Transcriptional Regulation of Adiponectin Gene Expression

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Transcriptional Regulation of Adiponectin Gene Expression

Directed by Professor Hyun Chul Lee

The Doctoral dissertation submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Table of contents

LIST OF FIGURES

Abstract

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Adiponectin is a hormone exclusively expressed in adipose tissue that enhances insulin sensitivity, regulates energy homeostasis the metabolism of carbohydrates and lipids. Plasma adiponectin concentration and mRNA expression are decreased in obese people, despite their increased adiposity, and in patients with type 2 diabetes with insulin resistance. To understand the mechanisms involved in regulating adiponectin gene expression, we isolated the human adiponectin promoter and analyzed the activities of promoters using 5' serial deletions. This led us to identify the negative cis-regulatory elements located within -174 to -152 bp in the human

adiponectin promoter in non-adipocytes. This site contained well known helix-loop-helix (HLH) or Myb factor binding sequences. Analysis of the Adiponectin promoter point mutants revealed that a HLH factor is a more plausible negative regulatory factor than a myb binding factor. To check if the human adiponectin promoter regulatory activity is different in adipocytes, we examined the expression of a reporter gene regulated by our deletion mutants after their transient transfection in both 3T3L1 pre-adipocytes and fully differentiated 3T3L1 adipocytes. Transcriptional mechanism of the human adiponectin promoter was different between adipocytes and non-adipocytes. Electrophoretic mobility gel shift assay (EMSA) revealed the DNA binding activity of the putative negative regulatory factor is sequence-specific, and is decreased during adipocyte differentiation in a time dependent manner.

Key word : Adiponectin, Promoter, Negative regulatory element, HLH factor, Adipocytes

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I. INTRODUCTION

Adipose tissue has been regarded as a storage site for extra energy in the form of triglyceride. But now, it is considered as an important endocrine organ that plays a key role in the integration of endocrine metabolic and inflammatory signals for the control of energy homeostasis by secreting several hormones known as adipocytokines [1-2]. These adipocytokines include leptin, tumor necrosis factor-α (TNF-α), plasminogen-activator inhibitor type I (PAI-1), adipsin, resistin, and adiponectin [3-8].

Adiponectin (also known as Acrp30, AdipoQ, apM1, and GBP28) was discovered by Lodish et.al. from 3T3L1 adipocytes [9]. It is exclusively expressed in white

adipose tissue. The molecule has a 30 kDa molecular mass (247 amino acids) consisting of four domains, an amino-terminal signal sequence, a variable region, a collagenous domain (cAd), and a carboxy-terminal globular domain (gAd) (Fig 1) [10].

Adiponectin is abundantly present in human plasma (5 − 30 μg/ml) [11]. Plasma adiponectin concentrations and mRNA expression are decreased in obese people, despite increased adiposity, and in patients with type 2 diabetes with insulin resistance [11-14]. Adiponectin increases insulin sensitivity by the activation of insulin signaling and enhancing glucose uptake [15]. And administration of full-length adiponectin lowers plasma glucose levels by suppressing hepatic glucose production in obese and diabetic mice [16]. Adiponectin is also implicated in protection from atherosclerosis in light of its expression is reduced in patients with coronary artery disease [17-18]. Yamauchi et al. have cloned two mouse adiponectin receptors, termed adiponectin receptors 1 and 2, both of which have seven transmembrane domains, which are abundantly expressed in skeletal muscle and liver, respectively [19].

The human and mouse adiponectin promoters are TATA-less but contain a classical CCAAT box and a 1.3-kb fragment located upstream of the transcriptional start site of the human adiponectin gene, or the 1.13 kb counterpart of mouse [20-21].

Many factors have been reported to be positive or negative effectors of adiponectin expression. For instance, PPAR-ɣ, LRH-1, C/EBP and ADD1/SREBP1c increase adiponectin gene expression in adipocytes. Moreover, tumor necrosis factor ɑ (TNFɑ), NFATc4 and ATF3 decrease adiponectin gene expression [22-27].

To further understand the mechanisms involved in regulating adiponectin gene expression we isolated ~1.27 bp of the human adiponectin promoter and analyzed the activities of promoters with 5' serial deletions. In this study, we identified an important suppression site of the human adiponectin promoter in non-adipocytes. Transcriptional mechanism of the human adiponectin promoter differed between adipocytes and non-adipocytes. The binding activity of this putative negative regulatory factor to the human adiponectin promoter was decreased during adipocyte differentiation in time dependent manner.

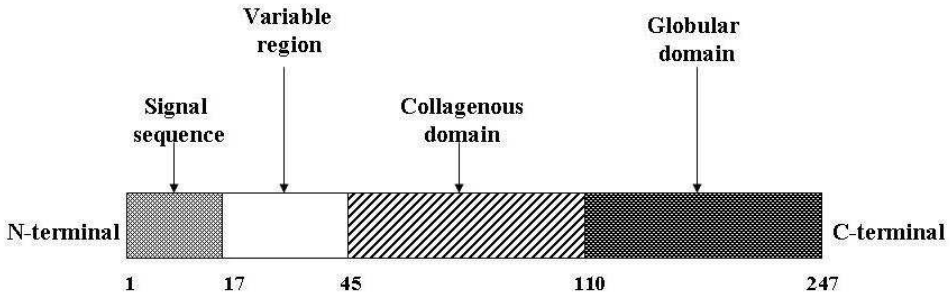

Fig 1. Structure and domains of adiponectin. The molecule has a 30kDa molecular mass (247 amino acid) consisting of four domains an amino-terminal signal sequence, a variable region, a collagenous domain (cAd), and a carboxy-terminal globular domain (gAd).

II. MATERIALS AND METHODS

1. Cell Culture

NIH3T3, Huh7 and HEK293 cells were maintained at 37℃, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Jeil Biotech. Inc., Daegu, South Korea) supplemented with 10% fetal bovine serum (GIBCO-BRL, Carlsbad, CA), penicillin (200 IU/ml), and streptomycin (100 lU/ml).

2. 3T3L1 differentiation

3T3L1 preadipocytes (ATCC, Manassas, VA, USA) were maintained at 37℃, 5% CO_2 in DMEM supplemented with 10% FBS, penicillin (200 IU/ml), and streptomycin (100 lU/ml). For inducing differentiation, 2-day postconfluent cells (designated day 0) were fed DMEM supplemented 10% FBS, 0.5 mM 3-isobutyl-1-methyl-xanthine, 2 μg/ml dexamethasone, and 1 μg/ml insulin for 2 days. Every 2 days thereafter, the cells were incubated with fresh DMEM supplemented with 10% FBS and 1 μg/ml insulin.

3. Cloning of the Human Adiponectin promoter and Construction of a Luciferase Repoter

Human genomic DNA was isolated from HEK293 cells using a PCR. The human adiponectin promoter spanning -1273 to +3 bp was amplified by polymerase chain reaction (PCR). The primers used were as follows: forward, 5'-GAA GAT CTG GAC ATT AGC AGG AAG CTG AG-3', reverse, 5'-CCC AAG CTT CAG AAC CAC AGA CTG CAG T-3'. The primers included BglII (forward primer) and HindIII (reverse primer) restriction sites. The PCR products were digested with BglII and HindIII, and subcloned into pGLB-CMV/enhancer, pGL3-basic (Promega, Madison, WI, USA) and pGL3-enhancer (Promega). pGLB-CMV/enhancer was subcloned CMV enhancer from pcDNA3 (Invitrogen, Carlsbad, PA) into the NheI site of pGL3-basic (Promega).

4. Deletion and mutation of human adiponectin promoter

Deletion mutants of the Adiponectin promoter region were generated by PCR and inserted into pGLB-CMV/enhancer, pGL3-basic (Promega) and pGL3-enhancer (Promega). The primers used were as follows;

Forward primer, APF2 : 5’- GAA GAT CTG TTG AAT GTT GCC ACT TCA AG -3’ , APF3 : 5’- GAA GAT CTG GCC ACA AGC TTT AAG AAT TC -3’ , APF4 : 5’- GAA GAT CTG GAG ACC TCC TGC TGG ACC ACT G -3’ , APF5 : 5’- GAA GAT CTG ACT AGG GCT CCA CTT GGC -3’ , APF6 : 5’- GAA GAT CTA GTT GGC CAA TGG GAA ATG AC -3’ , APF51 : 5’- GAA GAT CTA AGC TGG GTT GTA CCA GGT TC -3’ , APF52 : 5’- GAA GAT CTC AGG CTG TGG GCA ACT GCC AG -3’ , APF522 : 5’- GAA GAT CTA GGG GCA TGT GCC TGC CCA C -3’ reverse primer : 5’- CCC AAG CTT CAG AAC CAC AGA CTG CAG T -3’

5. Transient transfection

NIH3T3 , Huh7, and HEK293 cells were grown in 12-well plates and transfected using Tfx-50 (Promega) as follows; briefly, before transfection, the cell medium was aspirated and the remaining mono-layer cells were then exposed to the cocktail solution containing 250μl DMEM, 2μg plasmids, and 4μl Tfx-50 reagent, and 1 hour later, supplemented with 1 ml of the culture DMEM. The transfected cells were grown for 24–48 h. 3T3L1 pre-adipocytes and fully differentiated 3T3L1 adipocytes were cultured in 10 cm tissue culture dishes and transfected by electroporation (0.25

kV and 960μF) with 100μg DNA. Cuvettes were using Electroporation cuvettes plus model No. 640 (BTX Harvard apparatus, Holliston, MA, USA). Following electroporation, the cells were re-plated on tissue culture plates and recovered after 48h.

6. Luciferase activity

For luciferase and β-galactosidase assays, cell extracts were prepared according to the manufacturer's protocol (Promega). Briefly the transfected cells were exposed to lysis buffer for 10 min and the lysed cell extracts were saved after brief centrifugation. The luciferase assay was carried out by luminometer (MicroLumat, LB96P, EG&G BERTHOLD). The internal control β-galactosidase assays were used by UV spectrophotometer (UV-1601 PC, SHIMADZU, Japan) and optical density was determined at 420 nm.

7. Preparation of Nuclear Extracts

To prepare nuclear extracts, 10cm culture dish cell rinsed 2 times using PBS and harvested cell pellets. The pellets were suspended in 1 ml buffer A (10 mM Hepes,

pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol) and incubated on ice for 15 min. After incubation, added 15 μl 10% NP-40 and centrifugation at 12,000g for 5 min. The supernatant was removed and the pellet was resuspended in 200 μl buffer C (20 mM Hepes, pH 7.9, 0.4M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and protease inhibitor cocktail (Roche applied science, Indianapolis, USA) by vortexing for 5 s, and then shaking incubated 4℃ for 30 min. After centrifugation at 12,000g for 10 min on 4℃, the supernatant was collected and quantified with the Bradford's reagent (Sigma-Aldrich, St. Louis, USA).

8. Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSAs) were performed according to the methods previously described [28]. In brief, the following oligo nucleotides were annealed and labeled with [γ-32P]ATP (Amersham bioscience, Piscataway, USA); the wild type AP binding site (upper strand: 5'-GGC TGT GGG CAA CTG CCA GGG GCA-3' and lower strand: 5'-TGC CCC TGG CAG TTG CCC ACA GCC-3') , the mutant APM3 binding site (upper strand: 5'-GGC TGT GGG CAC ATG CCA

GGG GCA-3', and lower strand: 5'-TGC CCC TGG CAT GTG CCC ACA GCC-3') , APM5 binding site (upper strand: 5'-GGC TGT GGG CAA CTA CCA GGG GCA-3' and lower strand: 5'-TGC CCC TGG TAG TTG CCC ACA GCC-3') , USF binding site (upper strand: 5'-CCC CCA CCA CGT GGT GCC TGA -3' and lower strand: 5'-TCA GGC ACC ACG TGG TGG GGG-3') , and control oligo (upper strand: 5'-CCC AAG CTT ATG GTT TCT AAA CTG AGC CAG CTG C-3' and lower strand: 5'-TGC TCT AGA TTA CTG GGA GGA AGA GGC CAT CTG G-3').

The binding reaction was pre-incubated for 10 min on ice in a final volume of 20 μl containing binding buffer (25mM Hepes(pH 7.9), 100mM KCl, 5mM DTT, and 10% glycerol), 2 μl poly (dI–dC) , and nuclear extract. And then, the mixture add labeled probe (>50,000 cpm), and incubation for 30 min on ice. Incubated samples were loaded on a 6% nondenaturing polyacrylamide gel (30:0.8 acrylamide/ bisacrylamide) and running using 1x TBE at 200 V at 4℃. The dried gel was exposed to film on an intensifying screen overnight at -70℃. For competition assays, unlabeled oligonucleotides were added in the pre-incubation mixture in 1–100-fold molar excess. For supershift assays, antibody was included in the preincubation mixture.

9. Cell staining

For β-galactosidase activity of cultured cells with X-gal, transfected cells were washed with PBS, fixed with 4% paraformaldehyde (10 min, room temperature), and washed twice with PBS. Subsequently the cells were incubated in the staining solution containing 1 mg/ml X-gal, 2mM $MgCl_2$, 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN_6)–3H_2O$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 for 3–5 h at 37℃. The activity of β-galactosidase was visualized as the blue color of the 3,50-dichromo-4,40-dichloroindigo molecule resulting from cleaving the X-gal substrate by the β-galactosidase in the cells. The progress of differentiation was followed microscopically using oil red O staining. Briefly, cells were washed with PBS, fixed with 4% paraformaldehyde (1 h, room temperature), and washed twice with PBS. And stained with 0.2% Oil Red O in 60% isopropanol for 5 min. After staining, cells were photographed with a Nikon 300 microscope and Nikon F-601 camera.

10. RT-PCR analysis

For investigating the expression of adiponectin mRNA in 3T3L1 pre-adipocytes and 3T3L1 adipocytes, reverse transcription (RT)- PCR was performed. Total RNA

was taken from cultured 3T3L1 pre-adipocytes and adipocytes using Trizol reagent (Invitrogen) according to the manufacturer's recommended protocol. 2 μg total RNA was employed for the synthesis of single-stranded cDNA using Superscript II Reverse transcriptase (Invitrogen). For amplification of mouse adiponectin, primers were used ; Adipo/F : 5'- AAG GAC AAG GCC GTT CTC T -3' , Adipo/R : 5'- TAT GGG TAG TTG CAG TCA GTT GG – 3' . The PCR condition was 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s for 30 cycles. As an internal standard, cDNA for the housekeeping gene β–actin was amplified using specific primers. PCR products were separated by electrophoresis on 1.5% agarose gel and visualized by ethidium bromide staining with UV light using Gel Doc 1000 (Bio-Rad Laboratory, Hercules, CA, USA).

11. Western blot analysis

For western blot analysis, 3T3L1 pre-adipocytes and adipocytes were rinsed with ice-cold PBS and scraped on ice into lysis buffer that contained 50 mM Tris-HCl (pH 8), 120 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.5% NP-40, protease inhibitor cocktail (Roche applied science). The cell lysates were then shaking for 30 min in

4℃. Cell debris were removed by centrifugation (12,000 × g for 15 min in 4℃), the supernatant was collected and quantified with the Bradford's reagent (Sigma-Aldrich). Protein sample boiled for 5 min in Laemmli sample buffer, fractionated by SDS−polyacrylamide gel in a 12% polyacrylamide gel and electrotransferred onto nitrocellulose membrane (BIO-RAD, Hercules, CA, USA). Membranes were incubated 2h at 4°C in a blocking solution of 5% non-fat powdered milk in T−phosphate-buffered saline (phosphate-buffered saline, pH 7.5, containing 0.1% Tween 20). Adiponectin antibody (Affinity bioreagents, Golden, CO, USA) and β-actin antibody (Sigma-aldrich) were used as primary antibodies, diluted 1:1000. Horseradish peroxidase-conjugated goat anti-mouse IgG (Santa-Cruz biotechnology, CA, USA) was used as secondary antibody (at dilution of 1:5000). Development of the western blot was performed using an enhanced chemioluminiscence western blotting analysis system (Amersham).

III. RESULTS

1. Expression of Adiponectin during adipocyte differentiation.

To investigate the regulation of adiponectin gene expression, we examined the adiponectin mRNA and protein expression level during 3T3L1 differentiation. Total RNA was extracted from 3T3L1 pre-adipocytes after 8 days differentiation of the cells. Adiponectin expression was not detected in pre-adipocytes, but abundantly increased during differentiation (Fig. 2B). Protein expression was also not detected in pre-adipocytes and increased only after differentiation (Fig. 2A). Differentiation into adipocyte was necessary to induce increasing expression of adiponectin mRNA and protein. It means that, adiponectin expression is regulated by positive regulatory mechanism in adipocytes, or negative regulatory mechanism in pre-adipocytes, or both.

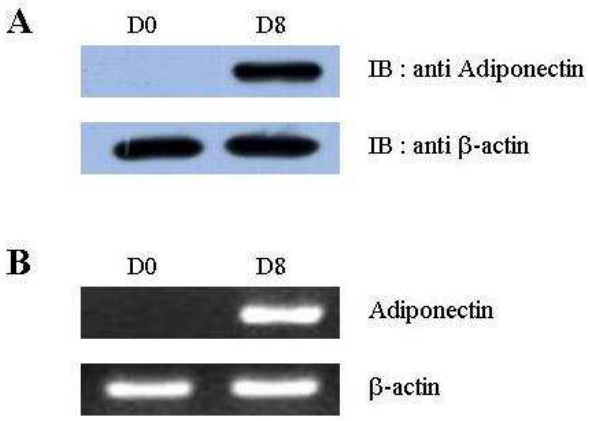

Fig 2. Protein and mRNA expression of adiponectin during adipocyte differentiation. Expression of adiponectin protein (A) and mRNA (B) in the 3T3L1 pre-adipocytes and fully differentiated 3T3L1 were examined by western blot and RT-PCR, respectively.

2. Functional analysis of the human adiponectin promoter.

To understand mechanisms involved in regulating adiponectin gene expression, we cloned ~1.27 kb of the human adiponectin promoter from genomic DNA by PCR (Fig 3). We prepared a series of human adiponectin promoter deletion constructs in the presence of the CMV enhancer and transfected them into NIH3T3, Huh7, and 293 cells. Fig 4A, a shown diagrammatic representation of different deletion constructs used. As shown in Fig 4B, in NIH3T3 cells the pGLB-CMV/AP-F1 (-1273/+3) construct had very low reporter activity (as measured by luciferase assay), and the other deletion mutants ; pGLB-CMV/AP-F2 (-1139/+3), pGLB-CMV/AP-F3 (-709/+3), pGLB-CMV/AP-F4 (-403/+3), and pGLB-CMV/AP-F5 (-234/+3) resulted similar low reporter activity. Whereas, pGLB-CMV/AP-F6 (-116/+3) construct had the highest reporter activity. Reporter activity of pGLB-CMV/AP-F6 (-116/+3) construct was increased 8 folds as compared to the pGLB-CMV/AP-F5 (-234/+3). Similar patterns of reporter activity were observed Huh7 and HEK293 cells (Fig 4C and D). These results suggest that the strongly negative cis-regulatory elements located within pGLB-CMV/AP-F5 (-234/+3) to pGLB-CMV/AP-F6 (-116/+3).

Fig 3. Sequence of the 5′-flanking region of the human adiponectin promoter. The sequence of the ~1.27bp human adiponectin promoter (-1273 to +3) is shown and numbered from the transcription initiation site.

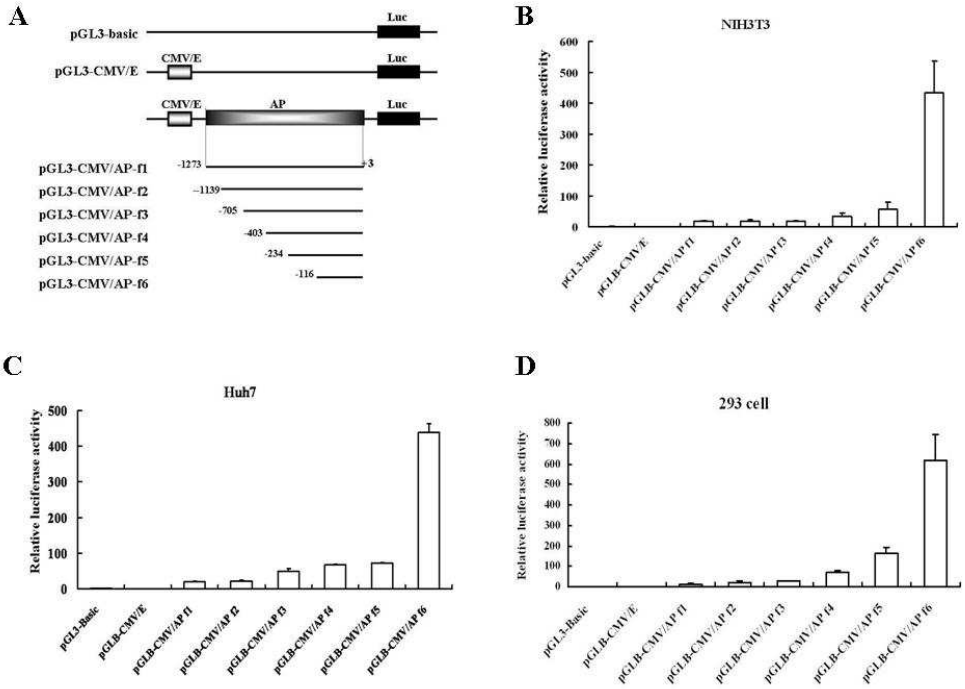

Fig 4. Identification of the negative regulatory promoter region of the adiponectin gene in NIH3T3, Huh7 and HEK293 cell. (a) schematic representation of a series of deletion constructs of human adiponectin promoter. (b), (c) and (d) Reporter assay showing Human adiponectin promoter activity. pGL3-basic vector and pGLB-CMV/E constructs used as control. Each plasmids were transiently transfected into (B) NIH3T3, (C) Huh7 and (D) HEK293 cells. The luciferase activity was normalized with β-galactosidase activity. The data are presented as the mean (±S.D.) of three independent experiments.

3. Location of repressor element between -174 to -152 bp of the human adiponectin promoter.

As shown in Fig 4, the strong negative cis-regulatory elements located between pGLB-CMV/AP-F5 (-234/+3) to pGLB-CMV/AP-F6 (-116/+3). To define the regulatory DNA region involved in negative regulating adiponectin gene expression, more deleted between pGLB-CMV/AP-F5 (-234 bp) to pGLB-CMV/AP-F6 (-116 bp). Fig 5A, shows a diagrammatic representation of different deletion constructs used ; pGLB-CMV/AP-F51 (-203/+3), pGLB-CMV/AP-F52 (-174/+3), pGLB-CMV/AP-F522 (-151/+3). As shown in Fig 5B, in NIH3T3 cells the pGLB-CMV/AP- F522 (-151/+3) construct had highest reporter activity. These results suggest that the negative cis-regulatory elements located within pGLB-CMV/AP-F52 (-174/+3) to pGLB-CMV/AP-F522 (-151/+3).

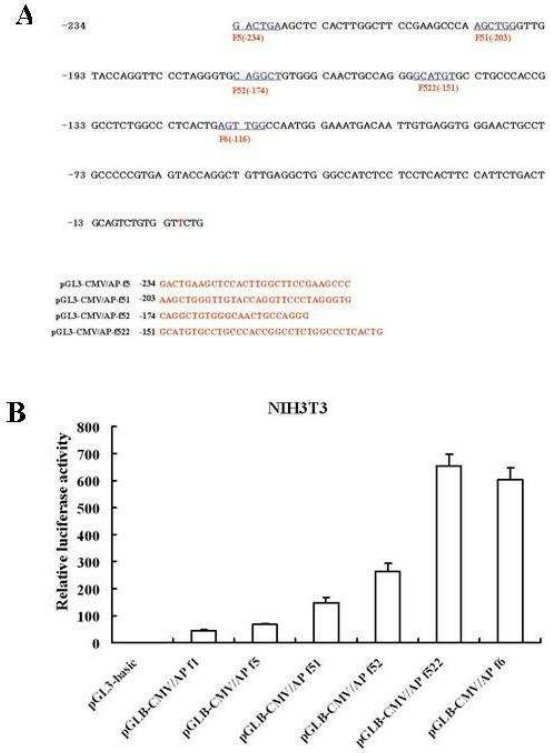

Fig 5. Location of repressor element between -174 to -152 bp of the human adiponectin promoter. (A) schematic representation of more deletion constructs between -174 bp to -152 bp of human adiponectin promoter. (B) Reporter assay showing Human adiponectin promoter deletion mutant activity. pGL3-basic vector used as control. Each plasmids were transiently transfected into NIH3T3 cells. The luciferase activity was normalized with β-galactosidase activity. The data are presented as the mean (±S.D.) of three independent experiments.

This site (-174 to -152 bp) is well conserved between human and mouse adiponectin promoter (Fig 6). Especially, CAACTG sequence (-163/-158) is nearly completely conserved. This sequence is well known E-box or Myb factor binding sites (Fig 7A). Therefore, we investigated the effects of mutations in these response elements on promoter activity. NIH3T3 were transfected with mutant constructs that pGLB-CMV/AP-F52(M1) (CAACTG → aAACTG) , pGLB-CMV/AP-F52(M2) (CAACTG → CgACTG) , pGLB-CMV/AP-F52(M3) (CAACTG → CAcaTG) , pGLB-CMV/AP-F52(M4) (CAACTG → CAACgG) , and pGLB-CMV/AP-F52(M5) (CAACTG → CAACTa). As shown in Fig 7B, the promoter activities in cells transfected with pGLB-CMV/AP-F52(M3) construct was a very little change . But, reporter activities of the other mutant plasmids were increased. These results suggest that the pGLB-CMV/AP-F52(M3) is less important than any other mutants in human adiponectin promoter activity. It means that, HLH factor is more possible negative regulatory factor than myb binding factor in human adiponectin promoter.

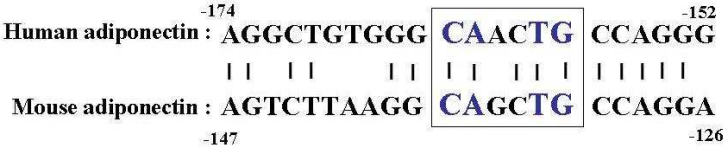

Fig 6. Sequence comparison putative negative regulatory element binding region between human and mouse adiponectin promoter. The sequence are well conserved.

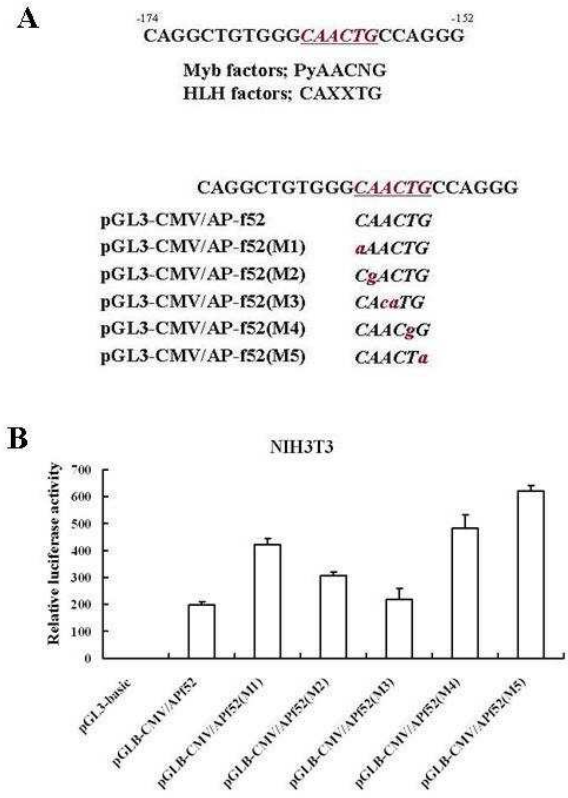

Fig 7. Mutagenesis of putative response element sequence in human adiponectin promoter. (a) Schematic representation of point mutation constructs between -163 bp to -158 bp of human adiponectin promoter. (B) Reporter assay showing human adiponectin promoter point mutants activities. pGL3-basic vector used as control. Each plasmids were transiently transfected into NIH3T3 cells. The luciferase activity was normalized with β-galactosidase activity. The data are presented as the mean (±S.D.) of three independent experiments.

4. Comparisons of putative response element mutant and wild type the human adiponectin promoter.

As shown in Fig 7, the negative cis-regulatory elements bound CAACTG sequence (-163 to -158 bp) in the human adiponectin promoter. Especially, the mutants, pGLB-CMV/AP-F52(M4) and pGLB-CMV/AP-F52(M5) are important. To define this result, we transfected the mutant construct pGLB-CMV/AP-f1(M) in the NIH3T3 cells. This construct has one point mutation in pGLB-CMV/AP-f1 plasmid, which is -159bp site T to G mutation in human adiponectin promoter (Fig 8A). As shown in Fig 8B, in NIH3T3 cells the pGLB-CMV/AP-F1(M) construct was increased 2.5 folds as compared to the pGLB-CMV/AP-F1. These results suggest that just one point mutation can change the human adiponectin promoter activity and this sequence CAACTG is very important in regulation of adiponectin expression.

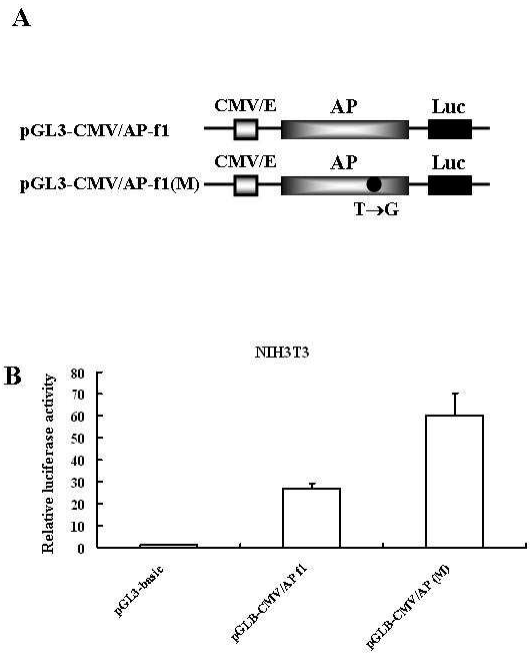

Fig 8. Comparisons of putative response element mutant and wild type the human adiponectin promoter. (A) Schematic representation of one point mutation constructs -159 bp site of human adiponectin promoter. (B) Reporter assay showing human adiponectin promoter one point mutant activity. pGL3-basic vector used as control. Each plasmids were transiently transfected into NIH3T3 cells. The luciferase activity was normalized with β-galactosidase activity. The data are presented as the mean (±S.D.) of three independent experiments.

Our all constructs involved CMV enhancer. Actually, concerned about CMV enhancer additional effect was removed. As shown in Fig 4A lane 2, pGLB-CMV/E construct activity was very low. To rechecking the enhancer effect, we constructed no enhancer plasmids ; pGLB-AP-F52 and pGLB-AP-F522, and SV40 enhancer involved plasmids ; pGLE-AP-F52 and pGLE-AP-F522 (Fig 9A). As shown in Fig 9B, in NIH3T3 cells the similar pattern of reporter activities were observed CMV enhancer involved constructs (Fig 9B). It means that, this negative regulatory effect of human adiponectin promoter is not CMV enhancer activity.

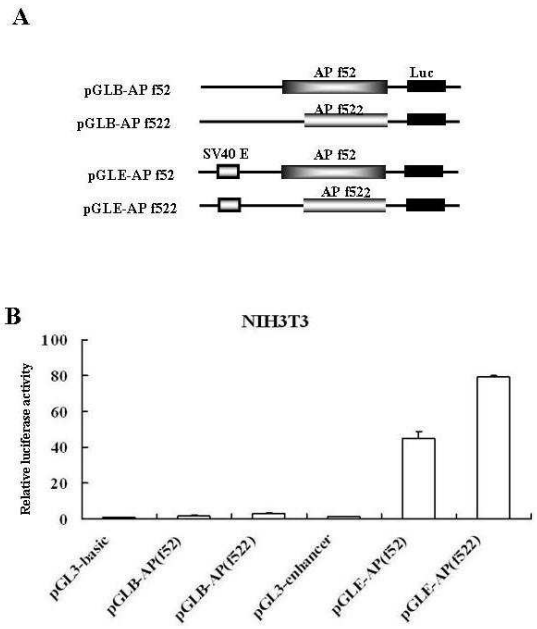

Fig 9. Reporter activity of the human adiponectin promoter involved different enhancer. (A) Schematic representation of no enhancer involved human adiponectin promoter and SV40 enhancer involved human adiponectin promoter. (B) Reporter assay showing human adiponectin promoter involved no enhancer of SV40 enhancer. pGL3-basic vector and pGL3-enhancer vector used as control. Each plasmids were transiently transfected into NIH3T3 cells. The luciferase activity was normalized with β-galactosidase activity. The data are presented as the mean (±S.D.) of three independent experiments.

5. Expression of the human adiponectin promoter in adipocytes.

To identify the human adiponectin promoter regulatory activity is different in adipocytes, we examined similar studies in 3T3L1 pre-adipocytes and fully differentiated 3T3L1 adipocytes. We performed transient transfection using electroporation method, because transfection efficiency of the 3T3L1 adipocytes is very low. To determine the optimal electroporation condition in 3T3L1 adipocytes, we examined many different voltage condition electroporation with β-galactosidase gene (data not shown) and stained X-gal staining. Fig 10, shows the optimal electroporation condition ; 250V, 960μF , 100μg DNA. As shown in Fig 11, in 3T3L1 pre-adipocytes reporter activity of the human adiponectin promoter deletion mutant was similar in NIH3T3 cells result (Fig 5B). In 3T3L1 pre-adipocytes also the pGLB-CMV/AP- F522 (-151/+3) construct had higher reporter activity than the pGLB-CMV/AP- F52 (-174/+3) construct.

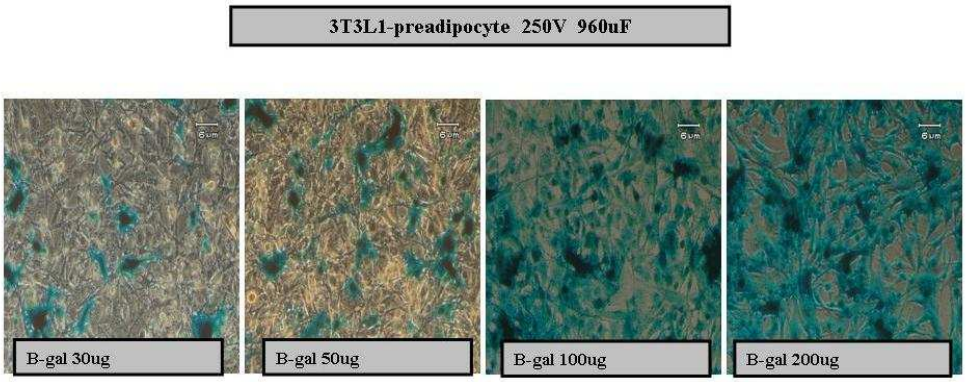

Fig 10. Determine the optimal electroporation condition in 3T3L1 pre-adipocytes. 3T3L1 pre-adipocytes were cultured in 10 cm tissue culture dishes and transfected by electroporation (0.25 kV and 960 μF) with several amounts of DNA. Cuvettes were using Electroporation cuvettes plus model No. 640 (BTX Harvard apparatus , USA). Following electroporation, the cells were re-plated on tissue culture plates and recover for 48h. For checking the efficiency, transfected cells were stained with x-gal staining.

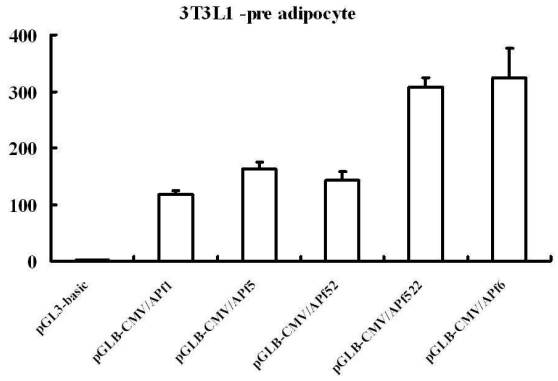

Fig 11. Reporter activity of the human adiponectin promoter in 3T3L1 pre-adipocytes. Reporter assay showing human adiponectin promoter deletion mutants. pGL3-basic vector was used as a control. Each plasmid was transiently transfected using electroporation into 3T3L1 pre-adipocytes. The luciferase activity was normalized with β-galactosidase activity. The data are presented as the mean (±S.D.) of three independent experiments.

3T3L1 pre-adipocytes (ATCC, Manassas, VA) inducing differentiation, 2-day postconfluent cells were fed 2 μg/ml dexamethasone, and 1μg/ml insulin, 0.5 mM 3-isobutyl-1-methyl-xanthine for first 2 days. Every 2 days thereafter, fresh DMEM supplemented with 10% FBS and 1 μg/ml insulin were added to the cells. The state of differentiation was followed microscopically using oil red O staining (Fig 12). We used differentiated adipocytes of day 8 for the next experiment. As shown in Fig 13, in 3T3L1 adipocytes reporter activity of human adiponectin promoter deletion mutant was different in 3T3L1 pre-adipocytes results (Fig 11). In 3T3L1 adipocytes, the pGLB-CMV/AP-F1 construct and pGLB-CMV/AP-F6 construct had similar high reporter activity. These results suggest transcriptional mechanism of human adiponectin promoter was different between adipocytes and non-adipocytes.

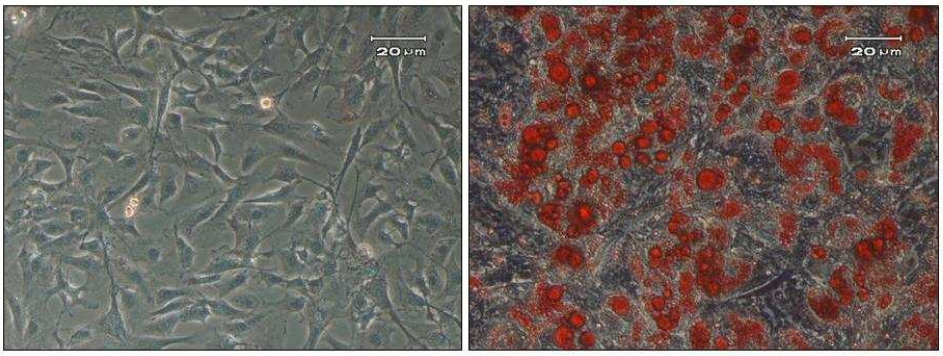

Fig 12. Oil red O staining. 3T3L1 pre-adipocytes underwent differentiation for 8 day with 0.5 mM 3-isobutyl-1-methyl-xanthine, 2 μg/ml dexamethasone, and 1μg/ml insulin. The state of differentiation was followed using oil red O staining.

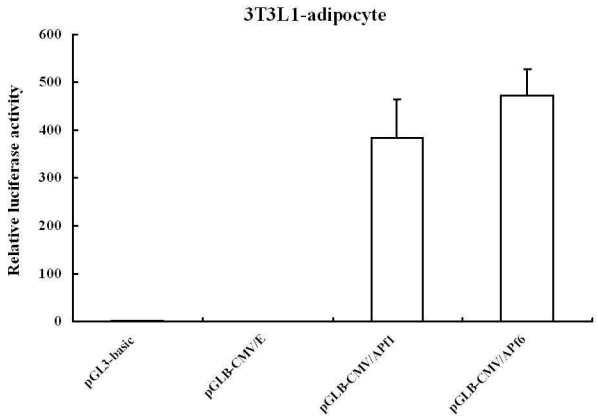

Fig 13. Reporter activity of the human adiponectin promoter in 3T3L1 adipocytes. Reporter assay showing human adiponectin promoter deletion mutant. pGL3-basic vector and pGLB-CMV/E construct used as controls. Each plasmid was transiently transfected using electroporation into 3T3L1 adipocytes. The luciferase activity was normalized with the β-galactosidase activity. Data are presented as the mean (±S.D.) of three independent experiments.

6. Electrophoretic mobility gel shift assay (EMSA) of nuclear extracts prepared from 3T3L1 pre-adipocytes and adipocytes.

The effect of the site specific mutation on negative regulatory factor binding activity was investigated using an adiponectin promoter deletion mutant probe the wild type AP probe , the mutant APM3 probe and APM5 probe , USF binding probe , and, as a control, the LF-B1 probe. Nuclear extracts were prepared from 3T3L1 pre-adipocytes. As shown in Fig 14, the putative negative regulatory factor bound to the wild type AP oligonucleotide probe in 3T3L1 pre-adipocytes (lane 1). As a further test of its sequence-specific binding characteristics, competition assays were performed in the presence of unlabeled wild type AP oligonucleotide as competitors. The binding complex began to fade away in the presence of a 10-fold molar excess of the intact form (lane 3) and was hardly detected in a 100-fold molar excess (lane 4). However, this binding complex didn't completely disappear even in a 100-fold molar excess of the mutated form (lane 5 to lane 10). This result strongly supports the notion that the DNA binding activity of the negative regulatory factor is sequence-specific. LF-B1 was used as a negative control.

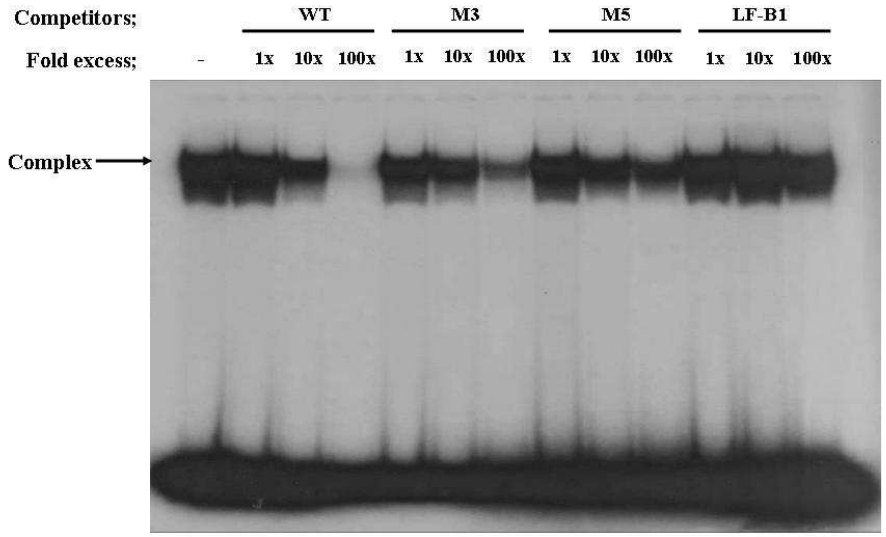

Fig 14. Effect of site-specific mutations on putative negative regulatory factor binding activity. Electrophoretic mobility gel shift assay (EMSA) was performed on the negative regulatory elements binding site (-173 to -149 bp). Wild type (WT) and mutants (M3, M5), negative control (LF-B1) probes were incubated with 3T3L1 pre-adipocytes nuclear extract. For competition assay, pre-incubation was carried out at a 1−100 fold molar excess with unlabeled oligonucleotides.

As shown in Fig 11 and Fig 13, reporter activity of the human adiponectin promoter deletion mutant in 3T3L1 adipocytes was different in 3T3L1 pre-adipocytes. To define the different binding pattern of the negative regulatory factor between 3T3L1 pre-adipocytes and 3T3L1 adipocytes, we performed EMSA with wild type AP probe. As shown in Fig 15, putative negative regulatory factor bound to the wild type AP oligonucleotide probe in 3T3L1 pre-adipocytes (lane 1), however, this binding complex completely disappeared in 3T3L1 adipocytes (lane 2). The housekeeping gene USF probe was used as a nuclear extract quality control. This result suggests that the putative negative regulatory factor bound human adiponectin promoter in 3T3L1 pre-adipocytes, but the expression was diminished or the binding pattern was changed of this factor in 3T3L1 adipocytes. To investigate the binding activity of the putative negative regulatory factor in the human adiponectin promoter during adipocyte differentiation of 3T3L1 pre-adipocytes, nuclear extracts were prepared from 3T3L1 differentiation day1, day3, day5, day7, day9 (Fig 16). Our results suggest that binding activity of putative negative regulatory factor to the human adiponectin promoter was decreased differentiation time dependently.

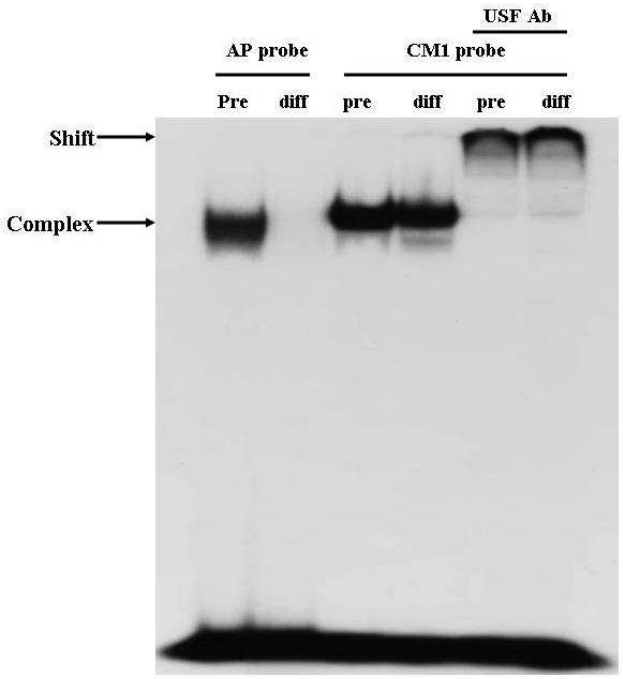

Fig 15. Different binding activity between 3T3L1 pre-adipocytes and 3T3L1 adipocytes. Electrophoretic mobility gel shift assay (EMSA) were performed on the wild type (WT) and control probe USF (CM1). Probes were incubated with 3T3L1 pre-adipocytes and 3T3L1 adipocytes nuclear extract. CM1 probe is used as an extract quality control, and the supershift assay used an USF antibody.

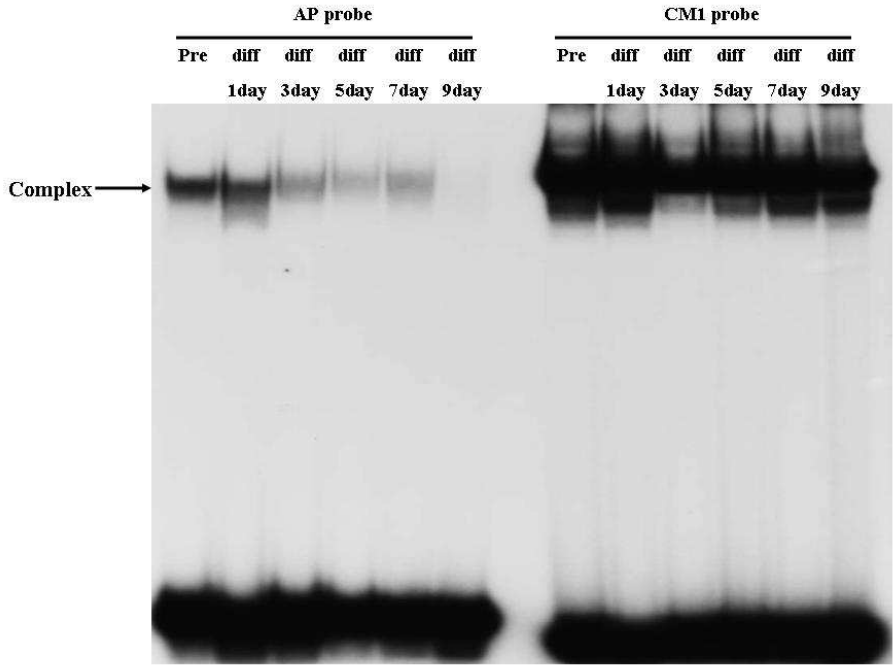

Fig 16. The binding activity of putative negative regulatory factor to the human adiponectin promoter was decreased during differentiation time dependently. Electrophoretic mobility gel shift assay (EMSA) were performed on the wild type (WT) and control probe USF (CM1). Probes were incubated with 3T3L1 pre-adipocytes and differentiated 3T3L1 adipocytes (day 1 , day 3 , day 5 , day 7 , day 9) nuclear extract. CM1 probe is used as a extract quality control, and supershift assay was used USF antibody.

IV. DISCUSSION

Adiponectin is expressed exclusively by fully differentiated adipocytes and associated with many metabolic diseases. One interesting feature is that, the plasma adiponectin concentrations and mRNA expression were decreased in obese people, despite increased adiposity, and in patients with type 2 diabetes with insulin resistance [11-14].

Several factors such as PPAR-ɣ, LRH-1, C/EBP and ADD1/SREBP1c, TNFα, NFATc4 and ATF3 have been reported to be regulate adiponectin gene expression. [22-27]. However, the molecular mechanisms that regulate the negative expression of the adiponectin gene are largely unknown.

In this present studies, to understand this negative regulatory mechanisms in adiponectin gene expression, we first cloned ~1.2 kb of the human adiponectin promoter and analyzed the activities of human adiponectin promoter deletion constructs into NIH3T3, Huh7, and 293 cells. Actually, 1.2 kb human adiponectin promoter construct had very low reporter activity. But, the pGLB-CMV/AP- F522 (-151/+3) construct increased reporter activity. These results suggest that the negative cis-regulatory elements located within -174 to -152 bp in human adiponectin

promoter (Fig 5).

This site is well conserved between human and mouse adiponectin promoter. It involves well known HLH or Myb factor binding sequences (CAACTG: -163/-158). Analysis of the adiponectin promoter point mutants revealed HLH factor is a more possible negative regulatory factor than Myb binding factor in the human adiponectin promoter.

Adiponectin is a protein secreted exclusively in adipose tissue and abundantly present in plasma. Plasma adiponectin concentrations were decreased in obese people, despite increased adiposity [11-14] . This results suggest the transcriptional mechanism of human adiponectin promoter was different between adipocytes and non-adipocytes. Our results support this suggestion (Fig 11 and Fig 13).

Electrophoretic mobility gel shift assay (EMSA) is strongly supports the notion that the DNA binding activity of the negative regulatory factor is sequence-specific. Putative negative regulatory factor bound to human adiponectin promoter in 3T3L1 pre-adipocytes, but the expression was diminished or the binding pattern was changed for this factor in 3T3L1 adipocytes. Moreover, the binding activity of the putative negative regulatory factor to the human adiponectin promoter was

decreased during differentiation time dependently (Fig 16).

Our next goal is to find the binding negative regulatory element. The first method is analyzing well-known HLH factors possessing suppression ability. We examined some HLH factors, for instance, HES, MAD, MAX ,MYX [29-32]. However these factors did not affected in human adiponectin promoter activity (data not shown). The next step we wish to employ is the isolation and identification of target protein(s) using affinity chromatography and mass spectrometry. In brief, annealing of the complementary oligonucleotides (-174 to -152 bp site in human adiponectin promoter) and polymerizing of the complementary 5'-phosphorylated oligonucleotides. And then, coupling of the ligated DNA to the activated Sepharose. This sequence-specific DNA-sepharose resin mixes 3T3L1 pre-adipocytes nuclear extract and incubates. After many times washing, proteins bound to the matrices were eluted and separated by SDS-PAGE gel, and then, visualized by silver staining. Prominent protein bands specific to active matrices were excised from each gel and are being identified by mass spectrometry [33-34].

In summary, we have discovered strongly negative cis-regulatory elements located within -174 to -151 bp in the human adiponectin promoter and the possible

binding factor is likely to be a HLH factor. The suppression of the transcriptional mechanism of the human adiponectin promoter was different between adipocytes and non-adipocytes. Moreover, the binding activity of the putative negative regulatory factor to the human adiponectin promoter was decreased during differentiation time dependently. Further study to find the binding negative regulatory element is needed to clearly define the role of adiponectin gene negative expression in metabolic diseases.

V. CONCLUSION

To identify the negative regulate mechanism of adiponectin gene expression, we isolated ~1.27bp of the human adiponectin promoter. We analyzed the activities of the promoter deletion and point mutant constructs into many cells and performed EMSA.

We obtain the following results.

1. The negative cis-regulatory elements located within -174 to -151 in human adiponectin promoter.
2. HLH factor is more possible negative regulatory factor than myb binding factor in the human adiponectin promoter.
3. Transcriptional mechanism of human adiponectin promoter was different between adipocytes and non-adipocytes.
4. The DNA binding activity of the negative regulatory factor is sequence-specific.
5. That binding activity of putative negative regulatory factor to the human adiponectin promoter was decreased during differentiation time dependently.

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Abstract (in Korean)

아티포넥틴 유전자의 전사 조절

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박영미

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아디포넥틴은 지방세포 특이적으로 발현하는 호르몬으로 인슐린 민감성을 증가시키고, 에너지 항상성을 조절하며, 탄수화물과 지방의 대사 등에 관여한다. 혈액내의 아디포넥틴 농도나 mRNA의 발현은 지방의 증가에도 불구하고 비만한 사람에서 감소하며, 인슐린 저항성을 가진 제2형 당뇨병 환자에서 감소한다. 본 실험에서는 이러한 아디포넥틴 발현의 조절 기전을 이해하기 위하여 인간 아디포넥틴 프로모터를 분리하여 그 deletion mutant 들의 활성을 측정하였다. 이 실험에서 우리는 비지방세포에서 인간 아디포넥틴 프로모터의 -174 에서 -152 bp 사이에 억제 조절에 관여하

는 인자가 존재한다는 것을 발견하였다. 이 부위에는 HLH 또는 myb 인자가 결합 할 수 있는 부위로 잘 알려진 sequence가 존재한다. 우리는 아디포넥틴 프로모터의 point mutant 들의 활성 측정을 통해 myb 인자보다는 HLH 인자가 결합할 가능성이 높다는 것을 보였다. 인간 아디포넥틴 프로모터의 전사가 지방세포와 비 지방세포에서 서로 다르다는 것을 확인하기 위해 electroporation 방법을 이용하여 3T3L1 pre-adipocytes 와 분화시킨 3T3L1 adipocytes에서 인간 아디포넥틴 프로모터의 활성을 측정한 결과 지방세포와 비 지방세포에서 서로 다른 전사 기전을 갖는다는 것을 확인하였다. 또한 EMSA (Electrophoretic mobility gel shift assay) 실험을 통하여 우리가 확인한 인간 아디포넥틴 프로모터 내의 억제 부위에 결합할 것으로 예상하는 억제 인자가 sequence 특이적으로 결합하며, 지방세포가 분화함에 따라서 시간에 의존적으로 그 결합이 감소한다는 것을 발견하였다.

핵심 단어 : 아디포넥틴, 프로모터, 억제 조절 인자, HLH factor, 지방 세포