The APM-1 interacts with SREBP-1 to increase transcription activity of SREBP-1 responsive genes

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ABSTRACT

The APM-1 interacts with SREBP-1 to increase transcription activity of SREBP-1 responsive genes

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APM-1 (also called Zbtb7c) is a BTB/POZ-domain class, proto-oncogenic transcription factor which shows the highest sequence homology with FBI-1. FBI-1, newly identified proto-oncogene was shown to be expressed, induced during preadipocyte differentiation by promoting growth arrest and terminal differentiation. SREBP-1 is major transcription regulator of adipocyte

differentiation and the many genes involved in cholesterol and fatty acid biosynthesis. We investigated whether APM-1 is modulating transcription controlled by SREBPs. APM-1 potently affected transcription activation by SREBP-1 on the artificial pGL2-6x(SRE)-Luc promoter in 293A cells. We found that APM-1 and SREBP-1 interact directly *in vivo* by co-immunoprecipitation and GST fusion protein pull-down assay. The zinc finger of APM-1 interacts directly with the bZIP DNA binding domain of SREBP-1. EMSA showed that the binding affinity of SREBP-1 to SRE and SRE/E-box is decreased by APM-1. However the interaction between APM-1 and SREBP-1 synergistically activate transcription of FASN gene by promoting GC-box binding of Sp1. We also found that Fatty Acid Synthase promoter, one of the transcriptional target genes of SREBP-1 and central enzyme in lipogenesis, was synergistically activated by APM-1 and SREBP-1. APM-1 is expressed during the differentiation of 3T3-L1 preadipocytes into adipocytes. Thus, we expected that APM-1 may be one of the important transcription regulators of SREBP-1 responsive genes in cancer and adipose tissue.

Key words: APM-1, SREBP-1, Sp1, SRE, E-box, Transcription, Fatty Acid Synthase, Adipocyte Differentiation

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I. INTRODUCTION

The BTB/POZ domain originally found in Drosophila melanogaster bric-à-brac, tramtrack and broad complex transcription regulators and in pox virus zinc finger proteins, is an evolutionarily conserved protein-protein interaction domain with about 1,000 distinct BTB/POZ entries in available sequence

databases.[1-2] The BTB/POZ domain regulatory proteins have various cellular regulatory functions. In particular, some of the POK proteins with BTB/POZ domain and Krüppel like zinc finger are major determinant in differentiation, development, apoptosis, ion channel activity and oncogenesis.[3-4]

SREBPs (sterol-regulatory-element-binding proteins) are important regulators of mammalian lipid metabolism.[5-6] SREBP-1a and SREBP-1c, which are expressed from overlapping mRNAs.[7] The two SREBP-1 proteins are identical except at the extreme N-termini. SREBP-1a and -1c have different N-terminal but are identical from the common second exon onwards. SREBP-1a has 28 unique amino acids from its first exon and SREBP-1c has only four (in addition to the initiator methionine residue). SREBP-1a is a potent activator of all SREBP-responsive genes, including those that mediate the synthesis of cholesterol, fatty acids, and triglycerides. SREBP-1c preferentially enhances transcription of genes required for fatty acid synthesis but not cholesterol synthesis. SREBPs are synthesized as membrane-bound precursor proteins and proteolytically processed to yield the N-terminal transcription factor domain that enters the nucleus. In the nucleus, these transcription factors activate most genes required to produce cholesterol and fatty acids. As long as intracellular cholesterol concentrations are sufficient, SREBPs remain bound to mainly the endoplasmic reticulum (ER) as a trimer

composed of SREBP, the SREBP cleavage-activating protein, and the insulin-inducing gene. Once the cholesterol contents in ER membranes decline, the SREBP-SREBP cleavage-activating protein complex can no longer bind to insulin inducing gene, thereby exiting the ER and reaching the Golgi apparatus where the SREBPs are proteolytically processed by two Golgi-associated membrane bound proteases. Concomitant with the well regulated proteolytic activation of SREBPs on the ER-Golgi membranes, the transcriptional activities of nuclear SREBPs are also modulated in diverse ways.[8-9] The liberated mature SREBPs then enter the nucleus where they activate transcription of various genes in the fatty acid and cholesterol metabolic pathways such as LDL receptor, ATP-citrate lyase, Fatty acid synthase.[10-12] In SREBP target gene promoters, SRE, NF-Y or Sp1 binding site is frequently found and NF-Y and Sp1 directly bind to SREBP.[13] Through the binding, NF-Y and Sp1 seem involved in recruitment of basic transcription machinery. SREBP-1 and Sp1 activate transcription synergistically by binding to SRE, E-box and GC box, respectively.[14-15]

Fatty acid synthase (FASN), one of the main lipogenic enzymes and SREBP-1 target gene promoters in *de novo* lipogenesis in mammals, catalyzes all the reactions in the conversion of acetyl-CoA and malonyl-CoA to palmitate.[16-18] FASN gene transcription is under tight nutritional and hormonal

control in lipogenic tissues, namely liver and adipose tissue. The transcription factors, stimulatory proteins 1 and 3 (Sp1 and Sp3), nuclear factor Y (NF-Y) and sterol regulatory element binding protein-1 (SREBP-1) have cognate binding sites on the FASN gene.[19] Also, tumor-associated FASN, by conferring growth and survival advantages rather than functioning as an anabolic energy-storage pathway, appears to necessarily accompany the natural history of most human cancers. A recent identification of cross-talk between FASN and well-established cancer-controlling networks begins to describe the oncogenic nature of FASN-driven lipogenesis. FASN expression was higher in tumors than in surrounding normal tissues, depending on the FASN status of the tumor and tissue type. FASN up-regulation in tumour cells seems to be part of a more general change in the genetic program controlling lipogenesis, as evidenced by the concomitant increase of other SREBP-1c regulated enzymes of the same lipogenic pathway.[20-21] Therefore, if the same disturbances in signaling pathways responsible for oncogenic transformation contribute to increased lipogenesis in cancer cells, tumor-associated FASN could be viewed as a mere downstream manifestation of an early and common deregulation of upstream regulatory circuits.[22-23]

The Krüppel-like factor (KLF) proteins belong to a family of transcription factors that bind to GC-GT rich sites and CACC boxes of a large range of

gene promoters. Binding of these factors is mediated by a highly conserved DNA-binding motif of C_2H_2 zinc fingers localized at their C-terminus, which suggests that these proteins could have a redundant activity.[24] However, recent results show that each KLF has its own function and regulation. KLF transcription factors act as transcriptional activators or repressors depending on the specificity of promoters to which they bind and the cellular context. Moreover, they interact with various co-activators or co-repressors, regulating via different mechanisms, the transcription of their target genes. More recent investigations suggest that members of the Krüppel-like zinc finger transcription factors (KLFs) family are important regulators of development, cellular differentiation and growth, adipogenesis and in tumor development. Recent studies also suggest a potential role for KLFs in adipogenesis. Specifically, transcription of the Krüppel -like factor KLF5 is activated by C/EBPβ and C/EBPδ and, in concert with these C/EBPs, contributes to induction of PPARɣ2.[25] Studies also suggest a role for other members of the KLF family including KLF6 and KLF15 in promoting adipogenesis.[26-27] KLF15 is highly expressed in adipose tissue and can induce GLUT4 expression. Upon further investigation, other groups that KLF2/LKLF was also highly expressed in white and brown adipose tissue in mice. It is likely that additional factors of parallel pathways are induced early and converge on

PPARγ at a stage downstream of C/EBPβ and C/EBPδ, such as the helix-loop-helix (HLH) transcription factor ADD-1/SREBP-1c. A potential role for SREBP-1c in regulating adipogenesis derives from studies showing that its expression is significantly enhanced in 3T3-L1 adipocytes in response to insulin.[28-29] One of the Krüppel-like zinc finger family, FBI-1 recently was identified as a possible active participant in the adipocyte differentiation process in both mice and human, and suggested that APM-1 may be promoting growth arrest and terminal differentiation during adipocyte differentiation.[30] APM-1 contains a POZ-domain and four Krüppel-like zinc fingers and is similar to the recently characterized proto-oncogene, FBI-1, in two key functional domains: the POZ-domain (81% similarity) and the four Krüppel-like zinc fingers (88% similarity).[31-32]

Recently, we found that APM-1 is one of the major controllers of Arf-Mdm2-p53-p21 pathway and represses transcription of p21 genes. Our data shows that the transcription repression of p21 by APM-1 involved p53, corepressors, and histone modifications at the promoter. These results suggest that APM-1 acts as a proto-oncogene in the presence of p53, and induces cellular transformation, and promotes cell proliferation, and tumor growth (unpublished). Moreover, the link between SREBP-1 and APM-1 on FASN promoter may lead to greater understanding of lipid synthesis and the cell

cycle in cancer.

In the present study, we investigated that APM-1 affects the transcriptional activity of SREBP-1. By using GST-pull down, co-immunoprecipitation experiments, we observe that APM-1 ZF domain and bZIP DNA binding domain of SREBP-1 directly interact with each other *in vitro, in vivo*. Furthermore, through cotransfection of APM-1 and SREBP-1 in human embryonic kidney 293 cells, we showed that these two factors synergistically activate the FASN promoter. These data suggested that the binding affinity of SREBP-1 to SRE and SRE/E-box is regulated by the zinc finger of APM-1 and Sp1 through EMSA and ChIP. Moreover, APM-1 is expressed during the differentiation of 3T3-L1 preadipocytes into adipocytes. Thus, we expected that APM-1 may be one of the important regulators of adipocyte differentiation by interacting SREBP-1 on FASN promoter.

II. Materials and Methods

1. Plasmids preparation

Fatty acid synthase promoter-luciferase reporter genes, FAS 1 (FAS −150 /+65), FAS 2 (FAS −135/+65), FAS 3 (FAS −150/−73 TATA) were kindly provided by Dr. Timothy F. Osborne of University of California, Irvine. pCMV-SREBP-1a, and 1c were generous gifted from Dr. Kyung-Sup Kim of Yonsei Medical School (Seoul, Korea). pGL2-C/EBPα promoter–luciferase reporter gene was prepared by cloning human C/EBPα promoter DNA fragment into pGL2-basic vector (Promega, WI). pcDNA3.1-APM-1, pcDNA3.0-Flag-APM-1, and pcDNA3.0-Flag-APM-1 ZF plasmids were prepared by cloning mouse brain cDNA or human cDNA fragments into pcDNA 3.1 or pcDNA 3.0 plasmid (Invitrogen, CA). To prepare recombinant GST-POZ_{APM-1} and GST-ZF_{APM-1} fusion proteins, cDNA fragments encoding POZ-domain (a.a. 1-132) and zinc fingers (a.a. 365-468) were cloned into pGEX4T1 or pGEX4T3 (Amersham Biosciences, NJ). The pcDNA3.1-SREBP-1a was prepared by cloning from pCMV-SREBP-1a into the pcDNA3.1 plasmid (Invitrogen, CA). To prepare the recombinant GST-bHLH/$LZ_{SREBP-1}$ fusion protein expression vector, the pCMV-SREBP-1a was cloned into pGEX4T3 vector BamHI / XhoI (Amersham Biosciences, NJ).

The oligonucleotide PCR primers used for bHLH/$LZ_{SREBP-1}$ are forward, 5'-GATCGGATCCGCCCCGGCCTCTGCCCAGAGC-3' and reverse 5'-GATCCTCGAGTCACAGAGATTTGCTTTT GTGGACAG-3'.

2. RT-PCR of total RNA isolation from 3T3-L1 mouse preadipocytes and differentiated 3T3-L1 adipocytes.

Total RNA was isolated from 3T3-L1 preadipocyte and differentiated adipocytes using TRIzol reagent (Invitrogen, CA, USA). cDNAs were synthesized using 5 μg total RNA, random hexamer (10 pmol), and SuperScript Reverse Transcriptase II (200 units) in 20 $\mu \ell$ using reverse transcription kit (Invitrogen, CA, USA). PCR were performed by using following amplification condition: 94℃ denaturation for 5 min, 27 cycles of amplification reaction, 94℃ for 30 sec, 55℃ for 30 sec, 72℃ for 30 sec, final extension reaction at 72℃ for 10 min. The primers used for APM-1 were forward 5'-TTTCCTGACCTGCCCGGTGGG-3' and reverse 5'-CTAGTTGTTGGCTTCAGACAT-3'. The primers used for mouse FAS were forward 5'-AACCATGGAGGAGGTGGTGAT-3' and reverse, 5'-TGCCAGCAAGC TGGAGGAGCA-3'. The primers used for mouse C/EBPβ were forward 5'-CAAGCTGAGCGACGAGTACA-3' and reverse 5'-CAGCTGCTCCACC TTCTTCT-3'. The primers used for mouse PPARγ_2

were forward 5'-CCAGAGCATGGTGCCTTCGCT-3' and reverse 5'-CAGCAACCATTGGGTC AGCTC-3'. The primers used for mouse C/EBPα were forward 5'-GAA CAGCAACGAGTACCGGGT-3' and reverse 5'-GCCATGGCCTTGACCA AGGAG-3'. The primers used for mouse a-FABP were forward 5'-ACCATGTGTGATGCCTTTGTG-3' and reverse 5'-TCATGCCCTTTCATAAACTCT-3'. The primers used for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were forward 5'-ACCACAGTCCAT GCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3'.

3. Cell culture

Human embryonic kidney 293A cells were cultured in Dulbecco' modified eagle medium (DMEM, Gibco-BRL, MD) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL, MD). Murine 3T3-L1 preadipocytes were maintained at low passage, and grown to confluence in DMEM, supplemented with 10% calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin, 0.8 mg/ml Biotin (all from Invitrogen), in a humidified atmosphere of 5% CO_2, 90% air at 37℃. Differentiation was induced by placing two-day post-confluent cultures in DMEM supplemented with 10% fetal bovine serum (FBS) (Invitrogen, CA) and antibiotics as above, for up to 8 days. The medium for differentiating 3T3-L1 cells was supplemented

with 0.525 mM methylisobutylxanthine (Sigma, MO), 1 μM dexamethasone (Sigma, MO) and 0.167 μM insulin (Roche, NJ). Forty-eight hours later this medium was replaced with medium supplemented only with 0.167 μM insulin (Roche, NJ).

4. Lenti-viral APM-1 stable cell lines

Stable 3T3-L1 cells overexpressing APM-1 were prepared by infection of 3T3-L1 cells with a recombinant LentiM1.4-APM-1 tagged with His- and Myc- peptides (Vectorcorea, Daejeon, Korea). After 2~3 days of infection, virus-containing medium was replaced with DMEM medium supplemented with 10% calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin, 0.8 mg/ml Biotin with puromycin (Sigma, MO).

5. Transient transfection

Human embryonic kidney 293A cells were seeded onto 6-well plates and grown for 16 hrs before transfection. The cells were transfected with 1.2 ㎍ of plasmid DNA mixture using Lipofectamine Plus reagent (Invitrogen, CA, USA) according to the manufacturer's protocols. Briefly, the plasmid DNA and 3 ㎕ of PLUS reagent were mixed in 100 ㎕ Opti-MEM (Gibco BRL, MD) and then added 100 ㎕ of containing 3 ㎕ of Lipofectamine reagent

were added. The cells were washed with PBS and supplied with 800 ㎕ of serum free Opti-MEM. After 15 min of incubation, Lipofectamine-DNA mixtures were added into wells. The cells were transfected for 3 hrs with the plasmid, then grown in DMEM supplemented with 10% FBS. The cells were harvested 36 hrs after transfection and lysed in 70 ㎕ of reporter lysis buffer (Promega, WI), and cell debris was removed by centrifugation and the supernatant was collected. Luciferase assays were conduct with 5 ㎕ of and 50 ㎕ of luciferase assay reagent (Promega, WI). Luciferase activities were normalized with β-galactoidase activities of the lysates.

6. Co-immunoprecipitation and western blot analysis

HEK 293A cells were cotransfected with 2.5 ㎍ pcDNA3.0-FLAG APM-1 and, 2.5 ㎍ pcDNA3.1-SREBP-1a plasmids using Lipofectamine Plus (Invitrogen, CA). The cells were harvested 48 hrs after transfection and and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 1% NP-40, 1 mM PMSF, and protease inhibitor mixture (1 tablet/50 ㎖). Lysates were clarified by centrifuging at 13,000 rpm for 15 min. 500 ㎍ of lysates were incubated with Protein A agarose (20 ㎕) and antibodies (2 ㎍), against Myc-Tag (9B11, Santa Cruz, CA), Flag-Tag (Sigma, MO) for overnight with gentle rotation at 4℃. The following days, immunoprecipitates were washed 5 times

in 1/3 diluted lysis buffer. After the last wash, immunoprecipitates were boiled in 20 ㎕ 5x SDS loading buffer, resolved by 12% SDS-PAGE, and transferred to Immun-Blot™ PVDF membrane (Bio-Rad, CA) and blocked with 5% skim milk (BD biosciences, NJ) in TBST (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, and 0.001% Tween 20) for 10 min. Blotted membranes were incubated with Ab- Flag (Sigma, MO), Ab-Myc (9B11, Cell signaling, MA), or Ab-α-GAPDH (Calbiochem, CA) overnight at 4℃. Membranes were further incubated with horseradish peroxidase-conjugated anti-mouse IgG (Vector, CA) or anti-rabbit IgG (Vector, CA), and developed with the ECL reagents (PerkinElmer, CA).

7. GST fusion protein purification, *in vitro* transcription and translation and GST fusion protein pull-down assays

The *E. coli* BL21 (DE3) transformed with either GST or GST-bHLH/$LZ_{SREBP-1}$ proteins expression vector, was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 hrs at 37℃. After *E. coli* pellets were lysed and sonicated in lysis buffer (1x PBS, 1 mM PMSF, 2 mM EDTA, and 0.2 mg/㎖ lysozyme), recombinant proteins were purified by affinity chromatography using glutathione-agarose 4 beads (Peptron, Daejeon, Korea). Purified proteins were resolved on a 15% SDS-PAGE gel to

quantitate and assess purity. The same volume of protein-agarose bead complex was used for all GST-fusion protein pull down assays. Recombinant GST, GST-POZ_{APM-1}, and GST-ZF_{APM-1} fusion proteins were prepared from *E. coli* BL21 (DE3) grown overnight at 18℃ in a medium containing 0.2 mM IPTG. The *E. coli* were lysed and purified using glutathione-agarose 4 bead affinity chromatography (Peptron, Taejeon, Korea). The purified proteins were then resolved with 12% SDS-PAGE to quantitate and assess purity. APM-1 and SREBP-1 polypeptides were prepared by incubating 1 μg of pcDNA3.1-APM-1 expression plasmid and pcDNA3.1-SREBP-1 expression plasmid with TNT Quick-coupled Transcription/Translation Extract (Promega, WI), containing 40 ㎕ of TNT Quick Master Mix and 2 ㎕ of [^{35}S]-methionine (1175.0 Ci/mol), (PerkinElmer Life Sciences, MA) at 30℃ for 90 min. Polypeptide expression levels were then analyzed by running 1 ㎕ of the total mixture through 12% SDS-PAGE and autoradiography. For GST-fusion protein pull-down assays, GST-fusion protein-agarose bead complexes were incubated with 10 ㎕ of *in vitro* translated [^{35}S]-methione–labeled SREBP-1 or APM-1 at 4°C for 4 hrs in the HEMG buffer (40 mM HEPES pH 7.9, 100 mM KCl, 0.2 mM EDTA, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 10% glycerol, 1.5 mM DTT, protease inhibitor mixture (1 tablet/50 ㎖)). The reaction mixtures were centrifuged, the pellets were washed thoroughly, and the bound

proteins were separated using 12% SDS-PAGE. They were then exposed to X-ray film using an image-intensifying screen (Kodak, NY).

8. Electrophoretin Mobility Shift Assay (EMSA)

The oligonucleotide probes (500 picomoles each in 83.3 mM Tris-HCl, pH 8.0, 16.7 mM $MgCl_2$, 166.7 mM NaCl) were annealed by heating at 93℃ for 5 min and cooling down slowly to room temperature. After diluting the solution containing the annealed oligonucleotides with water to 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl, 100 picomoles of annealed oligonucleotides for EMSAs were labeled with [α-^{32}P]-ATP and Klenow enzyme (Roche, Mannheim, Germany) by incubating 30 min. at 37℃. α-^{32}P labeled, double-stranded oligonucleotides were purified with SephadexTM G-50 (Amersham Biosciences, Uppsala, Sweden). Sequences of various oligonucleotides used in EMSA are as follows (only the top strand is shown): FAS SRE: 5'-GATCCGGGCATCACCCCACCGACG-3', FAS SRE/E-Box: 5'-GATCGTCAGCCCATGTGGCGTGGC-3'. Each binding reaction was carried out in 20 $\mu\ell$ of binding buffer containing 10 mM HEPES (pH 7.9), 60 mM KCl, 5 μM $ZnCl_2$, 1 mM dithiothreitol, 1% BSA, and 7% glycerol, and purified recombinant His-tagged SREBP-1 (200 ng), GST-APM-1 ZFDBD (75～675 ng). The protein-DNA complexes were resolved from free

probe by 6% non-denaturing polyacrylamide gel electrophoresis at room temperature in 0.5x TBE (89 mM Tris-Borate, 2 mM EDTA, pH 8.3) buffer. The dried gels were exposed to X-ray film at -70℃ with a Kodak intensifying screen (Kodak, NY, USA).

9. Chromatin immunoprecipitation (ChIP) Assays.

To investigate whether *in vivo* indirect molecular interaction through the SREBP-1 between APM-1 and the SREBP-1 binding site on the FASN promoter is actually occurring, ChIP assay was performed. Subconfluent 293A cells on a 10 cm dish were transfected with pcDNA3.0-Flag APM-1 or pcDNA3.1-SREBP-1a using Lipofectamine Plus (Invitrogen, CA) and grown for additional 48 hrs. 293A cells were treated with formaldehyde (final 1%) to cross-link APM-1 and SREBP-1a protein to the FAS promoter. Cells were washed with cold phosphate-buffered saline and lysed with SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0). The lysate was sonicated to shear DNA into fragments of 500~1000 bp. The sonicated supernatant was diluted 10-fold with ChIP dilution buffer (1% SDS, 1% Triton X-100, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, 1.2 mM EDTA) and incubated with antibodies against mouse Flag antibody (Sigma, MO) or rabbit SREBP-1a antibody overnight at 4℃ with rotation. To collect DNA-SREBP-1-antibody

or DNA-SREBP-1a-APM-1-antibody complex, salmon sperm DNA/protein A/G -agarose slurry was added to the mixture. The mixture was incubated for 1 hrs at 4℃ with rotation and pelleted in a DNA/protein A/G-agarose complex by brief centrifugation (4,000 rpm) at 4℃. After extensive washing of the pellet with washing buffers (Low salt immune complex wash buffer: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCI (pH 8.0), 167mM NaCI) (High salt immune complex wash buffer: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCI (pH 8.0), 0.5M NaCI) (LiCI immune complex wash buffer: 0.25M LiCI Wash buffer, 1% NP-40, 1% sodium deoxycholate, 1mM EDTA, 10mM Tris-HCI (pH 8.0) (TE buffer: 1% SDS, 0.1M $NaHCO_3$) recommended by the manufacturer, the pellet was dissolved with 500 ㎕ of elution buffer and spun to remove agarose. Supernatant was treated with 20 ㎕ of 5 M NaCl and heated to 65℃ for 4 hrs to reverse protein-DNA cross-link. After treatment with EDTA and proteinase K, the supernatant was extracted with phenol/chloroform and precipitated with ethanol to recover DNA. PCR reactions of immunoprecipitated DNA were carried out using the two sets of oligonucleotide primers designed to amplify the proximal promoter regions of FAS promoter. (bp -174 to -35: forward primer: 5'-GGGTCCCGGCTCGGC CG-3' and reverse primer: 5'-CCCCGGGCGGCCACGCC-3').

10. Oil Red O staining.

Culture dished were washed in PBS (pH 7.4), and cells were fixed in 3.7% formaldehyde for 15 minutes. Oil Red O was prepared by diluting a stock solution (0.5g of Oil Red O) (Sigma, MO) in 100 ml of isopropanol with water (60:40 (vol/vol)), followed by filtration. Cells were incubated in Oil Red O solution at room temperature. After staining, plates were washed twice in water and photographed.

III. RESULTS

1. APM-1 overexpression increases SREBP-1 and FAS mRNA expression

Recently, FBI-1 was identified as a possible active participant in the adipocyte differentiation process in both mice and human, and suggested that FBI-1 may be important in adipogenesis. Park et al. (2005) tested expression level of FBI-1 in adipose tissue of lean control and obese C57BL/6J mice. Total RNA was extracted from abdominal white adipose tissues of lean control and obese *ob/ob* mice. RT-PCR analysis of the total RNA showed that the expression of FBI-1 is increased in *ob/ob* C57BL/6J mice compared to control lean C57BL/6J mice (Park et al.). In NIH/3T3 and stable NIH/3T3-APM-1 cells established by infection with recombinant LentiM1.4-APM-1, APM-1 overexpression increased SREBP-1 and FASN mRNA expression (Fig. 1A). APM-1 contains a POZ-domain and four Krüppel-like zinc fingers and is similar to the recently characterized proto-oncogene, FBI-1 (Fig. 1B) in two key functional domains: the POZ-domain (81% similarity in amino acid sequence) and the four Krüppel-like zinc fingers (88% similarity) (Fig. 1C). APM-1 has a putative nuclear localization sequence (a.a. 470-486) juxtaposed to the fourth zinc finger. Phylogenic analysis revealed that FBI-1 is most closely related to APM-1 (Fig. 1D).

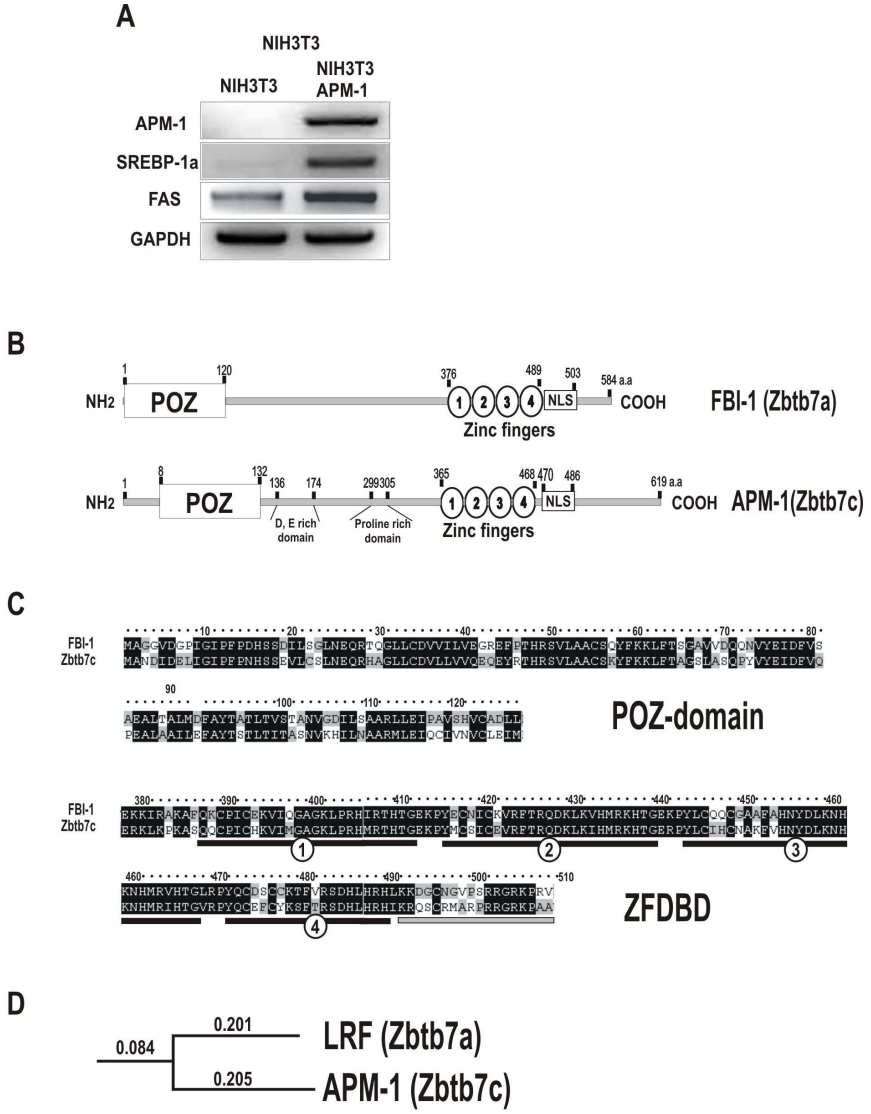


Figure 1. APM-1 increases transcription of SREBP-1 and FASN gene in NIH/3T3 cells. (A) RT-PCR analysis of APM-1, SREBP-1, FASN in the NIH/3T3 and stable NIH/3T3-APM-1 cells established by infection with recombinant lentivirus. APM-1 overexpression increased SREBP-1 and FASN mRNA expression. (B) Structure comparison of APM-1 and FBI-1 protein.

Two proteins contain a POZ domain, four zinc fingers, and a nuclear localization sequence. The two proteins are highly homologous in two domains, the POZ-domain (81%) and the zinc-finger domain (88%). POZ domain, open box; Circles, zinc fingers; NLS, nuclear localization sequence. (C) Amino acid sequence comparison of APM-1 and FBI-1 using GeneDOC program (http://www.nrbsc.org/gfx/genedoc/index.html). Conserved amino acids are shaded black and unconserved amino acids are light shaded black. APM-1 is similar to FBI-1 in two key functional domains: the POZ-domain (81%) and the four Krüppel-like zinc fingers (88%). (D) Phylogenic tree analysis revealed that FBI-1 is the most closely related to APM-1.

2. The APM-1 and APM-1 ZF increase transcription activation of artificial 6x (SRE)-tk*-Luc promoter by SREBP-1c

APM-1 shows high amino acid sequence similarity to FBI-1 which was proposed to be important in adipocyte differentiation. APM-1 increased transcription of SREBP-1 and FASN gene in the stable NIH/3T3 cells. APM-1 might be involved in the regulation of adipocyte differentiation, adipogenesis, and in the expression of adipogenic genes. To test these possibilities, we prepared a reporter plasmid construct which contains six copies of SREBP response element (SRE) of FAS gene or LDLR gene. The SREBP response elements were placed just upstream of the modified thymidine kinase (tk) minimal promoter and luciferase gene (Fig. 2A). The modified tk minimal promoter contains two mutated Sp1 binding GC boxes that do not bind Sp1. We co-transfected human kidney 293A cells with artificial 6x (SRE)-tk*-Luc promoter and expression vectors for SREBP-1 and APM-1. SREBP alone activates transcription potently and APM-1 increased transcription by SREBP-1 by 1.5- 3 folds. In contrast APM-1 alone does not have any effect on the transcription of reporter gene. The data suggest that APM-1 increased SREBP activity important in transcription significantly (Fig. 2A). Also, the ZFDBD of APM-1 is important region interacting with SREBP-1 *in vivo* (Fig. 5B-D). We investigated whether the ZFDBD of APM-1 has any affect on transcriptional

activity of 6x [SRE]-tk*-Luc promoter by SREBP-1a. Indeed, the ZFDBD of APM-1 could slightly increase transcription of the reporter gene by SREBP-1a (Fig. 2B).

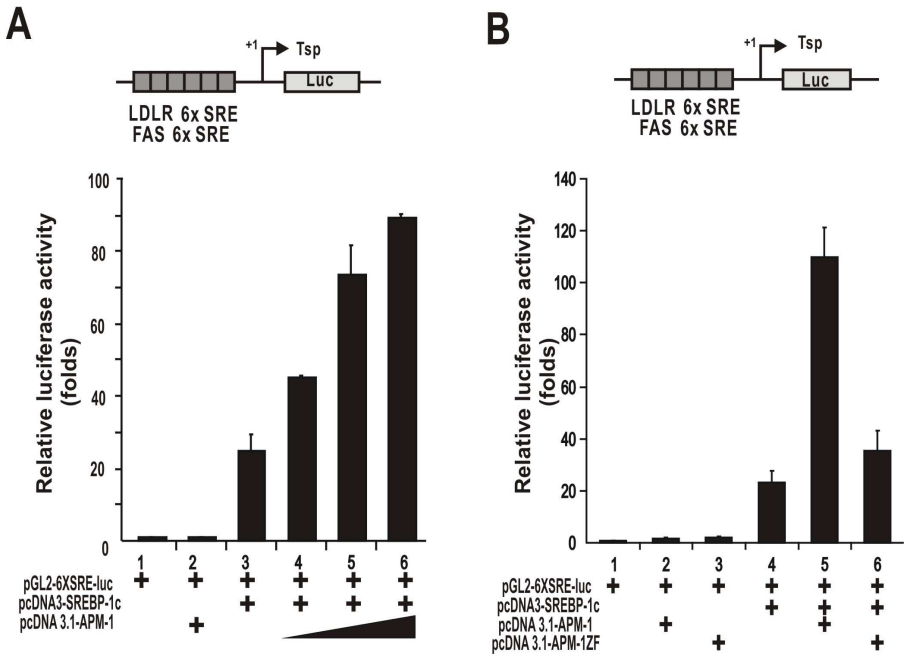


Figure 2. APM-1 and APM-1 ZF increase transcription activation of 6x [SRE]-tk*-Luc by SREBP-1 in human embryonic kidney 293A cells. (A) APM-1 enhances transcription activation of the 6x [SRE]-tk*-Luc by SREBP-1. Structure of 6x [SRE]-tk*-Luc promoter construct. tk* indicate the *tk* (thymidine kinase) minimal promoter with the mutations at the two Sp1 binding sites. 293A cells were cotransfected with 6x [SRE]-tk*-Luc reporter plasmid and expression vectors of SREBP-1 (25 ng/well) and increasing amount of APM-1 (25 ng, 300 ng, 600 ng). (B) The ZFDBD of APM-1 also enhances transcription activation of 6x [SRE]-tk*-Luc reporter promoter by SREBP-1. 293A cells were cotransfected with 6x [SRE]-tk*-Luc reporter plasmid and expression vectors of SREBP-1 (25 ng/well) and APM-1 (600 ng). Luciferase activities were normalized with the β-gal concentration and data presented are the average of three independent assays. Bars represent standard deviations.

3. The APM-1 enhances transcription activation of FAS gene by SREBP-1a in 293A cells

In proximal promoter (bp −150∼ +65) of rat FAS genes, SRE, GC-box, and SRE/E-box (recognized by bHLH type transcription factors) are present. APM-1 increases the transcription of test reporter plasmid with SRE element. APM-1 may influence the transcription of endogenous SREBP-1 responsive gene, such as FASN. We investigated whether APM-1 can activate transcription of lipogenic FASN gene that was shown to be activated by SREBPs. We co-transfected rat FAS promoter luciferase fusion reporter plasmids: pGL2-FAS1-Luc (bp −150 ~ +65), pGL2-FAS2-Luc (bp −135 ~ +65), pGL2-FAS3-Luc (bp −150 ~ −73), and pGL3-FAS-Luc (−2.7 kb) with expression vectors of SREBP-1a or APM-1 into HEK293A cells.

APM-1 itself has no effect on the transcription of the all FAS promoter constructs, but APM-1 significantly enhanced transcription by SREBP-1a on the FAS1, FAS2 promoter. However neither SREBP-1a nor APM-1 affected transcription of the FAS3 promoter (Fig. 3B). The sequence alignment of the upstream regulatory sequences (bp −160 ~ −12) of three mammalian FAS genes (Human, Mouse, Rat) showed that the proximal regions are highly conserved and have multiple binding sites for SREBP, thyroid nuclear receptor and Sp1 (Fig. 3A).[33] This suggests that our finding on the

transcriptional activation of SREBP-1 responsive gene by APM-1, must be true also in both human and rodent FAS gene.

We tested whether APM-1 and SREBP-1a can function similarly in activation of human FAS promoter with longer regulatory sequence (−2.7 kb). We co-transfected human embryonic kidney 293A cells with pGL3-FAS-Luc promoter (−2.7 kb) and the expression vectors for SREBP-1 and APM-1. SREBP-1 alone activates transcription potently by 8 folds and APM-1 further increased transcription activation by SREBP-1 by 4.5～5 folds (Fig. 3C). APM-1 alone does not have any effect on the transcription of reporter gene. Previously, others have shown that SREBP-1 binding site at −65 ~ −45 bp (5'-CAGCCCATGTGGCGTG-3') is the most important in the transcriptional regulation by SREBP. To investigate whether APM-1 is really up-regulating transcription by affecting SREBP bound on the SRE (bp −65 ~ −45) of the FAS gene, we transfected the HEK 293A cells with the plasmid mixture of pGL3-FAS-Luc Wt promoter (−2.7 kb) or pGL3-FAS-Luc Mt promoter (−2.7 kb) and expression vectors of APM-1 and SREBP-1a . Luciferase activity analysis revealed that mutation of the SRE/E-box site significantly decreased transcription by SREBP-1 or APM-1, suggesting that the −65 SRE/E-box (bp −65 ~ −45) is crucial for increasing transcription activity by SREBP-1 and APM-1 on the FASN promoter.

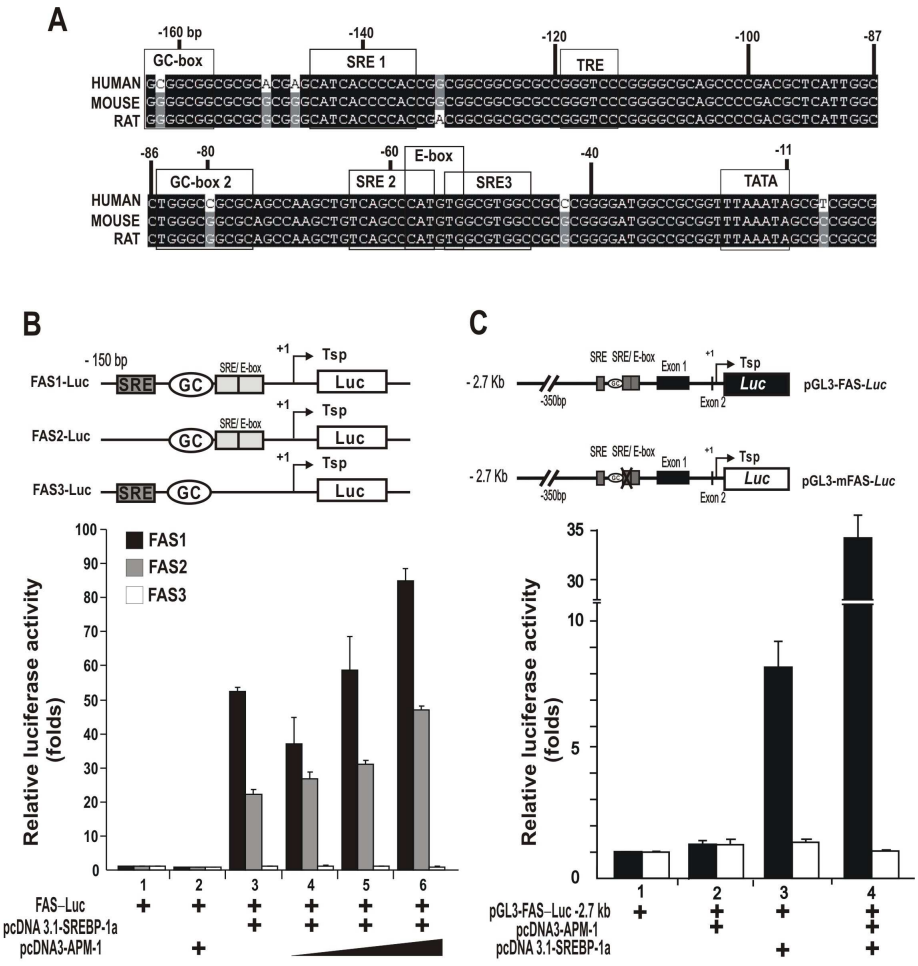


Figure 3. APM-1 increased transcription activation of rat and human FASN genes by SREBP-1. (A) Sequence comparison of human FAS promoter to mouse, rat FAS promoters. E-box consensus, CATGTG; GC-box consensus, GGGCGG; Sp1, stimulatory protein 1; SRE, sterol response element; TATA, TATA-box; TRE, thyroid responsive element. (B) Structures of pGL2-FAS 1-*Luc* reporter promoter construct (bp −150 ~ + 65), pGL2-FAS 2-Luc reporter promoter construct (bp −135 ~ + 65) and pSyNi-FAS 3-Luc reporter promoter construct (bp −150 ~ −73) are shown. SRE, SREBP binding

element; GC, Sp1 binding GC box; E-box, SREBP binding CATGTG element; Tsp, transcription start site. APM-1 enhances transcription activation of the FAS1, 2 promoters by SREBP-1. The 293A cells were co-transfected with pGL2-FAS-Luc reporter plasmids and expression vectors of SREBP-1 (25 ng/well) and APM-1 (25 ng, 200 ng, 400 ng). (C) Structures of pGL3-FASN-Luc (−2.7 kb) Wild type or Mutant type with mutation at one of the functional SRE (bp −65 ~ −45) are shown. 293A cells were transfected with pGL3-FASN-Luc (WT, MT), SREBP-1 or APM-1. Luciferase activities were normalized with the β-gal concentration and data presented are the average of three independent assays. Bars represent standard deviations.

4. APM-1 directly interacts with the bZIP DNA binding domain of SREBP-1 via its zinc fingers

Three SREBPs have been identified, SREBP-1a and SREBP-1c are produced from a single gene through the use of alternate promoters, and SREBP-2 is from a separate gene. APM-1 contains a POZ-domain and four Krüppel-like zinc fingers and has a putative nuclear localization sequence (a.a., 470∼486) juxtaposed to the fourth zinc finger (Fig. 4A). Immuno-cytochemical staining of APM-1 showed that it is located primarily in the nucleus (Fig. 4E), suggesting it may have a role as a transcription factor. Transcription analysis showed that APM-1 affects transcriptional activation potential of SREBP-1 on the four test promoters, pGL2-6x (SRE)-Luc, pGL2-FAS1-Luc (bp −150/+65), FAS2-Luc (bp −135/+65) and pGL3-FAS-Luc (−2.7 kb) constructs. This raised a possibility that APM-1 might interact with SREBP-1 to give strong transcription activation.

To determine whether the molecular interaction between APM-1 and SREBP-1 is direct, we performed a GST fusion protein pull down assay using recombinant SREBP-1 bHLH and *in vitro* translated [^{35}S]-methionine labeled APM-1 protein. GST-SREBP-1 bHLH fusion protein was incubated with APM-1, pulled down and the precipitates were separated by a SDS-PAGE. Autoradiography of the gel showed that full length APM-1 interacts with the

bHLH domain of SREBP-1 (Fig. 4B). Also we performed GST fusion protein pull down assay using GST-POZ or GST-ZF of APM-1. The GST or GST-fusion protein were incubated with [^{35}S]-methionine labeled SREBP-1 and pulled down. [^{35}S]- methionine labeled SREBP-1 was readily detectable in the precipitate pulled with GST- ZF of APM-1 but not with GST-POZ of APM-1 (Fig. 4C). These results demonstrate that the molecular interaction between the ZF domain of APM-1 and basic Helix-loop-Helix Leucine Zipper (bHLH ZIP) domain of SREBP-1 direct *in vitro.*

We further investigate whether the two proteins could interact with each other *in vivo* by co-immunoprecipitation. First, HEK293A cells were cotransfected with the expression vectors of Flag-tagged APM-1 and Myc-tagged SREBP-1a. The cell lysates were immunoprecipitated with anti-FLAG-M2 antibody. After extensive washing, the immunoprecipitates were resolved by 12% SDS-PAGE and analyzed for the presence of FLAG-APM-1 and Myc-SREBP-1 by Western blotting. As predicted, FLAG-APM-1 was readily detectable. Myc-SREBP-1 was also clearly detected in the immunoprecipitate by anti-FLAG-antibody (Fig. 4D). These results clearly showed that APM-1 is interacting with SREBP-1 *in vivo*. APM-1 and SREBP-1a were colocalized in CV-1 cells suggesting the possibility of molecular interaction (Fig. 4E).

Lastly, we investigated whether APM-1 and SREBP-1a are co-localized in nucleus. African green monkey kidney CV-1 cells were transiently transfected with the expression vectors of Flag-APM-1 and His-SREBP-1a and analyzed by immunocytochemistry using anti-Flag and anti-SREBP-1 antibodies. When APM-1 is expressed alone, APM-1 is mainly detected in nucleus. However SREBP-1a is localized in nucleus and also in cytoplasm at low level. When cells were co-transfected with the two expression vectors, both APM-1 and SREBP-1a are primary detected in nucleus, suggesting the two may interact with each other possible interaction between the two transcription factors (Fig 4E). Both GST fusion proteins pull down, immunocytochemistry and co-immunoprecipitation suggested that SREBP-1a and APM-1 could interact with each other.

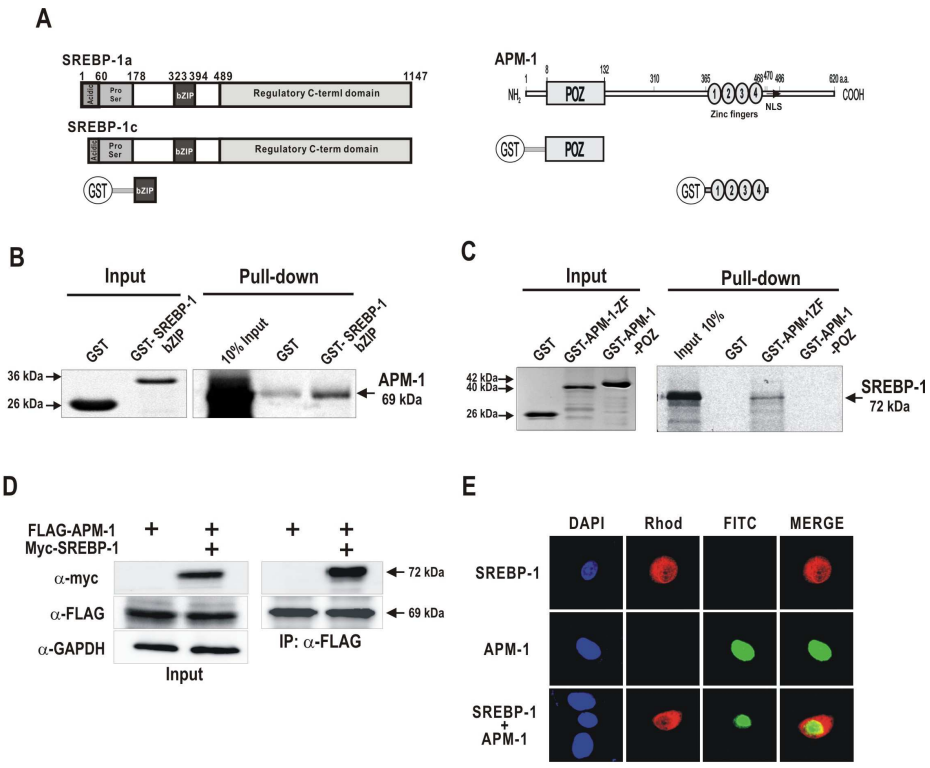


Figure 4. APM-1 interacts directly with the bHLH DNA binding domain of SREBP-1. (A) Structures of SREBP-1a, -1c and GST-bHLH domain of SREBP-1a fused with GST protein. The SREBP-1c is identical to SREBP-1a except for a shortened NH_2-terminal acidic domain (24 a.a. in SREBP-1c versus 42 a.a. in SREBP-1a). The acidic NH_2-terminal sequence and basic HLH domain are highly similar among SREBP-1a, -1c, and 2. Acidic, Acidic-domain; Pro/Ser, Pro/Ser-rich-domain; Ser/Gly/Pro, Gly/Pro/Ser-rich-domain; Gln, Glutamine-rich-domain; bHLH, Basic helix-loop-helix domain. Structures of APM-1, GST-POZ_{APM-1} domain and GST-ZF_{APM-1} fusion construct used for GST fusion protein pull-down assays. APM-1 contains a BTB/POZ domain at its N-terminus and Krüppel-like zinc fingers at its C-terminus. POZ, POZ-domain; ZF, Krüppel-like zinc fingers; NLS, nuclear

localization signal. (B) [^{35}S]-methionine labeled APM-1 polynucleotide was prepared by *in vitro* transcription and translation and incubated with recombinant GST or GST-bHLH of SREBP. The reaction mixtures were spun, analyzed by a SDS-PAGE, and the gel was exposed to X-ray film. The input represents 10% of the labeled protein used for the pull-down. The APM-1 and the bHLH domain of SREBP-1a interact directly *in vitro*. (C) [^{35}S]-methionine labeled SREBP-1 polypeptide was prepared by *in vitro* transcription and translation and incubated with recombinant GST or GST-POZ_{APM-1} and recombinant GST-ZF_{APM-1}. The ZF of APM-1 and bZIP DNA binding domain of SREBP-1a interacts directly *in vitro*. (D) Co-immunoprecipitation assay, 293A cells were transiently transfected with the mammalian expression vectors of Myc-SREBP-1 and FLAG-APM-1. The cell lysates were immunoprecipitated with antibody against anti-FLAG tag and the precipitates were analyzed by Western blot analysis using anti-Myc antibody. APM-1 and SREBP-1a also interacts *in vivo*. (E) CV-1 cells were transfected with expression vector of FLAG-APM-1 or SREBP-1. FLAG and SREBP-1 were detected by FITC (green) or Rhodamine (red) labeled antibodies. Nucleus was detected by soaking with DAPI (blue) solution. Co-localization of the APM-1 and SREBP-1 was indicated by yellow color of two merged overlapping fluorescence colors.

5. Direct interaction between the zinc-finger DNA binding domain of APM-1 and the bHLH of SREBP-1 reduces DNA binding of SREBP-1 to the SRE and SRE/E-box probes of the FAS gene

We investigated the consequence of protein-protein interaction between the APM-1 and the bHLH of SREBP-1a by EMSA. Using recombinant His-SREBP-1a and [^{32}P]-labeled SRE and SRE/E-box oligonucleotide probes of FAS gene promoter, we carried out EMSA in the presence of increasing amount of synthetic APM-1 prepared by *in vitro* transcription and translation. APM-1 itself does not show DNA binding activity.

Interestingly, the APM-1 reduced the DNA binding activity of SREBP-1a. Decreased SREBP binding to the SRE or E-box in the presence of APM-1 is intriguing. However these conditions could not explain the increased transcriptional activation of 6x [SRE]-tk*-Luc or FAS gene promoter by SREBP-1 and APM-1 (Fig. 5A). Because Sp1 binds to regulatory elements in the FAS promoter, and acts as a major activator of transcription in the genes with SRE and GC-box, we suspected that Sp1 binding to the FAS promoter might be modulated by SREBP-1 and APM-1.

Therefore we investigated the dynamics of Sp1, SREBP-1, and APM-1 using ChIP assay. Interestingly, When SREBP-1 was induced, binding of Sp1 to the GC-boxes decrease. On the other hand, when APM-1 was

overexpressed, the binding of Sp1 to the GC-boxes strongly was increased on the endogenous FASN gene and transfected pGL3-FASN-Luc Wt (-2.7 kb) promoter construct. In contrast, we were not able to observe the changes in the transcription factor binding on the mutant FASN promoter at SRE/E-box, pGL3-FASN-Luc Mt (-2.7 kb). On this particular construct, Sp1 binding was weak and was not greatly affected by cotransfected SREBP and APM-1 (Fig. 5C).

The result suggests that in the presence of both APM-1 and SREBP-1a, promoter occupancy of SREBP-1 and Sp1 might be both increased compared to the normal situation. This mode and intensity of transcription factor binding resulted in 2-3 fold higher FASN gene transcription than the condition where only SREBP-1 is transfected.

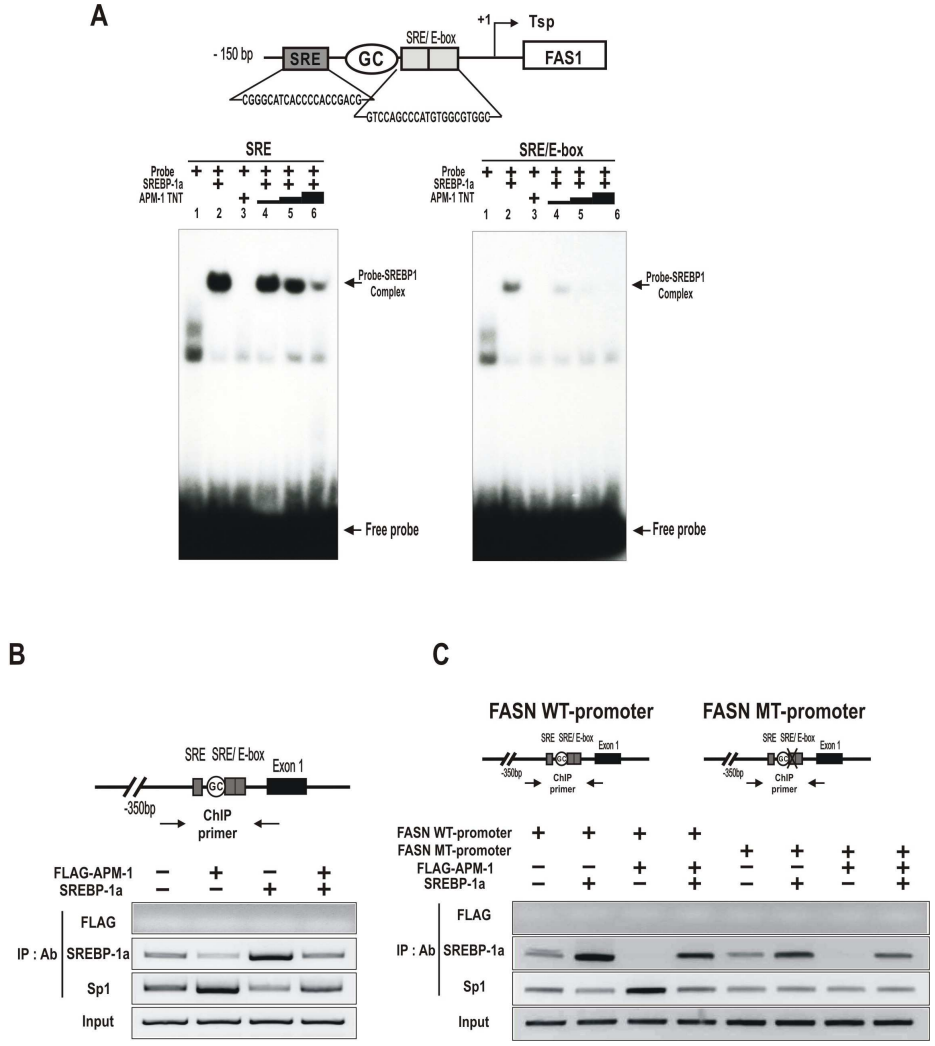


Figure 5. APM-1 decreases SREBP-1 binding to the SRE and the E-box probes of the FAS gene promoter. (A) EMSA. The APM-1 decreased SREBP-1a binding to the SRE and the SRE/E-box. The SREBP binding oligonucleotide probes (SRE or SRE/E-box) of the FASN gene promoter were incubated with recombinant SREBP-1a protein (200 ng), in the presence or absence of *in vitro* synthesized APM-1. The protein-DNA complexes were separated from free probe by 4% non-denaturing polyacrylamide gel. The

dried gels were exposed to X-ray film at −70℃ with a Kodak intensifying screen. (B) ChIP assay. 293A cells were transfected with the expression plasmids for SREBP-1a and FLAG-APM-1. After chromatin immunoprecipitation (IP) with anti-FLAG, anti-SREBP-1, anti-Sp1, PCR was performed with the primer set for the human FAS promoter containing two sterol regulatory elements (SREs) and one GC-box. (C) 293A cells were transfected with the expression plasmids for SREBP-1a, FLAG-APM-1 and pGL3b-FAS promoter. After chromatin immunoprecipitation (IP) with anti-FLAG, anti-SREBP-1, anti-Sp1, PCR was performed with the primer set for the human FAS promoter containing two sterol regulatory elements (SREs) and one GC-box.

6. APM-1 expression is regulated during adipocytes differentiation and facilitates differentiation of 3T3-L1 preadipocytes

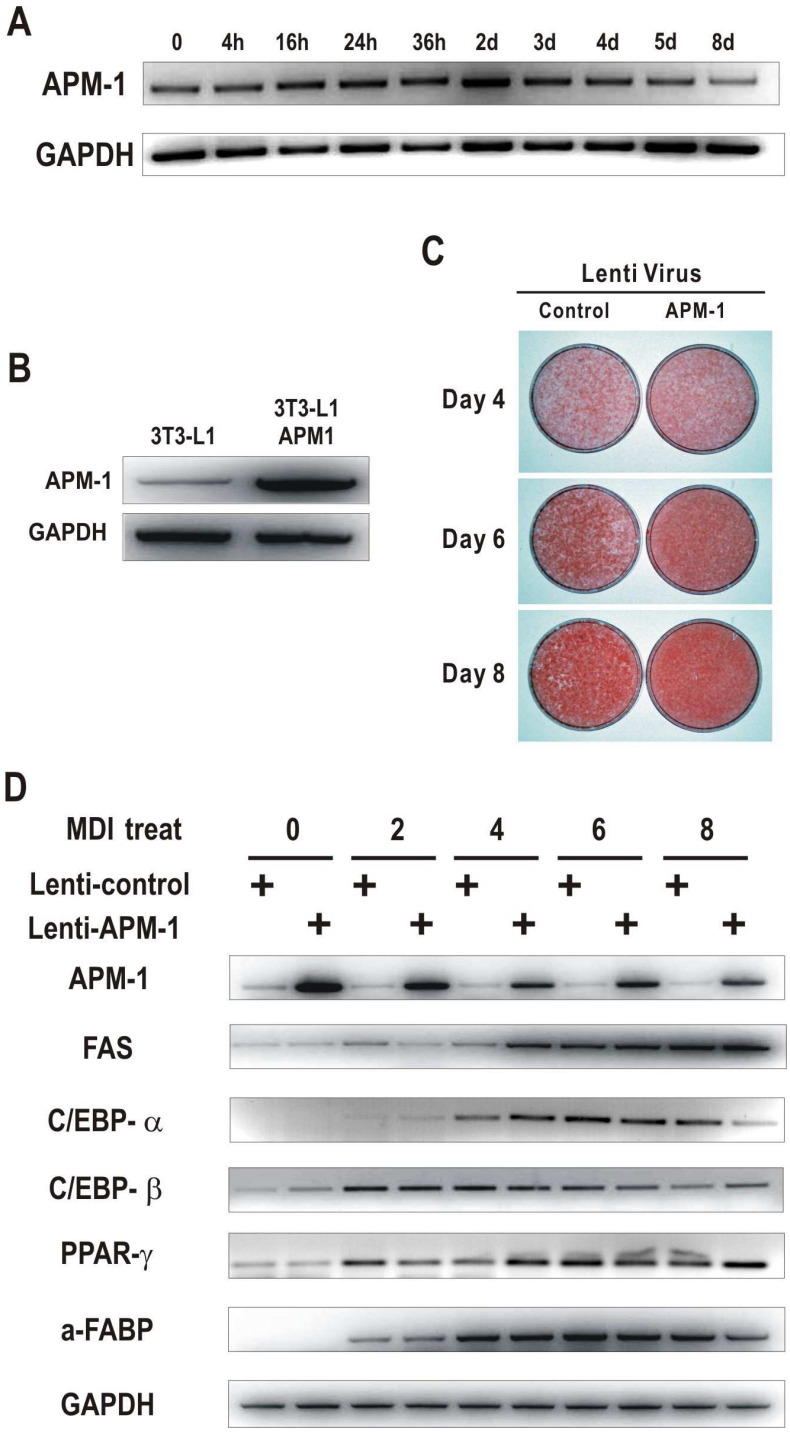
Our data showed that SREBP-1 and APM-1 interact with each other and such molecular interaction of SREBP-1 and APM-1 were shown to regulate transcriptional activity of SREBP-1 in FASN promoter. SREBP-1 was known to promote adipocyte differentiation and is involved in the insulin-mediated regulation of fatty acid synthase and leptin gene expression.[11] We investigated whether APM-1 is participating in the differentiation of adipocytes. Firstly, to determine the function of APM-1 in adipocyte differentiation, we examined mRNA expression of APM-1 during adipocyte differentiation.

3T3-L1 preadipocytes are a well-characterized in vitro model of adipocyte differentiation that can be differentiated into mature adipocytes upon exposure to the hormonal stimuli of insulin, IBMX, and Dexamethasone. During differentiation, expression of APM-1 mRNA was slightly increased beginning 16 hrs after initiation of differentiation, following the immediate induction of C/EBPβ and C/EBPα. APM-1 transcription gradually increased and reached maximum level at the day two of differentiation. Thereafter, the expression of APM-1 gradually decreased, but APM-1 mRNA still remained detectable during the rest of the differentiation process. These experiments suggest that expression of APM-1 is regulated during differentiation process (Fig. 6A).

To investigate whether APM-1 is one of the regulators of adipocyte differentiation, 3T3-L1 cells stably overexpressing mouse APM-1 was prepared by infection with recombinant Lentiviral overexpressing APM-1. APM-1 overexpression was confirmed in Lenti-APM-1 cells by RT-PCR (Fig. 6B).

We induced the 3T3-L1 control cells and the APM-1 overexpressing cells to differentiate by the hormonal inducer mixture of IBMX, DEX and insulin. Oil red O staining of the differentiated cells revealed that APM-1-overexpressing cells exhibited more intense staining at 6 days and 8 days of differentiation (Fig. 6C). APM-1 may promote either cell proliferation or accelerate adipocyte differentiation and more lipid accumulation.

We investigated expression pattern of molecular markers and key regulator of adipocyte differentiation at mRNA level in the control cells and stable 3T3-L1 APM-1 cells. The cells were induced to differentiate by hormonal mixture and total RNA was isolated 0 day, 2 days, 4 days, 6 days and 8 days after induction. RT-PCR analysis of the adipocyte markers such as C/EBPα, PPARγ, and FASN revealed that the markers were significantly increased in 3T3-L1 APM-1 stable cells. However, the other adipocyte markers, a-FABP was not affected by APM-1, compared with the control cells (Fig. 6D). APM-1 in 3T3-L1 cells appeared to activate adipocyte differentiation and lipid accumulation.


A
0
4h
16h
24h
36h
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APM-1
GAPDH
B
3T3-L1
3T3-L1 APM1
APM-1
GAPDH
C
Lenti Virus
Control
APM-1
Day 4
Day 6
Day 8
D
MDI treat
0
2
4
6
8
Lenti-control
Lenti-APM-1
APM-1
FAS
C/EBP- α
C/EBP- β
PPAR-γ
a-FABP
GAPDH

Figure 6. Expression pattern of APM-1 during 3T3-L1 preadipocytes and effects of APM-1 on differentiation and APM-1 promotes differentiation of 3T3-L1 preadipocyte. (A) Expression of APM-1 mRNA during differentiation of 3T3-L1 cells was analyzed by RT-PCR. 3T3-L1 cells were harvested at the indicated times (0 hr, 4 hrs, 16 hrs, 24 hrs, 36 hrs, 2 days, 3 days, 4 days, 5 days, 8 days). (B) RT-PCR analysis of APM-1 mRNA level in the 3T3-L1 control and stable 3T3-L1-APM-1 cells established by infected with recombinant lentivirus. (C) Control cells and stable 3T3-L1 APM-1 preadipocytes induced to differentiate and stained with Oil-red O at 4 days, 6 days and 8 days after induction of differentiation. (D) Expression pattern of molecular markers and key regulators of adipocyte differentiation at mRNA level. Control cells and stable 3T3-L1 APM-1 preadipocytes were induced to differentiate and total RNA was isolated 0 day, 2 days, 4 days, 6 days and 8 days after induction.

IV. **DISCUSSION**

FBI-1 (also called Pokemon, ZBTB7a), a member of the BTB/POZ zinc finger protein family, was suggested to play a role in the differentiation of adipocyte.[34-35] APM-1 is the BTB/POZ protein which is the most closely related to the FBI-1 in terms of protein structure and functions. More recently, we showed that APM-1 is a proto-oncogene that can promote cell proliferation (unpublished data).

We initially suspected that APM-1, recently characterized proto-oncogene, might participate in lipid metabolism and regulate some of the lipogenic genes because APM-1 promotes cell proliferation by repressing p21 gene expression and lipid synthesis is important in the cancer cell proliferation. Because FASN is critically involved in the lipid metabolism in cancer cells, we investigated whether APM-1 regulated FASN expression and also the molecular mechanism behind the regulation of FASN gene expression by APM-1. First, we prepared stable NIH/3T3 cells and 3T3-L1 cells, both overexpressing APM-1. We found that the mRNA of SREBP-1 and FASN was increased in stable NIH/3T3-APM-1 cells (Fig. 1B). By preparing 3T3-L1-APM-1 stable cells, we found that APM-1 could promote adipocyte differentiation. The promotion of adipocyte differentiation by APM-1 may be associated with

increased expression of PPARγ, FASN, and C/EBPα at day 4 of adipocyte differentiation after the cells were treated with mixture of IBMX, Dexamethasone and insulin (Fig. 6C,D). The present study establishes that APM-1 increases FASN gene expression during adipogenesis. Previous studies suggest that Krüppel-like factor (KLF) family members had potential roles in adipogenesis. KLF2 binds directly to the PPARγ$_2$ promoter and inhibits its transcription. KLF15 was shown to be induced at late phase of 3T3-L1 differentiation and positively regulated GLUT4 gene expression. Finally, KLF6 is one of the genes induced by adipogenic hormonal stimulation in 3T3-L1. The functions of these KLFs in adipocyte differentiation remain unknown. Although APM-1 has both Krüppel-like zinc-finger and BTB/POZ domain, it seems to be having similar transcription regulatory functions to other KLFs.

Because the transcription of FASN is increased by APM-1 and FASN gene expression principally controlled by SREBP-1, we suspected that APM-1 might regulate the FASN gene expression by affecting the transcriptional molecular events involving SREBP-1 and/or other transcription factors at the FASN promoter. Transcriptional regulation of FASN gene depends largely on the protein-protein interactions of the transcription factors that bind to the proximal promoter elements of FASN gene. The transcriptional regulation of

the proximal promoter is complex and involves complex protein-protein interactions among Sp1, SREBP-1, SRE, GC-box, and SRE/E-box.

The SREBP-1 which belongs to the basic helix-loop-helix leucine zipper transcription factor family, plays a major role in lipogenic genes expression including FASN. We prepared the artificial SREBP-1 responsive promoter fused with luciferase reporter gene, which has 6 copies of SRE of LDLR (5'-CATCACCCCAC-3') at the proximal promoter. APM-1 superactivated transcription of the test promoter by SREBP-1 by 2-3 folds. The data clearly showed that proto-oncogene APM-1 could superactivate transcription by the SREBP-1.

We also tested whether FASN gene expression can be up-regulated by APM-1 and SREBP-1 in HEK 293A cells. Using FASN promoters with SRE and GC-box (Fig. 2), we observed that APM-1 acts as transcriptional activator of SREBP-1 on FASN promoter. It appears that both the SRE/E-box at −65 bp and the SRE at −150 bp are important in transcription activation by APM-1 and SREBP-1, but the SRE/E-box at −65 appeared more important in transcription activation. FASN promoter constructs with much longer upstream regulatory sequence and also with mutation at the SRE/E-box at −65 bp could not be activated by either APM-1 or SREBP-1 (Fig. 3B).

We investigate the molecular mechanism of molecular events at the

proximal promoter of FASN gene; we carried out transcription factor binding by EMSA. The EMSA showed that the APM-1 decreased SREBP-1 binding to the SRE at -150 bp and the SRE/E-box at -65 bp. We found that the bHLH of SREBP-1 and zinc-finger of APM-1 interact with each other (Fig. 4). SREBP-1 binding to the two SRE elements was decreased to the control level, by in vitro translated APM-1 (Fig. 5A). The result is quite contradictory to our expectation because, while APM-1 increased the transcription of FASN and pGL2-6x(SRE)-Luc, it decreased SREBP-1 binding. We came up with the idea that there is a reasonable possibility that activity or binding of other transcription factor(s) that interacts with the proximal promoter elements (SRE, GC-box, SRE/E-box) of FASN gene may be affected by the presence of APM-1.

In the neighboring (usually 15 bases around the SRE) sequence of SREBP-1 target gene promoters, NF-Y site or Sp1 site is usually found. NF-Y and Sp1 directly bind to SREBP and seem involved in recruitment of basic transcription machinery.[39] In the proximal promoter of the FASN gene, there are binding sites for Sp1, Sp3 (GC-box, bp -91 to -83), nuclear factor Y (NF-Y) (bp -71 to -52), upstream stimulatory factor (USF) (SRE/E-box bp -65 to -45) and sterol regulatory element binding protein (SREBP-1) (SRE; SRE/E-box) in the proximal promoter. Previous reports have identified that

transcriptional activation of FASN gene by SREBP-1 depends on additional transcription factor such as Sp1, NF-Y, cAMP response element-binding protein or CCAAT/enhancer-binding protein α.

In particular, the Sp1 binding GC-box and the SRE/E-box are located very closely and Sp1 bound onto the GC-box was shown to be critical in the transcription of FASN gene by collaborating with the SREBP-1 bound just downstream as reported for the LDL receptor promoter and FASN promoter.[15,42] Accordingly, we investigated the transcription factor binding dynamics among Sp1, SREBP-1, and APM-1 on the proximal promoter of FASN gene by ChIP assays. Interestingly, ectopic SREBP-1 decreased Sp1 binding although ecopic SREBP-1 increased transcription. In contrast, ectopic APM-1 increased Sp1 binding but decreased SREBP-1 binding. When expression of SREBP-1 and APM-1 is high, Sp1 and SREBP-1 binding maintain certain ratio with more Sp1 binding compared to SREBP-1. This mode and binding intensity of transcription factors such as Sp1 and SREBP-1 resulted in 2-3 folds higher FASN gene transcription than the condition where only SREBP-1 is transfected. In these molecular event, the proximal regions SRE/E-box of FASN promoter seems to be the center of transcription activation because FASN Mt (-2.7 kb) construct resulted in no transcription activation by SREBP-1 and APM-1 (Fig 5C).

In well-nourished individuals the role of FASN is of minor importance owing to sufficient levels of dietary fat, which leads to the under-use of endogenous free fatty acids.[36-38] Most normal cells and tissues, even those with high cellular turnover, seem to preferentially use circulating lipids for the synthesis of new structural lipids. There is a newly interest in the ultimate role of fatty acid synthase, a key lipogenic enzyme catalysing the terminal steps in the de novo biogenesis of fatty acids in cancer pathogenesis. Tumour-associated FASN, by conferring growth and survival advantages rather than functioning as an anabolic energy-storage pathway, appears to necessarily accompany the natural history of most human cancers.[41]

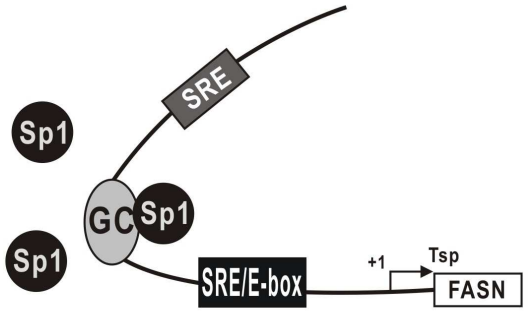
Because the expression of FASN gene is often increasing in cancer cells and increase expression of FASN is important in cancer cell proliferation. Interestingly, our recent investigation revealed that APM-1 is proto-oncogene. Furthermore, FBI-1, a cousin of APM-1, was shown to be a proto-oncogene and was suggested to be involved in adipogenic gene expression.

Although the expression of the FASN gene is increased in cancer, molecular mechanism is not clearly understood. Our data suggested that FASN expression can be increased by APM-1 in cancer. APM-1 is a potent proto-oncogene that represses of the Arf-Mdm2-p53-p21 pathway. The transcription repression of p21 gene by APM-1, involves direct molecular

interaction between p53 bound onto the distal promoter and APM-1 (unpublished).

In conclusion, our study revealed novel roles for proto-oncogene APM-1 in the transcription regulation of FASN gene expression in the cancer cells. We have clearly shown that APM-1 interacts with SREBP-1 which requires the bHLH DNA binding domains of SREBP-1 and the ZF domain of APM-1. APM-1 and SREBP-1 synergistically activate transcription of FASN gene by promoting GC-box binding of Sp1 in HEK 293A cells. The molecular mechanism of transcription superactivation discovered in this thesis is intriguing and gave critical information how proto-oncogene APM-1 is utilizing the cellular regulatory system of FASN promoter to provide cellular membrane components needed for rapid cancer cell growth and proliferation.

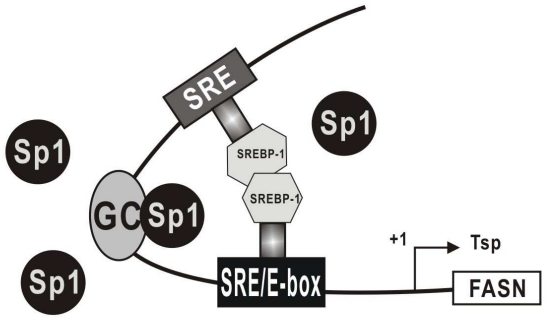
A



No or low SREBP

Sp1 binding
Basal FASN transcription

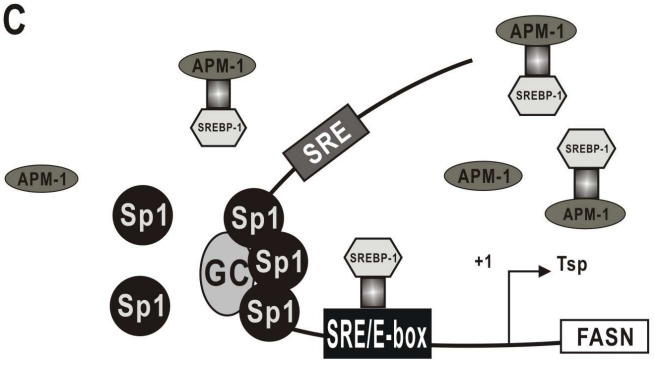
B



SREBP-1 induced

Low Sp1 binding
moderately activate
FASN transcription

C



SREBP-1
induced
&
High APM-1

High Sp1 binding
High FASN transcription

Cancer cells

Figure 7. Hypothetical mechanism of transcriptional regulation on FASN promoter by SREBP-1, Sp1 and APM-1. (A) No or low SREBP: Some Sp1 binds GC-boxes on FASN promoter and slightly appears basal level transcription. (B) SREBP-1 induced: A lot of SREBP-1 bind SRE or SRE/E-box on FASN promoter and quite activate transcription. (C) SREBP-1 induction and high expression of APM-1 in cancer cells: Interaction between SREBP-1 and APM-1 cause less chance to bind to SRE or SRE/E-box on FASN promoter. Accordingly, Sp1 has a more chance to access on the GC-box of FASN promoter. As a result, increased binding of Sp1 strongly activates transcription of FASN gene.

Ⅴ. Conclusion

1. NIH/3T3 stable APM-1 overexpression cells increased SREBP-1 and FASN mRNA expression.
2. The APM-1 and APM-1 ZF increase transcription activation of artificial 6x (SRE)-tk*-Luc promoter by SREBP-1c.
3. The APM-1 enhances transcription activation of endogenous FASN gene by SREBP-1a in 293A cells.
4. APM-1 directly interacts with the bHLH DNA binding domain of SREBP-1 via its zinc fingers.
5. Although the DNA binding activity of bHLH of SREBP-1 is decreased in the presence of APM-1, the DNA binding of Sp1 strongly is increased on FASN promoter.
6. APM-1 expression is regulated during differentiation of 3T3-L1 preadipocytes and stable APM-1 overexpression facilities adipogenesis.
7. Proto-oncogene APM-1 might be utilizing the cellular regulatory system of FASN promoter to provide cellular membrane component needed for rapid cancer growth.

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국문 요약

SREBP-1 과 상호작용을 통해 SREBP responsive 유전자들의 전사를 조절하는 APM-1

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김연숙

SREBP-1 은 basic helix-loop-helix (bHLH) family 의 전사인자로 특히 지방대사와 당대사에 관여한다. SREBP 전구체는 processing 과정을 거쳐 핵 내로 이동하고 SREBP responsive element (SRE) 또는 E-box 를 인식하여, SREBP responsive genes 의 전사를 조절한다. 이런 SREBP 가 활성화된 형태로 핵 내로 들어와서 무작위적으로 target genes 의 전사를 조절하는 것이 아니라, 다른 단백질들과 상호작용하여 조절하는 것으로 여겨진다. APM-1 은 FBI-1 과 매우 유사한 BTB/POZ domain 과 Krüppel like zinc finger DNA binding domain 으로 구성된 전사조절 기능을 가지고 있는 단백질이다. 이전 발표된 논문에 의하면 FBI-1 이

adipogenesis 에 관여하여 adipocyte differentiation 을 조절하는 단백질로 알려져 있어, APM-1 또한 이와 유사한 역할을 할 것이라고 생각된다. 본 연구를 통해, 지질대사에 중요한 조절 factor 인 SREBP-1 과 APM-1 과의 상호작용을 GST 융합 단백질 pulldown assay, co-immunoprecipitation 을 통해 직·간접적으로 결합한다는 사실을 보였다. 이러한 단백질-단백질 상호작용으로 SREBP-1 의 표적유전자인 fatty acid synthase 와 같은 지방 대사에 관여하는 핵심 효소들의 전사에 영향을 조사하였다. FASN promoter 를 이용한 transient transfection assay 를 통하여 APM-1 이 SREBP-1 에 의한 전사를 활성화할 수 있다는 사실을 발견하였다. ChIP assay 결과를 통해, APM-1 이 과발현 되었을 때 FASN promoter 에 존재하는 Sp1 결합 부위인 GC-box 에 Sp1 이 결합을 증가시켜 전사를 촉진함을 발견하였다. 또한 APM-1 이 과발현한 3T3-L1 지방세포의 분화과정 동안, FASN, PPARγ의 mRNA 이 증가되고, 지방 축적이 많아졌음을 Oil-red O 염색을 통해 관찰 하였다. 이러한 결과들을 통해 BTB/POZ domain 을 가진 발암유전자인 APM-1 이 SREBP-1 와의 상호작용에 의해 FASN 와 같은 SREBP responsive genes 전사를 조절하여, 암세포에서의 지방 합성에 중요한 역할을 할 것이라고 생각한다.

핵심되는 말: 전사조절, 지방세포 분화, APM-1, SREBP-1, Sp1, FASN promoter