit dendritic and T cells to the site of microbial invasion and act as potent adjuvant factors in enhancing antigen-specific Ig production [104, 105]. Neutrophil defensins were revealed to enhance lung epithelial wound repair and mucin gene expression [106]. Decreased defensin activity has been linked to lung pathogenesis of cystic fibrosis (CF) [107, 108], but promoted defensin expression is observed in diseased skin from patients with psoriasis and atopic dermatitis [109].

Fig. 2-1. A model of defensin activity in an infected epithelium. Epithelial cells synthesize antimicrobial defensins both constitutively and in response to infectious and inflammatory stimuli. Defensins are able to kill ingested microbes as well as attract dendritic cells and memory T cells, setting the stage for the adaptive phase of the immune response.
Defensins are divided into α- and β-defensin subfamilies according to the positions of six highly conserved cysteine residues that participate in disulphide linkages [110]. Among the four human β-defensins (HBD1-4) characterized in various epithelial tissues, HBD2 is detected in epithelial cells of the skin, lung, gingiva, trachea and reproductive tract. It usually shows very low expression level under normal physiological conditions, but is induced to high level in response to the invasiveness of yeast and both Gram-negative and Gram-positive bacteria [111, 112], and the stimulations of lipopolysaccharide [113-115] and a number of pro-inflammatory cytokines [116-119]. Multiple pathways, including NF-κB pathway [111-113, 120] and MAPK pathway [121, 122], were reported to be involved in HBD2 regulation.

Genomic analysis of HBD2 revealed a promoter region containing several putative transcription factor binding sites, including NF-κB, activator protein (AP)-1, AP-2, and NF-IL-6, which are known to be involved in the regulation of inflammatory responses [116]. Additionally, the putative binding site (core sequence GGAA) of Ets transcription factors was also identified in this promoter, but it has not been investigated whether the Ets family of transcriptional factors is involved in HBD2 regulation.

MEF (myeloid ELF1 like factor) [123], an Ets transcriptional factor, located on chromosome Xq26.1 with an observed protein size about 98 kD. It belongs to the ELF subfamily, which also contains ELF1, NERF, ESX/ESE1. These members have a number of shared functions in addition to their unique ones. ELF1 has been shown to regulate the expression of several T-cell specific (CD4, TCRζ, TCRα) and B-cell specific (tyrosine kinases blk, lck, lyn) genes, indicating that ELF1 may have certain roles in adaptive immune response [124]. In contrast, MEF seems to play a role in innate immune system. It is required in epithelial cells to regulate the expression of lysozyme, which is an important component of innate immunity against common pathogens [125]. Furthermore, MEF also directly transactivates perforin in NK cells. MEF/- mice have a profound reduction in the number of NK-T and NK cells. Purified MEF/- NK cells secrete only minimal amounts of IFNγ and cannot be targeted to tumor [126]. These indicate a specific role of MEF in the development and function of NK cells and in innate immunity.

Besides lysozyme and perforin, by far the genes that are identified to be transcriptionally controlled by MEF include GM-CSF [127] and IL-3 [128] in hematopoietic cells, as well as IL-8 and MMP-9 in epithelial cells. The role of MEF as a tumor suppressor candidate in epithelial cells was established by the findings that it can repress the transcription of IL-8 and MMP-9, which are up-regulated by ETS2 [83].

The basic fragments needed for MEF transcriptional function include the nuclear
translocation domain (namely NLS), ETS domain for DNA-binding and transactivation domains [129]. The potent transactivation domain is located in the N-terminus of MEF sequence (Fig. 2-2).

![Diagram of MEF domains](image)

Fig. 2-2. Schematic diagram indicating the basic fragments needed for the transcriptional function of MEF.

In this study, the effects of MEF on the transcriptional activity and expression of HBD2 are investigated in human epithelial cells. The results indicate that MEF is a potential activating factor for transcriptional regulation of HBD2.

2.2 Materials and methods

2.2.1 Cell culture

A549 cells (human pulmonary adenocarcinoma cells), MEF-stably transfected A549 cells, NIH3T3 cells (mouse fibroblastoma cells) and HEK293 cells (human kidney cells) were grown in DMEM supplemented with 10% FBS at 37 °C in a humidified 5% CO₂ and 95% air atmosphere.

HeLa cells (human cervical adenocarcinoma cells) were grown in Minimum Essential Medium supplemented with 10% FBS at 37 °C in a humidified 5% CO₂ and 95% air atmosphere.

2.2.2 Plasmid constructs

HBD2(-247) was cloned by PCR using a Genome Walker Kit (Clontech). 5’-primer, HBD2(-247) and 3’-primer, HBD2(+22+44) were used for PCR. The PCR product was cloned into PCR2.1 vector using the Original TA Cloning Kit (Invitrogen). After confirming the sequence, it was cloned into the SacI and XhoI sites of pGL2-basic vector. HBD2(-247)MUT is a WT HBD2(-247) construct with a mutant EBS. It was created from HBD2(-247) using a Transformer Site-directed Mutagenesis Kit (Clontech). Mutation primer, HBD2(-247)mut, which contains a mutant EBS, and a selection primer,
Trans Oligo *ScaI/StuI* were used.

HBD2(-89) was prepared by PCR using HBD2(-247) as a template. 5’-primer, HBD2(-89)*XhoI* and 3’-primer, HBD2(+22+44) were used. The PCR product was cloned into the *XhoI* sites of pGL2-basic vector.

The expressing vectors of Ets family transcription factors and antisense MEF were prepared as described in 1.2.2.

The sequences of primers used in this study are shown in Table 2-1.

All constructs were verified by DNA sequence.

2.2.3 Transfection and Luciferase Assay

Transient transfections of plasmid DNA were performed with TransIT-LT1 (Panvera) according to the manufacturer’s recommendations. Briefly, TransIT-LT1 and Opti-MEM were mixed thoroughly and incubated at room temperature for 15 min. Then DNA was added to the diluted TransIT-LT1 reagent (ratio: 1μg DNA/3 μl LT1). After being mixed gently, the mixture was incubated at room temperature for 15 min. Then the mixture was directly added to the cells cultured on 24-well plates, without changing the medium. Forty-eight hours after transfection, the medium was removed and cells were harvested for luciferase assay. Co-transfection of the pRL-CMV (Promega), which expresses Renilla luciferase, verified that differences in firefly luciferase reporter gene expression were not due to differences in transfection efficiency. Luciferase activity was measured as described in 1.2.4. Values are shown as means±S.E. (n=4).

2.2.4 RT-PCR

A549 cells and MEF-stably or transiently transfected A549 cells were grown to about full confluence after 48 h incubation. Cells were harvested and total RNA was extracted using Isogen (NIPPON GENE). RT-PCR was performed with 0.5 μg RNA using a RNA PCR Kit (Takara) according to the manufacturer’s instructions: 30 ℃ for 10 min, 42 ℃ for 60 min, 99 ℃ for 5 min, and 5 ℃ for 5 min for reverse transcription; 95 ℃ for 1 min, 60 ℃ for 1 min, 72 ℃ for 1.5 min, 40 cycles. Primers, HBD2-1A and HBD2-1S were used. Expression of GAPDH was checked as an internal control. 5’-primer, GAPDH-up and 3’-primer, GAPDH-down were used.

The sequences of primers used in this study are shown in Table 2-1.
2.2.5 Western blotting analysis

Whole cell lysates were subjected to SDS-PAGE using 7.5% gel, then the proteins were transferred to PVDF membrane. After blocking with 5% skim milk at 4 °C overnight, membranes were incubated with affinity-purified rabbit antisera to MEF (1/200; TransGenic Inc.) at room temperature for 2 h. The membranes were subsequently incubated with HRP-conjugated goat anti-rabbit IgG (1/10000; Seikagaku Corporation) at room temperature for 1 h. Finally chemiluminescence was detected using the ECL Kit (Amersham Life Science).

2.2.6 Preparation of nuclear extract

A549 cells (1 × 10^6) were washed, collected and pelleted with PBS(-) by centrifugation at 1500 g for 5 min, at 4 °C. The pellet was resuspended in 400 µl of cold buffer containing 10 mM Hepes-KOH (pH=7.9), 10 mM KCl, 0.1 mM EDTA (pH=8.0), 0.1 mM EGTA (pH=8.0), 1 mM dithiothreitol and 0.5 mM phenylmethyl-sulfonyl fluoride by gentle pipetting. The cells were then allowed to swell on ice for 15 min, after which 25 µl of 10% Nonidet P-40 solution was added, and the tube was vigorously vortexed for 10 sec. The homogenate was centrifuged at 15,000 rpm for 1 min, at 4 °C. The nuclear pellet was resuspended in 50 µl of ice cold buffer containing 20 mM Hepes-KOH (pH=7.9), 0.4 M NaCl, 1 mM EDTA (pH=8.0), 1 mM EGTA (pH=8.0), 1 mM dithiothreitol and 1 mM phenylmethyl-sulfonyl, the tube was vigorously vortexed for 15 min at 4 °C. Then the nuclear extract was centrifuged at 15,000 rpm for 5 min, at 4 °C and the clear supernatant was collected, quickly frozen in liquid nitrogen, and stored at -80 °C until further use. Protein concentration was determined using the Bradford assay (Bio-Rad) and bovine serum albumin standards (Sigma).

| Table 2-1. Sequences of oligonucleotides used for PCR, RT-PCR and mutagenesis. |
|---------------------------------|---------------------------------|
| Name   | Sequence (5’-3’)               |
| HBD2(-247) | GAGGAATTCTCTGGTCCCAAG        |
| HBD2(-247)mut | AAGAGCAGGAGGTAGGATTTTCT   |
| HBD2(-89)Xhol | CCGCTCGAGAATACCAGTCTGAACTCTA |
| HBD2(+22+44) | CCATGAGGCTTGTATCTCTTC       |
| HBD2-1A  | GGAGCCCTTTCTGAACTCCGCA      |
| HBD2-1S  | CCAGCCATCAGCCATGAGGGT       |
| GAPDH-up | CGGGAAGCTTGTATGCAATGG        |
| GAPDH-down | GGCAGTGATGCGCATGGACTG       |
| Trans Oligo Scal/Stul | GTGACTGGTGAGGCCTCAAACAGTC    |
2.2.7 Electrophoretic Mobility Shift Assay (EMSA)

The double-strand oligonucleotides, EBS(wt), EBS(mut), EBS(mut*), were synthesized for use in the EMSA experiments. The sense sequences of each pair are listed in Fig. 2-9A. The probe, EBS(wt), was labeled with $\gamma^{32}$pATP (22 TBq/mmol) using T4 polynucleotide kinase (TAKARA). Pre-incubation of 5-10 µg nuclear extract with 2 µg poly(dI-dC) (Amersham Pharmacia Biotech), excessive unlabeled EBS(wt), EBS(mut) or EBS(mut*), antibody to MEF or ETS2 (Santa Cruz Biotechnology) or ESE2 (Santa Cruz Biotechnology) was carried out on ice for 30 min in buffer containing 10 mM Tris-HCl (pH=7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.05% Nonidet P-40, 5% Glycerol and 1 mg/ml bovine serum albumin. Radiolabeled oligonucleotide probes (0.04 pmol, $5 \times 10^4$ cpm) were then added to the reaction mixtures and incubated at room temperature for 30 min. The reaction products were then analyzed by electrophoresis on a 5.5% polyacrylamide gel, followed by BAS2000.

2.3 Results

2.3.1 MEF up-regulates HBD2 promoter activity.

HBD2(-247), the promoter construct used in this study, contains the main elements responsive to inflammatory factors and the EBS (Fig. 2-3). To determine which ETS factor is involved in HBD2 regulation, first diverse Ets factors were transfected into A549 cells together with HBD2(-247) promoter. The following luciferase assay showed that MEF activated HBD2(-247) promoter more strongly than the other Ets factors in A549 cells. Similar result was also observed in HeLa cells (Fig. 2-4A). In addition, in mouse fibroblast cell line (NIH3T3), which does not endogenously express MEF, MEF transfection induced much higher HBD2(-247) activity (Fig. 2-4B). The positive involvement of MEF was further confirmed by transfecting the antisense MEF RNA construct, which blocked the endogenous MEF. As shown in Fig. 2-4C, HBD2(-247) transactivity was inhibited by transfecting antisense MEF RNA into MEF-stably transfected A549 cells, which have high background of MEF expression. Antisense MEF RNA also blocked the endogenous MEF, resulting in a down-regulation in HBD2 promoter activity (Fig. 2-5). The efficiency of DNA transfection and MEF expression levels in each cell line were examined by western blotting (Fig. 2-6).
Fig. 2-3. Schematic representation of HBD2(-247) containing the indicated putative cis-elements.
Fig. 2-4. MEF up-regulates HBD2 promoter activity. A549 cells, HeLa cells, NIH-3T3 cells and MEF-stably transfected A549 cells were respectively transiently transfected with the HBD2 promoter construct HBD2(-247) (0.2 µg/well) and the indicated ETS transcription factors (0.4 µg/well) or antisense MEF (0.4 µg/well), using TransIT-LT1. Forty-eight hours after transfection, cells were harvested for luciferase assay.

Fig. 2-5. Antisense MEF down-regulates HBD2 promoter activity. HEK293 cells were transiently transfected with the HBD2 promoter construct HBD2(-247) (0.2 µg/well) and MEF (0.4 µg/well) or antisense MEF, using TransIT-LT1. Forty-eight hours after transfection, cells were harvested for luciferase assay.
2.3.2 MEF up-regulates endogenous HBD2 expression.
To test the effect of MEF on endogenous transcription level of HBD2, the HBD2 mRNA level was examined in MEF-transiently transfected and MEF-stably transfected A549 cells (Fig. 2-7). Both of them showed higher HBD2 mRNA level than untransfected A549 cells, suggesting that MEF also promotes the transactivation of endogenous HBD2 gene.

2.3.3 The transactivity of MEF on HBD2 promoter is EBS-dependent.
The experimental results shown above indicate that MEF up-regulates HBD2 proximal promoter activity. To investigate whether the EBS in HBD2(-247) is involved in MEF regulation, a shorter promoter construct, HBD2(-89), was first developed, in which EBS was deleted. Interestingly, MEF no longer potently transactivated the promoter activity (Fig. 2-8A). Next, the nucleotide mutations were introduced into EBS in HBD2(-247) and then the transactivity of MEF on this mutant promoter and WT promoter was
compared. As shown in Fig. 2-8B, MEF increased WT HBD2(-247) activity in a dose-dependent manner, and this increase was greatly attenuated by the loss of EBS, indicating that MEF may exert its transactivity by binding to the EBS in HBD2(247). It should be noted that MEF still retains some transactivating ability on HBD2(-247)MUT, although to a lesser extent in comparison with WT construct. Thus, it is likely that MEF may simultaneously regulate this promoter through other unidentified mechanism(s).

2.3.4 MEF binds to EBS in HBD2 promoter.

Having demonstrated the ability of MEF in transactivating HBD2 promoter activity, it was next examined whether MEF protein directly interacts with the proximal EBS by using [$\gamma^{32}$P]-labeled fragment of HBD2 promoter sequence, EBS(wt), as probe, which contains intact EBS (Fig. 2-9A). EMSA indicated that the upper band resulted from the
binding between MEF protein and probe, because the antibody against MEF attenuated this band (Fig. 2-9B). The interaction of the polyclonal antibody with MEF protein may interfere with the binding of MEF with probe DNA, so that the super-shifted band could not be observed. The lower bands are supposed to correspond to other Ets family factors competing for the EBS or some proteins having affinity to the probe sequence, because these bands became stronger after the MEF binding was blocked by its antibody. The antibody to ETS2 and ESE2, used as controls, had no effect on all of these bands. In addition, competitor assay was performed using unlabeled probe sequence HBD(wt) and sequences with EBS mutations, HBD2(mut) and HBD2(mut*) (Fig. 2-9A). In HBD2(mut*), besides the identified EBS, the other two sites similar to EBS sequence, GGAG and GGAT, were also mutated. Compared to HBD2(mut) and HBD2(mut*), unlabeled HBD2(wt) attenuated the upper band more strongly, thereby confirming the binding between the probe with WT EBS and MEF protein (Fig. 2-9C).

![Fig. 2-9. Binding of MEF to EBS in HBD2 promoter. EBS(wt) probe (0.04 pmol) was used for each lane. A: Sequences of oligonucleotide probe and competitors. EBS(mut) and EBS(mut*) have mutated nucleotides indicated in boldface letters. B: Anti-MEF (0.12 µg), anti-ETS2 (0.2 µg) and anti-ESE2 (0.2 µg) antibodies were individually used for antibody reaction. C: As indicated, compared to the probe, an excess of unlabeled competitor oligonucleotides was added to the reaction mixture for the competitor experiment.](image-url)
2.4 Discussion

2.4.1 Data summary and analysis

Defensins compose a group of biological effectors, which can protect various tissues from microbial invasion and infection through direct killing as well as recruiting immune cells (T cells and dendritic cells). Thus defensins play a critical role in innate immunity system and the link between innate and acquired immunities. Among the defensin family, the induction of HBD2 has been extensively studied recently. A variety of exogenous stimuli and physiological factors have been shown to induce HBD2. NF-κB pathway appears to be the most well-known pathway involved in transducing these signals to the transcriptional regulation of HBD2 [113, 130]. In addition, it is reported that AP-1 and intracellular calcium are also involved in HBD2 expression in airway epithelial cells [131].

The data presented here indicate that MEF enhanced not only HBD2 promoter activity but also its endogenous transcription. In support of this notion, expressing antisense MEF RNA attenuated HBD2 promoter activity in both stably MEF-transfected cells and those without exogenous input of MEF. Furthermore, the EBS in HBD2 promoter appears to be essential for its binding with MEF protein and the consequent promoter transactivation. Together, these results indicate that MEF acts as a novel transactivator of HBD2 in epithelial cells, which functions through a mechanism independent of NF-κB pathway. Thus MEF may have the potential to enhance basic HBD2 expression under non-irritated physiological conditions.

Although MEF constitutively localizes in nucleus [129], its activity may vary under different circumstances or in different cell contexts. A recent study suggested that, in hematopoietic cells, its transcriptional activity was largely restricted to the G1 phase of the cell cycle [132]. This phenomenon is due to its change in phosphorylation status, which may also occur in epithelial cells. Our study also indicates that MEF may respond to some exogenous stresses through a change of its subnuclear localization, which, in turn, results in the change of its transcriptional activity [133]. So it is interesting to check if this regulatory mechanism is also applicable to HDB2 expression.

2.4.2 MEF in innate immunity

Human immune system is primarily composed of two components: innate and adaptive immune systems. The innate immune system serves as the first line of defense because it is immediately ready to combat a wide range of pathogens without requiring prior exposure to these infectious agents. One of the important effectors of the innate immune
system is the natural killer (NK) cells. NK cells execute their defensive role by killing infected cells using cytolytic or cytotoxic granules which are released onto the surface of the target cells, and the effector proteins that can penetrate the cell membrane. One of these effector proteins, the perforin (PFP, pore-forming protein) polymerizes into pore-forming aggregates in the plasma membrane of target cells leading to osmotic lysis, granzyme entry and subsequent apoptosis.

The role of MEF in immune system is illustrated by the defects observed from the MEF-deficient mice. Two major defects in lymphoid development in MEF-/- mice are impairment in NK and NK-T cell development and lack of NK cell function. Deficiency of MEF expression in mice resulted in about 70% reduction of the number of NK-T cells in thymus, spleen and liver tissues, and simultaneously 60% reduction in the number of NK cells in the spleen. MEF-/- NK cells are unable to lyse cell targets, which is largely due to the lack of perforin expression in these cells. This defect is cell type-specific as MEF-/- cytotoxic T lymphocytes (CTLs) can be stimulated to express perforin and can lyse antigen-bearing cells. Therefore, constitutive perforin expression, which is crucial for the readiness of NK cells, requires MEF expression whereas the inducible expression of perforin in CTLs does not [124, 126]. So MEF is a key transcriptional regulator for the constitutive perforin expression as well as for the normal function of NK cells.

Antimicrobial molecules secreted by various tissues constitute another essential component of innate immunity [102, 103]. In epithelial cells, MEF is a major transcriptional activator of antimicrobial molecule, lysozyme [125], which protects us from ever-present danger of bacterial infection by hydrolyzing the β(1-4) glycosidic bonds between N-acetylMuramic acid and N-acetylglucosamine present in the cell wall of Gram-positive bacteria [134]. Our present study provides a new evidence of the linkage between MEF and the antimicrobial peptide, HBD2. Taken together, it is implied that MEF also plays important roles in innate immunity of epithelial cells (Fig. 2-10).

![Diagram](image)

**Fig. 2-10.** MEF functions as a transcription factor, whose downstream target genes involved in innate immunity include antimicrobial peptides: lysozyme and defensin, and cytolytic molecule: perforin.

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Part II. The post-translational protein-protein interaction and modifications are involved in functional regulation of MEF.

Besides previously shown fragments needed for transactivation, MEF protein simultaneously contains a number of discrete functional domains: an acid domain, an AML1 interaction domain, the ETS domain located in the center, a serine/threonine rich region and a proline rich domain near its C-terminus (Fig. P2-1). The ETS domain of MEF has high homology with other proteins in the ELF subfamily. MEF does not contain HLH or PNT domain for protein-protein interaction.

The direct interaction between MEF and AML1 proteins, including the AML1/ETO proteins, was first found in t(8;21)-positive acute myeloid leukemia (AML) cells and the N-terminus of MEF was determined as the region responsible for its interaction with AML1. The synergistic transactivation of MEF and AML1B is abolished by the interaction between MEF and AML1/ETO. So it is implied that interference with MEF function by AML1/ETO may lead to dysregulation of genes important for myeloid differentiation, thereby contributing to the pathogenesis of t(8;21) AML [128].

The next protein that was found to interact with MEF is cyclin A. It was demonstrated that the transcriptional activity of MEF is most active during G1 phase of cell cycle. Cyclin A/Cdk2 associated kinase activity significantly impairs MEF-dependent transactivation and that cyclin A physically interacts with MEF protein. This interaction results in the phosphorylation of MEF within three Thr/Ser residues (Ser641, Thr643, Ser648) located near its C-terminus and elimination of these three phosphorylation sites decreases the phosphorylation of MEF to only 20% of that seen within the native protein [132].

Recent studies indicate that in epithelial cells MEF physically interacts with another nuclear protein, PML, which induces the accumulation of MEF in PML nuclear bodies.
The interaction promotes MEF transactivity, resulting in the up-regulation of endogenous lysozyme expression. Amino acids 348-517 of MEF are required for accumulation of MEF in PML nuclear bodies and the transactivation of lysozyme enhanced by PML. Moreover, the C-terminal proline rich region spanning 477-517 of MEF (Fig. P2-1) is shown to be the putative region for interaction between MEF and PML by the use of mammalian two-hybrid system. In addition, heat shock can stimulate the accumulation of MEF in PML nuclear bodies and the transactivation of lysozyme promoter [133].

The main concerns of the research in this part are to evaluate the post-translational protein-protein interaction and modification of MEF, and the consequent impacts on its functions in epithelial cells.
Chapter 3. The regulation of Human-β defensin 2 by MEF is enhanced by promyelocytic leukemia protein.

3.1 Introduction

The mammalian nucleus is compartmentalized into highly organized structural and functional domains that have been reported to be related with various physiological processes such as transcription and DNA replication [135]. The PML nuclear bodies (PML NBs), also known as nuclear domain 10 (ND 10) or PML oncogenic domains (PODs), represent one of the best-studied examples of a subnuclear structure. PML NBs consist of an aggregation of several proteins, most notably PML and Sp100. The PML protein is essential for the nucleation and formation of the PML NBs as well as for the recruitment of other PML-NB associated proteins such as Sp100, Daxx and p53.

PML was first discovered as part of the PML-RAR-α oncogenic fusion protein with the retinoic acid receptor-α (RAR-α) in cells from patients with acute promyelocytic leukemia (APL), in which expression of PML-RAR-α provoked the striking disaggregation of the PML NBs [136]. This effect is reversible by retinoic acid (RA, the physiological ligand of RAR-α), such that NB restoration correlates with the therapeutic effect of retinoid in causing disease remission. The PML NBs also represent preferential targets for early events during DNA viral infections (for example, herpes simplex virus, Epstein-Barr virus or adenovirus), which, like APL, lead to structural alteration of NBs [137]. These indicate that the integrity of PML NBs is altered in human diseases. A variety of functions have been suggested for PML NBs, including tumor suppression, innate immunity and transcription regulation through titration, modification and compartmentalization of functional proteins [138].

It has been previously reported that MEF is constitutively localized in the nucleus under normal or stress conditions [129]. In addition, it was revealed that MEF physically interacts with PML and is recruited to the PML NBs, resulting in an increase in MEF transactivation of the lysozyme promoter in epithelial cells [133]. The aim of the present study was to investigate the collaborating effect of PML on MEF transactivation of the HBD2 promoter, which was revealed to be regulated by MEF in Chapter 2.

3.2 Materials and methods

3.2.1 Cell culture
HeLa cells and HEK293 cells were cultured as described in 2.2.1.

### 3.2.2 Plasmid constructs

Human β-defensin 2 promoter construct, HBD2(-247) was prepared as described in 2.2.2.

MEF in pCB6 was prepared as described in 1.2.2.

pcDNA3-Flag-PML was kindly provided by Dr. Mitsuyoshi Nakao.

pSG5-Sp100 was kindly provided by Drs. J.-S. Seeler and A. Dejean.

MEF in pEGFP-N1 (GFP-fused MEF) was prepared as described previously [129].

### 3.2.3 Transfection and luciferase assay

Transient transfections of plasmid DNAs were performed with TransIT-LT1 (Panvera) according to the manufacturer's recommendations. Co-transfection of various plasmids was performed with 0.2 µg of reporter plasmid and the indicated combinations of 0.1 µg of MEF, 0.5 µg of PML and 1 µg of Sp100 plasmids. Empty vector was added where necessary to ensure a constant amount of input DNA. Each sample was co-transfected with 10 ng of phRG-TK vector (Promega Corp.), which expresses Renilla reniformis luciferase to verify that differences in firefly luciferase reporter gene expression were not caused by differences in transfection efficiency. First, TransIT-LT1 and Opti-MEM were mixed thoroughly and incubated at room temperature for 15 min. Then DNA was added to the diluted TransIT-LT1 reagent (ratio: 1µg DNA/3 µl LT1). After being mixed gently, the mixture was incubated at room temperature for 15 min. Then the mixture was directly added to the HeLa cells cultured on 12-well plates, without changing the medium. Forty-eight hours after transfection, the medium was removed and cells were harvested for luciferase assay. Luciferase assay was performed in the same way as described in 1.2.2. Values are shown as means±S.E. (n=3).

### 3.2.4 Immunofluorescence

HeLa cells were grown on poly-L-lysine-coated 35-mm glass-bottomed dishes and transfected with the pEGFP-MEF, pcDNA3-Flag-PML, or a combination of these, fixed in 3.7% paraformaldehyde, and permeabilized with 0.5% Triton X-100 in PBS(-) for 15 min at room temperature. Fixed cells were subsequently blocked for 60 min at room temperature with PBS(-) containing 1 mg/ml bovine serum albumin and incubated with a 1:100 dilution of primary antibody, mouse anti-PML (Santa Cruz Biotechnology), for 1 h at room temperature. Cells were washed three times with PBS(-) and then stained with 1:100 dilution of TRITC-conjugated anti-mouse for 1 h at room temperature. Cells were
washed three times with PBS(-) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Immunofluorescence analyses were carried out using Fluoview FV-500 confocal laser scanning microscope (Olympus, Tokyo, Japan).

3.2.5 RT-PCR
HeLa cells were seeded in 6-well plates and transfected with the indicated combinations of 0.25 µg of MEF and 1.25 µg of PML expressing plasmids. Empty vector was added where necessary to ensure a constant amount of input DNA. Total RNA was extracted from 1 x 10^6 cells using Isogen (Nippongene). RT-PCR experiments were performed with an RNA PCR Kit (ReverTra Dash; TOYOBO) according to the manufacturer's instructions. The reverse transcription reaction was carried out at 42 °C for 30 min, 99 °C for 5 min, and 4 °C for 5 min. PCR was carried out at 98 °C for 1 min for 1 cycle, 98 °C for 15 s, 60 °C for 20 s, and 74 °C for 90 s for 15-40 cycles, and 74 °C for 20 min for 1 cycle. The following primers were used: for HBD2, 5'-primer, HBD2-1A and 3'-primer, HBD2-1S; for internal expression control, GAPDH, 5'-primer, GAPDH-up and 3'-primer, GAPDH-down.
The sequences of primers used in this study are shown in Table 2-1.

3.3 Results

3.3.1 MEF accumulates in the PML NBs and its transcriptional activity on the HBD2 promoter is enhanced upon PML over-expression.
To verify the localization of MEF, HeLa cells were transfected with pEGFP-MEF alone or together with pCDNA-PML. Fluorescence images showed that MEF was distributed throughout the nucleoplasm and only slightly co-localized with endogenous PML. Interestingly, MEF translocated into the NBs when PML was over-expressed. The staining of cells with PML antibody showed that PML and GFP-MEF were co-localized within these PML NBs (Fig. 3-1A).
The functional significance of this translocation was examined by luciferase assay using HBD2 promoter construct, HBD2(-247). As shown in Fig. 3-1B, MEF could activate the HBD2 promoter, thus confirming the finding demonstrated in Chapter 2. The co-transfection of PML and MEF appeared to synergistically up-regulate the HBD2 promoter activity in comparison with either PML or MEF alone.
Although the physiological functions of PML NBs remain largely unknown, many studies have suggested that they may play a role in the regulation of transcription. The data presented here indicate that PML enhances the activation potential of MEF in the context of HBD2 promoter.
3.3.2 Sp100 negatively affects the PML-enhanced MEF transactivation of the HBD2 promoter.

Sp100, another major constituent of the PML NBs, has been shown to interact with the Ets family member, ETS1, to stimulate its transcriptional activity [139]. So the possibility was checked in this study if Sp100 could affect the activation potential of MEF and increase its transactivation of the HBD2 promoter. As a result, Sp100 did not have any enhancing effects on the transcriptional activity of MEF as shown in Fig. 3-2A. Therefore, the enhancement of MEF activity on HBD2 promoter appears to be specifically mediated by PML. Interestingly, the PML-enhanced MEF transactivation of the HBD2 promoter was decreased by Sp100 over-expression (Fig. 3-2B).

3.3.3 Heat shock stress enhances the MEF transactivation of HBD2.

Cellular stresses affect PML NBs and it has been revealed that heat shock stress induced the translocation of MEF at both endogenous and exogenous levels, into endogenous PML NBs. Here it was investigated whether this heat shock-induced translocation of endogenous MEF correlates to an increase in MEF transcriptional activity. HEK293 cells were used for the heat shock experiment because this cell line has a relatively abundant expression of MEF. Heat shock treatment (42 °C for 90 min) of HEK293 cells resulted in an up-regulated HBD2 promoter activity (Fig. 3-3A).

Next, the transcriptional expression of endogenous HBD2 was investigated under the same heat shock condition by performing semi-quantitative RT-PCR. As shown in Fig. 3-3B, the HBD2 endogenous expression level in HEK293 cells increased after heat shock treatment. The increase in HBD2 transcription upon heat shock stress is not related to the change in the protein level of MEF, because heat shock can not alter MEF expression as shown in previous study [133].

Moreover, endogenous MEF translocalized into the PML NBs after heat shock treatment as determined by immunostaining of HEK293 cells with anti-MEF and anti-PML antibodies (Fig. 3-3C). Heat shock disperses the PML NB components, Daxx, Sp100, but not PML [140]. Thus it is likely that MEF and PML can still functionally interact with each other in the nuclear body. This explains, at least in part, the up-regulation of the HBD2 report gene.
Fig. 3-2. Sp100 negatively affects the PML-enhanced MEF transactivation of the HBD2 promoter. A. HBD2(-247), MEF and Sp100 were cotransfected into HeLa cells. Sp100 did not affect the activity of MEF on the HBD2 promoter. *P<0.03, as compared to pGL2-basic empty vector. B. HeLa cells were cotransfected with HBD2(-247) and MEF, PML, Sp100 or the indicated combinations of these. Forty-eight hours after transfection, cells were harvested for luciferase assay. Values are means±S.E. from triplicate platings. *P<0.03 and **P<0.01, as compared to the control; ***P<0.01, as compared to MEF; and $$$ P<0.01, as compared to MEF+PML.
Fig. 3-3. Heat shock stress enhanced the MEF transactivation of HBD2 promoter and increased the endogenous HBD2 transcription. A. HEK293 cells were transfected with HBD2(-247) or pGL2-basic empty vector. Twenty-four hours after transfection, cells were subjected to heat shock at 42 °C for 90min and then harvested for luciferase assay. ** denotes a significant difference from pGL2 (heat shock), with p<0.01 and ## denotes a significant difference from HBD2 (non-heat shock), with p<0.01. B. HEK293 cells were plated on 6-cm plates. At 80-90% confluent, cells were subjected to heat shock using the same conditions as above, after which total RNA was recovered and RT-PCR was performed as described in 3.2.5. C. Non-heat shock and heat shock-treated HEK293 cells were stained with MEF and PML antibodies.
3.4 Discussion

3.4.1 Data summary and analysis

Since MEF plays essential roles in innate immunity system, it may be one of the gene targets to enhance innate immune responses. Therefore, there is a need to fully elucidate the mechanisms underlying MEF regulation. The data presented in this study showed that the transactivity of MEF on HBD2 was enhanced by PML, which alone did not show activating effect. Furthermore, exogenous heat shock stress promoted the co-localization between endogenous MEF and PML NBs and consequently up-regulated both HBD2 promoter activity and its endogenous expression. Taken together, the results above suggest that the transcriptional activity of MEF on HBD2 may be regulated by PML especially under stress conditions, which differs from the mechanism by which HBD2 is induced upon the stimulations from various microbes or pro-inflammatory cytokines (Fig. 3-4).

![Diagram](image.png)

Fig. 3-4. MEF is involved in the transcriptional control of HBD2. Heat shock stress promotes the translocation of MEF into PML NB, and enhances the transactivity of MEF and the subsequent HBD2 expression. The regulatory mechanism is different from that related to the stimulations from microbes or inflammatory cytokines and mediated mostly by NF-κB pathway.

Concerning the relation of Sp100 and PML, previous studies have shown that cells lacking PML exhibited dispersion of PML NB-related proteins and the introduction of PML into PML-/- cells recruited all of these proteins including Sp100 into the NBs. Sp100 actually does not directly interact with PML. These findings suggest the presence of mediator proteins for connecting PML and Sp100 [141]. Considering the data of this study that PML-enhanced MEF transactivation of the HBD2 promoter was decreased by
Sp100 over-expression, it is reasonable to hypothesize that the binding affinity between PML and PML NB protein, Sp100, may be stronger than that between PML and MEF, and hence the over-expression of Sp100 disrupted the ability of PML to recruit MEF into PML NBs and enhance its activity. In contrast, the transactivity of another Ets family factor, ETS1, is enhanced through physically interacting with Sp100, although this interaction does not occur inside PML NBs, but throughout the nucleus. This suggests that ETS1 has the ability to alter NBs by recruiting the core component Sp100 away from the NBs [139].

3.4.2 PML and PML NB in transcriptional control

Although the biochemical function of PML remains largely unknown, consistent evidences are accumulating for a role of PML in transcriptional regulation. PML is a member of the RING finger family proteins (Fig. 3-5). Although PML does not directly bind to DNA and unlike transcriptional co-activators such as CBP and p300, it does not possess intrinsic histone acetylase activity, when tethered to DNA its RBCC domain displays a cryptic transactivating activity which is dependent on the presence of the RING finger [142]. Conversely, full-length PML inhibits transcription in some cases [143]. This suggests that the conformation of PML or its interactions with other proteins determine its transcriptional role: a transcriptional co-activator or co-repressor. For instance, PML co-activates Fos and the progesterone receptor through its RBCC domain [144, 145]. It also acts as a co-activator in the RARα-RXRα transcriptional complex, possibly through its interaction with transcriptional co-activator CBP or TIF1α, or through its participation in the transcriptionally active DRIP complex [146]. On the other hand, as a co-repressor of transcription, for example, it can suppress the transcriptional activity of Sp1 on the promoter of EGF receptor[147], and inhibit the ability of the retinoblastoma protein (pRB) to induce the target gene of glucocorticoid receptor [148].

Regarding the role of PML NB in transcriptional control, it might serve as a compartmentalized area, into which co-factors are recruited or co-factor complexes are
assembled to be utilized by transcription factors. In agreement with this model, RARα/RXAα actively repress transcription through interaction with co-repressor and HDAC, whereas when bound to retinoic acid, they can activate transcription through interaction with co-activators such as CBP, which directly interacts with PML and is found in PML NB. Transcriptional repressors such as Daxx and Tax are inactivated when recruited into the PML NB [149, 150].
Chapter 4. Post-translational modification of MEF by SUMO.

4.1 Introduction

The small ubiquitin-related modifier (SUMO) post-translationally modifies many proteins with roles in diverse processes including regulation of transcription, protein localization, chromatin structure and DNA repair [151, 152]. Four SUMO homologs have been described in mammals. SUMO1 [153-155] shows about 50% sequence identity to SUMO2/3, two of which are almost identical with about 95% sequence homology [156-158]. SUMO4 has a restricted expression pattern with high level reported only in kidney [159]. The modification by SUMO is a reversible, dynamic process, in which SUMO protein can be covalently attached to and released from substrate protein through an isopeptide bond between a C-terminal glycine in SUMO and a lysine in substrate. Like ubiquitination, a number of enzymes participate in the sumoylation machinery. At the beginning, SUMO is synthesized as a precursor protein that is C-terminally processed by a class of cysteine proteases, termed SUMO-proteases or sentrin-specific (SENPs/SUSPs) [160]. Subsequently, the conjugation to proteins involves the ATP-dependent dimeric SUMO activating E1 enzyme (AOS1/UBA2) and the E2 conjugating enzyme Ubc9. In the ubiquitin pathway, one additional factor, called E3 or ubiquitin protein ligase, is required in most cases for conjugation of ubiquitin to the target protein. Recently E3-like factors that stimulate SUMO conjugation have also been identified as pivotal players to promote the transfer of SUMO from the E2 to specific substrates in the SUMO pathway. To date, the three unrelated proteins having SUMO E3 ligase activity are RanBP2 [161], the PIAS proteins [162], and the polycomb group protein Pc2 [163]. Although SUMO E3 ligases are not required for sumoylation in vitro, they may be important in regulating substrate selection in vivo. Currently known targets for RanBP2-stimulated sumoylation are RanGAP1, the nuclear body component Sp100, HDAC4 and the ubiquitin ligase Mdm2 [164, 165]; for PIAS proteins, STATs, p53, Jun, LEF1 and nuclear hormone receptors [166-168]; for Pc2, the only identified targets so far are the transcription repressors CtBP1 and CtBP2 [163].

The various and important functions of SUMO are underlined by its diverse modification substrates. Since the identification of the first SUMO-modified protein, RanGAP [153], in 1996, more than 60 SUMO targets proteins have been reported so far and the number is still increasing. Most of these target proteins represent nuclear proteins, of which a significant number are either transcription factors or other proteins...
involved in DNA transactions (Fig. 4-1). Although much remains to be learned about the mechanisms by which SUMO modification alters substrate protein activity, the significance of SUMO modification has been established by several lines of evidences.

First, SUMO regulates subcellular and subnuclear localizations. It is indicated that SUMO influences the nuclear import or export trafficking of substrate proteins. As described in the background, the subcellular localizations of the two Ets factors, TEL and ELK1, are affected by SUMO modification [49, 55]. In addition, the SUMO-modified RanGAP1 [153, 169], Dictyostelium MEK [170], and SUMO-fused IKB kinase regulator NEMO [171] also show changed subcellular localization. On the other hand, SUMO can recruit proteins, like Sp3 and TEL, to specific subnuclear speckles [54, 172]. Another well-characterized example of SUMO-dependent changes in
subnuclear localization comes from the studies of the tumor suppressor PML. WT PML, which is post-translationally modified by SUMO [173], is present in a subnuclear structure, PML NB. Many other SUMO-modified proteins including transcription factors, chromatin modifiers, and proteins involved in genomic maintenance have also been found in PML NBs [138]. Mutation of the SUMO acceptor lysines in PML or over-expressing SUMO protease causes nuclear body components such as CBP, Sp100 and Daxx to re-localize in the nucleus although their own modification by SUMO is not required for them to localize in PML NBs [174, 175]. These findings suggest that SUMO-modified PML supports assembly and integrity of PML NBs.

Next, SUMO regulates gene expression. Many of the SUMO-modified proteins identified to date are promoter-specific transcription factors, co-activator, or co-repressors. Although in a few cases, like heat shock factor (HSF) [176], co-activators GRIF and SRC1, SUMO modification enhances transcription, in a majority of cases attachment of SUMO appears to repress the activity of transcriptional activators. The transcription factor Sp3, for example, has been shown to be SUMO modified in vivo and removal of SUMO by mutation of Sp3 acceptor lysine or introduction of a SUMO protease dramatically increases transcriptional activity of Sp3 [172, 177]. The similar regulation pattern by SUMO is also observed in other transcription factors, such as ELK1 [50], c-Myb [178], C/EBP proteins [179] and steroid hormone receptors [180, 181]. The common feature of these factors is a region previously defined to function as inhibitory domain, to which currently the sumoylation site has been mapped [182]. SUMO could negatively regulate transcription factor activity through altered interactions with DNA, chromatin or other proteins. As described in background, the transcriptional co-repressor HDAC2 is recruited to SUMO-modified Ets factor, ELK1 [51]. The acetyltransferase p300, which acts as a general transcriptional co-activator for a host of transcription factors, can exert context-specific repressive functions, which is shown to be dependent on sumoylation of a distinct lysine residue within a repressor domain of p300 and the consequent interaction with HDAC6 [183]. Understanding the protein-protein interaction change between the naïve and SUMO-modified protein will provide a clue on how SUMO mediates gene expression.

A consensus SUMO acceptor sequence, namely SUMO motif, has been identified, consisting of the sequence $ψKXE$, where $ψ$ is a large hydropobic amino acid, X is any residue, and K is the site of SUMO conjugation. The screening of MEF sequence by “SUMOplotTM Prediction” (http://www.abgent.com/sumoplot.html) found several potential SUMO motifs with different identities (Fig. 4-2). One is located in ETS domain; the other two are in C-terminus of MEF. Lysine 657 within IKME is predicted
with the highest possibility to be conjugated by SUMO.

In some cases, ubiquitin and SUMO will recognize the same lysine residue in some substrates, like IKBα and yeast PCNA, and competitively bring different functional impacts on the substrates [184]. Because the lysine residue can simultaneously undergo additional modification, such as acetylation, these modification machineries may interplay to make more complex regulatory networks.

4.2 Materials and methods

4.2.1 Cell culture
A549, HeLa and HEK293 cells were cultured as described in 2.2.1.

4.2.2 Plasmid constructs
C-MEF, His-tagged MEF C-terminus for expression in E. coli, was prepared by inserting MEF fragment 337-663 into the vector PET30a(+).

N-MEF, His-tagged MEF N-terminus for expression in E. coli was prepared by inserting MEF fragment 1-336 into the vector PET30a(+).

C-MEF(K657R) and C-MEF(K537R), in which the lysine 657 and 537 of MEF were mutated into arginine, were prepared by QuikChange II Site-directed Mutagenesis Kit (Stratagene) according to manufacturer’s instructions, using C-MEF as template and respective sense and antisense primers: the sequences of the sense primers are shown as MEF-P(K657R) and MEF-P(K537R) in Table 4-1.

N-MEF(K280R), in which the lysine 280 of MEF was mutated into arginine, was
prepared by Quikchange II Site-directed Mutagenesis Kit (Stratagene), using N-MEF as template and sense and antisense primers: the sequence of the sense primer is shown as MEF-P(K280R) in Table 4-1.

MEF(K280R), MEF(K537R) and MEF(K657R) in pEGFP and in pCB6 were prepared by Quikchange II Site-directed Mutagenesis Kit (Stratagene), using WT MEF in pEGFP and pCB6 as templates, and respective sense and antisense primers: the sense primers are shown as MEF-P(K280R), MEF-P(K537R) and MEF-P(K657R) in Table 4-1.

The construct of lysozyme promoter, Lys100, was prepared as previously described [125].

PTE1-E2-S1 and PTE1-E2-S2 were generally provided by Dr. Hisato Saitoh.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEF-P(K657R)</td>
<td>CCCCATTTCAACCCTACTTCCCTTCATCGAATGGAGCGCCCATGAC</td>
</tr>
<tr>
<td>MEF-P(K537R)</td>
<td>CTACAGCAGCCCCCTAGGGTCCGGGAGGGGCACTGAGGTCTCC</td>
</tr>
<tr>
<td>MEF-P(K280R)</td>
<td>CCAAGAGGCATACTGGCCGAGTGGAAGGGCAGGGCTGGTG</td>
</tr>
</tbody>
</table>

4.2.3 Protein recovery from E. coli

Individual MEF expression vector or a combination with PTE1-E2-S1 or PTE1-E2-S2 vector was transformed into E. coli, BL21 (DE3) STAR (Invitrogen), which was later plated on LB plate with proper selection antibiotics. Then the antibiotics-resistant colony was picked up and incubated at 37 °C with antibiotics for about 12 h, then induced with isopropyl-β-D-thiogalactose (IPTG) (Nacalai Tesque) at 25 °C. After 8 h incubation, E. coli was collected and lysate was recovered by sonication on ice. After centrifuging, the supernatant was mix with beads and rotated for 1 h, after which the beads were washed with E. coli lysis buffer 6 times.

4.2.4 Western blotting analysis

The lysates from E. coli purified by ProBond nickel-chelating resin (Invitrogen, indicated as Ni²⁺ beads in figures) or whole cell lysates or those immunoprecipitated by affinity-purified rabbit antisera to MEF (1/200; TransGenic Inc.) were subjected to SDS-PAGE. For detecting full length MEF, C-terminus and N-terminus of MEF, 6%, 15% and 7.5% gel were respectively used. Then the proteins were transferred to PVDF membrane. After blocking with 5% skim milk at 4 °C overnight or room temperature for 1 h, membranes were incubated with the primary antibodies at room temperature for 1 h.
The membranes were subsequently incubated with proper secondary antibodies. Finally, chemiluminescence was detected using the ECL Kit (Amersham Life Science). The respective primary antibodies used here are: affinity-purified rabbit antisera to MEF (1/200; TransGenic Inc.), rabbit polyclonal anti-HIS-probe (1/1000; Santa Cruz Biotechnology), mouse monoclonal anti-c-myc (1/100; Santa Cruz Biotechnology), mouse monoclonal anti-SUMO1 (1/1000; Zymed) and rabbit polyclonal anti-SUMO2 (1/1000; made and provided by Dr. Hisato Saitoh). The secondary antibodies used here are: HRP-conjugated goat anti-rabbit IgG (1/10000; Seikagaku Corporation) and HRP-conjugated goat anti-mouse IgG (1/10000; Jackson ImmunoResearch).

4.2.5 Transfection and luciferase assay

Transient transfections of plasmid DNA were performed with TransIT-LT1 (Panvera) according to the manufacturer’s recommendations. Briefly, TransIT-LT1 and Opti-MEM were mixed thoroughly and incubated at room temperature for 15 min. Then the DNA indicated in each figure was added to the diluted TransIT-LT1 reagent (ratio: 1µg DNA/3 µl LT1). After being mixed gently, the mixture was incubated at room temperature for 15 min. Then the mixture was directly added to the cells cultured on 24-well plates, without changing the medium. Forty-eight hours after transfection, the medium was removed and cells were harvested for luciferase assay. Luciferase assay was performed as described in 1.2.4.

4.2.6 Immunofluorescence

HeLa cells were grown on poly-L-lysine-coated 35-mm glass-bottomed dishes and transfected with the WT MEF or individual mutant MEF in pEGFP and Flag-PML in pcDNA3 or a combination of SUMO1 in pcDNA and Ubc9 in pCGN. Forty-eight hours later, cells were fixed in 3.7% paraformaldehyde, and permeabilized with 0.5% Triton X-100 in PBS(-) for 15 min at room temperature. Fixed cells were subsequently blocked for 60 min at room temperature with PBS(-) containing 1 mg/ml bovine serum albumin and incubated with a 1:100 dilution of primary antibody, mouse anti-PML (Santa Cruz Biotechnology), for 1 h at room temperature. Cells were washed three times with PBS(-) and then stained with 1:100 dilution of TRITC-conjugated anti-mouse for 1 h at room temperature. Cells were washed three times with PBS(-) and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Immunofluorescence analyses were carried out using Fluoview FV-500 confocal laser scanning microscope (Olympus, Tokyo, Japan).
4.2.7 Immunoprecipitation

The HEK293 cells collected from 6-well plate were washed with PBS(-) twice, then lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% SDS, 5% deoxycholate, and 5% Nonidet P-40). The lysates were diluted to 1:5 by adding dilution buffer (150 mM NaCl and 50 mM Tris-HCl, pH 8.0) and then mildly sonicated. After centrifuging for 20 min and incubating with protein G beads (Amersham Biosciences) at 4 °C for 1 h, the supernatants were incubated with 1.5 µg anti-MEF antibody at 4 °C for 2 h and then with protein G beads at 4 °C for 2 h. The beads were washed five times with washing buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.5% Nonidet P-40). Immunoprecipitates were suspended in a Laemmli sample buffer (2% SDS, 100 mM dithiothreitol, 60 mM Tris-HCl, pH 6.8, and 0.001% bromphenol blue). The samples were subjected to SDS-PAGE followed by western blotting with appropriate antibodies.

4.3 Results

4.3.1 MEF is sumoylated in E. coli.

MEF is a nuclear protein and several potential SUMO motifs are contained in its sequences, which are coincident with the general requirements to be covalently modified by SUMO (Fig. 4-2). To check the SUMO-conjugating potential of various predicted SUMO motifs in MEF, the intact or lysine-mutated N-terminus and C-terminus of MEF were expressed in E. coli together with the system which expresses the proteins needed for sumoylation: E1, E2 and SUMO1 or SUMO2 (Fig. 4-3).

First the sumoylation in C-MEF was examined. As shown in Fig. 4-4, C-MEF showed the band with size about 37 kD, which was recognized by both anti-MEF and anti-His antibodies. When C-MEF was co-expressed with E1, E2 and SUMO1 or SUMO2, several bands were found located at the upper positions. The same phenomenon was also observed for C-MEF(K537R), but not for C-MEF(K657R). Further, to answer if the up-shifted bands come from the SUMO conjugation, the anti-SUMO1 and anti-SUMO2 antibodies were applied for western blotting, respectively. Both SUMO1 and SUMO2 antibodies recognized the upper band with size about 50 kD, which disappeared in C-MEF(K657R), but not in C-MEF(K537R). The results confirmed that the C-terminal of MEF is sumoylated at the site of lysine 657, but not lysine 537 in E. coli system.

Next, the similar experiment was carried out to check the sumoylation in N-MEF, which contains one predicted SUMO motif. As shown in Fig. 4-5, both WT N-MEF and N-MEF(K280R) (about 75 kD) showed the same up-shifted bands when co-expressed
with E1, E2 and SUMO1. The band located around 100 kD is supposed to be the sumoylated N-MEF. This indicates that lysine 280 in N-MEF is not responsible for its sumoylation. Based on the current data, it is still difficult to identify the real sumoylation site in N-terminus of MEF.

4.3.2 MEF is sumoylated in mammalian cells.

The E. coli system used in the experiments above represents the optimized in vitro conditions for sumoylation-detecting, so it is necessary to check if SUMO modification of MEF also happens in mammalian cells. The substrate proteins of SUMO simultaneously undergo dynamic de-sumoylation, so usually the endogenous sumoylation stays at an undetectable level. To overcome this problem, MEF was over-expressed in HEK293 cells together with E2 enzyme, Ubc9, and SUMO1. The cell lysates were immunoprecipitated with MEF antibody, followed by western blotting analysis. As shown in Fig. 4-6A, the up-shifted MEF band was detected by MEF antibody only for the lysate with over-expression of Ubc9 and SUMO1. The co-precipitation of SUMO1 with MEF was detected in Fig. 4-6B, using anti-myc antibody recognizing myc-tagged SUMO1. These data suggest that MEF is also SUMO-modified in mammalian cells.

So far, the SUMO modification of lysine 657 of MEF has been demonstrated both in vitro and in vivo, so the following experiments will be mainly focusing on the functional modification of MEF induced by lysine 657 sumoylation.

Fig. 4-3. The E. Coli system was used to examine the SUMO modification of MEF in vitro. PTE1-E2-SUMO expresses both SUMO and SUMO conjugation enzymes, E1(AOS1/UBA2) and E2 (Ubc9). PET30-MEF expresses MEF protein.
Fig. 4-4. Detection of sumoylation site in C-terminus of MEF in E. coli. A. WT C-MEF or mutant C-MEF with mutation in SUMO acceptor site was transformed into E. coli together with SUMO1 or 2 and SUMO conjugation enzymes E1 and E2. Total lysate from each bacterial culture was incubated with Ni²⁺ beads, then the proteins bound to the beads were loaded to 15% gel for SDS-PAGE followed by western blotting using MEF antibody. B. The same proteins as those in A were subjected to SDS-PAGE followed by western blotting using MEF, HIS, SUMO1 or SUMO2 antibody, respectively.
Fig. 4-5. Detection of sumoylation site in N-terminus of MEF in E. coli. WT N-MEF or mutant N-MEF with mutation in SUMO acceptor site was transformed into E. coli together with SUMO1 and SUMO conjugation enzymes E1 and E2. Total lysate from each bacterial culture was incubated with Ni²⁺ beads, then the proteins bound to the beads were loaded to 7.5% gel for SDS-PAGE followed by western blotting using HIS antibody.

Fig. 4-6. MEF is sumoylated in mammalian cells. HEK293 cells were transfected with MEF (1µg) only or a combination with SUMO1 (3µg) and Ubc9 (3µg) in 6-well plate. Forty-eight hours after transfection, cell lysates were harvested for immunoprecipitation with MEF antibody or normal rabbit antibody (Cont.). Then the immunoprecipitated lysates were subjected to SDS-PAGE and western blotting was performed using MEF antibody (A) or myc antibody (B).
4.3.3 SUMO does not affect PML NB translocation of MEF.
SUMO is closely related with protein localization. So it was first investigated if SUMO can alter the subcellular localization of MEF, which is a nuclear protein and has the potential to translocate to PML NBs. GFP-fused MEF (green) constructs and PML antibody (red) were used for immunofluorescence. Without transfection of PML, both WT MEF and MEF(K657R) showed diffused nuclear localization. When PML was transfected into cells, both WT MEF and MEF(K657R) were recruited into PML NBs (Fig. 4-7A). When SUMO1 and Ubc9 were co-transfected into cells but without input of PML, both WT MEF and MEF(K657R) could merge with PML (Fig. 4-7B). This might be due to the increased integrity of PML NB after introducing the sumoylation system, but not the direct consequence of sumoylation of MEF itself. The data indicate that in cells the SUMO modification in C-terminus is not required for the nuclear and PML localization of MEF.

4.3.4 SUMO decreases the transactivity of MEF.
Lysozyme has been proven to be one of the target genes of MEF and its transcription is sensitively promoted by MEF, so the lysozyme promoter was used here to reflect the transcriptional activity of MEF. As shown in Fig. 4-8, the lysozyme promoter activity was greatly enhanced by MEF transfection, but the co-transfection of Ubc9 and SUMO1 hampered the transactivity of MEF both in A549 cells and NIH3T3 cells, which have low and no endogenous expression of MEF, respectively. The transactivity of WT MEF with intact C-terminal sumoylation site was inhibited to a greater extent, compared with the MEF(K657R). Interestingly, the MEF(K657R) showed lower inherent transactivity compared to WT MEF, which seemed to be inconsistent with the observation that Ubc9 and SUMO1 down-regulated the transactivity of MEF. This inconsistency might be caused by the existence of some other mechanism(s) that can interplay with the modification by SUMO.
Fig. 4-7. Sumoylation in C-terminus of MEF does not affect its localization in PML NBs. A. WT MEF or MEF(K657R) in pEGFP (0.4 μg) and PML (0.6 μg) (A) or SUMO1/Ubc9 (1 μg/1 μg) (B) were co-transfected into HeLa cells plated on glass-bottom dishes. Forty-eight hours after transfection, cells were fixed and stained with PML antibody for immunofluorescence. MEF in pEGFP was detected as green fluorescence, while PML was detected as red. Yellow or orange indicates colocalization.
Fig. 4-8. Sumoylation decreases the transactivity of MEF. Lysozyme promoter Lys100 (0.2 μg) and the indicated DNA (MEF or MEF(K657R) in pCB6: 0.1 μg; SUMO1: 0.2 μg; Ube9: 0.2 μg) were transfected into A549 or NIH3T3 cells plated on 12-well plate. Forty-eight hours after transfection, cells were harvested for luciferase assay.
4.4 Discussion

4.4.1 Data summary and analysis

It is illustrated in this study that the C-terminus of MEF is sumoylated in the lysine 657 located within the identified SUMO motif, IKME. It seems that the N-terminus of MEF is also modified by SUMO, but the predicted SUMO motif located in ETS domain is dispensable for this modification, suggesting the likeliness of some other lysine with the potential to be sumoylated. Inspection of the SwissProt database reveals that around 30% of all human proteins contain at least one site conforming to the minimal consensus ψKXE, but it appears unlikely that all these proteins are actually modified by SUMO. Indeed there is an increasing number of sumoylated proteins that are modified at sites that diverge from this consensus sequence.

Regarding the influence on transcription, although many studies have shown the involvement of SUMO in transcriptional control, currently there is still no clear and unified molecular basis for the mechanism by which SUMO modification regulates transcription factor activity. One obvious consequence of the modification by SUMO is that SUMO alters the surface of the target protein and might cause either general conformational changes or specific changes at critical interfaces, thereby influencing the ability of the protein to interact with its partners. One model, therefore, is that modification with SUMO promotes or inhibits protein-protein interaction and thereby regulates the assembly of transcriptional complex. Sumoylation might also block alternative lysine-targeted modifications such as acetylation or ubiquitination. For example, sumoylation of the NF-κB inhibitor IκBα occurs on a lysine residue that is also a target for ubiquitination and thus stabilizes IκBα, preventing its ubiquitination and subsequent degradation and, consequently, the activation of NF-κB [184]. Modulation of the level of acetylation is another possible way in which conjugation of SUMO could suppress transcriptional activity. For instance, the sumoylated lysine of Sp3 is also subject to acetylation [185]. Although the relation between these two modifications is still unclear, one could imagine a scenario in which the transcriptional activity of Sp3, and possibly other transcription factors, could be regulated by different modifications at the same lysine.

The data of this study imply that sumoylation may affect the transactivity of MEF on its target gene, but the detailed mechanism has not been revealed yet. The transactivity of MEF seemed to be suppressed by co-expressing SUMO1, but inconsistently MEF(K657R) showed a lowered transactivity compared to intact MEF. This inconsistency might be caused by the existence of other modification(s) and its interplay
with sumoylation. The possible candidates for this unidentified modification include phosphorylation and acetylation.

**4.4.2 Phosphorylation and acetylation**

Phosphorylation represents the best studied protein modification, by which a phosphate group is coupled to the serine, threonine or tyrosine residue of substrate protein. As described in background, several phosphorylation cascades impinge on the Ets family and are essential for mediating the functions of this group of factors. Different from the other Ets factors phosphorylated by MAP kinase cascades, MEF physically interacts with cyclin A and phosphorylated by cyclin A/Cdk2, resulting in the phosphorylation of three residues of MEF: Ser641, Thr643 and Ser648 [124] and suppression of its transactivity. Interestingly, the locations of these three phosphorylation residues are very close to the lysine residue to be sumoylated in the C-terminus of MEF. So the phosphorylation and sumoylation may have some mutual regulatory relation, such as reciprocal dependent ostracizing. In other words, losing or attaching one of them to the substrate MEF will lead to the alteration in the other one. So deletion of the SUMO attaching site in MEF(K657R) may simultaneously bring change to the phosphorylation status of MEF. The interplay between acetylation and sumoylation may influence the function of MEF in the similar way, although so far the acetylation of MEF has not been reported.

Lysine acetylation, or the transfer of an acetyl group from an acetyl coenzyme A to the ε-group of a lysine residue, has been shown to occur in many protein targets, including core histones, about 40 transcription factors and over 30 other proteins [186], since it was initially discovered with histone proteins about four decades years ago [187]. This post-translational modification is quite common and plays important roles in regulating the function of eukaryotic, viral and bacterial proteins [188]. Like other covalent modifications, lysine acetylation exerts its effects through "loss-of-function" and "gain-of-function" mechanisms. For transcriptional control, acetylation is generally associated with activation, whereas lack of acetylation tends to correlate with repression—two regulatory processes working in harmony to achieve appropriate levels of transcription. This modification is reversible *in vivo*, with its specificity and level being largely controlled by signal-dependent association of substrates with acetyltransferases and deacetylases. PCAF, p300/CBP are the well known acetyltransferases, which catalyze the acetylation of transcription factors including p53, c-Myb, GATA1, EKLF, E2F, and so on [189-193]. The acetylation correlates with the increased DNA consensus binding of p53 and c-Myb, and with increases in stability and half-life of E2F, finally leading to the promotion in the transactivity of these factors.
HDAC proteins work as deacetylases to antagonistically remove this modification.

As for the possibility of MEF acetylation, the related study showed that the cell treatment by Tricostatin (TSA), an acetylation enhancer, greatly enhanced the protein level of MEF in HEK293 cells, but did not affect its mRNA expression (data not shown). This observation is consistent with the function of acetylation, which can increase the half life and stability of substrate protein.
Summary

Considering the broad and critical roles the Ets family of transcription factor plays in a wide range of physiological and pathological activities, finding new Ets target genes and clarifying the related mechanisms will shed light on comprehensive understanding of the functions fulfilled by this group of transcription factors. Meanwhile, illustrating the mechanisms involved in post-translational protein-protein interaction, localization and modification will be helpful for grasping the subtle machinery by which Ets factors mediate the biological processes as well as their dysfunctions under various cellular or environmental contexts. In hope of devoting efforts to clarifying the functions and modifications of some particular Ets factors using epithelial cell models, and based on the results presented in this study the following conclusions can be summarized:

1. Two target genes regulated by Ets transcription factors were identified and the related mechanisms were clarified.

1.1 ETS2 is involved in protein kinase C-activated expression of granulocyte-macrophage colony-stimulating factor in human non-small lung carcinoma cell line, A549.

The involvement of GM-CSF in various important biological activities, such as the in vitro invasiveness and the local progression of squamous cell carcinoma in the lung, emphasizes the necessity to clarify its detailed regulatory mechanisms, including the specific transcription factor(s).

In this study, Ets family member, ETS2, was identified to positively regulate GM-CSF transcription in epithelial cells, and this effect was enhanced by PKC activator, PMA. PMA can promote the protein level expression of ETS2. No matter the individual activity of PMA and ETS2 or their combined effect was EBS-dependent. The two responsive EBS are respectively located -40 and -100 bp upstream of the transcription start site of GM-CSF promoter. ETS2 and PMA cooperatively up-regulated not only the promoter activity of GM-CSF but also its endogenous transcription. These data indicate that GM-CSF is up-regulated by ETS2, a target of PKC in NSCLC.

In this part, the utility of ETS decoy ODN against tumor cell activity was also partially investigated. It was shown that the ETS decoy containing three EBS effectively inhibited the promoter activity of GM-CSF, MMP-9 and IL-8, three of which have been elucidated
to be involved in tumor progression. Further, the ETS decoy lowered proliferating ability of A549, a tumor cell model. The tumor-suppressing capacity of the ETS decoy was implied based on the \textit{in vitro} experimental observations.

1.2 MEF up-regulates human beta-defensin 2 expression in epithelial cells.

HBD2 is an antimicrobial peptide, which is widely expressed in epithelial tissues and displays a potent killing activity in response to the invasiveness of a wide range of microorganisms and the stimulation of various molecules. MEF has been reported to be involved in innate immunity responses, such as activation of perforin and lysozyme transcription. The present study shows that MEF not only activated HBD2 promoter activity, but also increased the endogenous HBD2 transcription level. Moreover, the activated HBD2 promoter activity was attenuated by blocking MEF expression and the loss of the EBS in the HBD2 promoter. The interaction between the EBS and MEF protein was further confirmed by EMSA. Thus, the data of this study indicate that MEF may play an important role in regulating HBD2 expression in epithelial cells. Taken together, the special role of MEF in innate immunity has been established by a series of studies showing its involvement in regulating immunity-related genes, such as perforin, lysozyme and defensin.

2. Functional mediation of MEF by post-translational protein-protein interaction and modifications.

2.1 Promyelocytic leukemia protein enhances the transcriptional activity of MEF and the subsequent HBD2 expression.

It has been reported that PML physically interacts with MEF and hence synergistically augments the transactivity of MEF on lysozyme gene. So in this study it was investigated if this synergistic effort is also applicable to the transcriptional control of HBD2, another MEF target gene. The results of this study show that upon the PML transfection, MEF was recruited to PML NB and its transcriptional activity on HBD2 promoter was promoted. Upon the stress of heat shock, the endogenous MEF could also co-localize with PML NB, and the HBD2 expression was concomitantly enhanced. On the other hand, Sp100, a major component of PML NB, hampered the cooperative effect of PML and MEF, probably due to the competitive binding to PML. This part of study indicates that upon the environment stimulation, MEF is able to change its localization to adjust its transactivity through interacting with other protein. This suggests that the
protein-protein interaction and localization are influential ways to affect the potency of transcription factors, although their total protein quantity might not change in the cells.

2.2 MEF is post-translationally modified by SUMO.

MEF is a nuclear protein and three SUMO motif are found in the amino acid sequence of MEF, which indicate the high possibility of MEF as the substrate of SUMO modification. The in vitro research using the E. coli sumoylation system demonstrated that MEF is modified by SUMO with the lysine 657, but not lysine 537 in its C-terminus. The SUMO modification of MEF was also verified in mammalian cells. It seemed that the N-terminus of MEF was also sumoylation, but not through the predicted SUMO motif. The functional investigations indicate that SUMO modification of lysine 657 in MEF does not correlate its localization in PML NB, but probably contributes to the modulation of MEF transcriptional activity. Further investigations need to be carried out to elucidate the detailed mechanism.
References


36. Chakrabarti, S.R., et al., Modulation of TEL transcription activity by interaction with the


60. Marini, M., et al., Interleukin-1 binds to specific receptors on human bronchial epithelial cells and upregulates granulocyte/macrophage colony-stimulating factor synthesis and release. Am J Respir Cell Mol Biol, 1991. 4(6): p. 519-24 microM, demonstrated significant inhibition of GM-CSF release. Based on the rank order of potency of several glucocorticoids, and the fact that nonglucocorticoid steroids including testosterone and beta-estradiol (0.1 microM) had no effect, we suggest that this is a specific receptor-mediated effect. We conclude that human lung produces GM-CSF in vitro and that antiinflammatory steroids are potent and effective inhibitors of the production of this cytokine. This may contribute to the therapeutic efficacy of these drugs in pulmonary diseases.


104. Yang, D., et al., Beta-defensins: linking innate and adaptive immunity through dendritic and T


140. Nefkens, I., et al., Heat shock and Cd2+ exposure regulate PML and Daxx release from ND10 by


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**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
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<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>APL</td>
<td>acute promyelocytic leukemia</td>
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<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
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<tr>
<td>Cdk</td>
<td>cyclin-dependent kinase</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>ChK</td>
<td>checkpoint kinase</td>
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<tr>
<td>CLE0</td>
<td>conserved lymphokine element 0</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP-response element-binding protein</td>
</tr>
<tr>
<td>CtBP</td>
<td>E1A C-terminal-binding protein</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRIP</td>
<td>Vitamin D receptor (VDR) interacting protein</td>
</tr>
<tr>
<td>EBS</td>
<td>ETS binding site</td>
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<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EKLF</td>
<td>erythroid Kruppel-like factor</td>
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<td>ELF1</td>
<td>E74 like factor 1</td>
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<td>ELK1</td>
<td>Ets like protein 1</td>
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<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ERF</td>
<td>ETS2 repressor factor</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
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<td>ESE</td>
<td>epithelium-specific ets</td>
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<tr>
<td>Ets</td>
<td>E26 transformation-specific</td>
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<tr>
<td>4α-PDD</td>
<td>4α-phorbol-12,13-didecanoate</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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**Abbreviations**

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<thead>
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<th>Abbreviation</th>
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<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage-colony stimulating factor</td>
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<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
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<tr>
<td>GRIF</td>
<td>gamma-aminobutyric acid type A (GABA(A)) receptor interacting factor</td>
</tr>
<tr>
<td>HBD</td>
<td>Human beta-defensin</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HIPK2</td>
<td>homeodomain-interacting protein kinase 2</td>
</tr>
<tr>
<td>HTH</td>
<td>helix-loop-helix</td>
</tr>
<tr>
<td>IKB</td>
<td>inhibitor of NF-κB</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>kD</td>
<td>kilodalton</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>MEF</td>
<td>myeloid ELF1 like factor</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>MAP kinase</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK kinase</td>
</tr>
<tr>
<td>Mdm</td>
<td>mouse double minute</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>NES</td>
<td>nuclear export signal</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NSCLC</td>
<td>non-small cell lung cancer</td>
</tr>
<tr>
<td>NB</td>
<td>nuclear body</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>ODN</td>
<td>oligodeoxynucleotide</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>PCAF</td>
<td>p300/CREB-binding protein-associated factor</td>
</tr>
<tr>
<td>PDBu</td>
<td>phorbol 12, 13-dibutyrate</td>
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**Abbreviations**

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<th>Abbreviation</th>
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<tr>
<td>PEA3</td>
<td>polyoma enhancer A 3</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
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<td>PIAS</td>
<td>protein inhibitor of activated STAT</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
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<td>PML</td>
<td>promyelocytic leukemia</td>
</tr>
<tr>
<td>PNT</td>
<td>pointed</td>
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<td>PU.1</td>
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<td>RanBP</td>
<td>Ran-binding protein</td>
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<td>RanGAP</td>
<td>RanGTPase-activating protein</td>
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<td>ribonucleic acid</td>
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<td>RXR</td>
<td>retinoic acid X receptor</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>SENP/SUSP</td>
<td>sentrin/SUMO-specific protease</td>
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<tr>
<td>SRF</td>
<td>serum response factor</td>
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<tr>
<td>SRC</td>
<td>steroid receptor coactivator</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<tr>
<td>SUMO</td>
<td>small ubiquitin-related modifier</td>
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<tr>
<td>TCF</td>
<td>ternary complex factor</td>
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<td>tumor growth factor</td>
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<td>TIF1α</td>
<td>Transcriptional intermediary factor 1α</td>
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<td>tumor necrosis factor α</td>
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<td>Ubc9</td>
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<tr>
<td>uPA</td>
<td>urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>WB</td>
<td>western blotting</td>
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<tr>
<td>WT</td>
<td>wild type</td>
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