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Chito-Oligosaccharide Inhibits the De-Methylation of a ‘CpG’ Island within the Leptin (LEP) Promoter during Adipogenesis of 3T3-L1 Cells

Bojul Bahar¹, John V. O’Doherty¹, Alan M. O’Doherty¹, Torres Sweeney²*

¹ School of Agriculture and Food Science, University College Dublin, Dublin, Ireland, ²School of Veterinary Medicine, University College Dublin, Dublin, Ireland

Abstract
Chito-oligosaccharide (COS) is a natural bioactive compound, which has been shown to suppress lipid metabolic genes and lipid accumulation in differentiating adipocytes. Leptin has been identified as a key regulator of energy homeostasis and is known to be under epigenetic regulation during adipogenesis. Hence, the first objective of this experiment was to compare leptin gene (LEP) expression and leptin secretion during the different stages of adipogenesis and to investigate the effect of COS on these processes. As COS inhibited LEP expression during adipogenesis, the second aim was to investigate the methylation dynamics of a ‘CpG’ island in the proximal region of the LEP promoter during adipogenesis and to determine the effect of COS on this process. Mouse 3T3-L1 cells were stimulated to differentiate in the absence or presence of COS and the levels of leptin mRNA and protein were evaluated on days 0, 2, 4 and 6 post-induction of differentiation (PID). The extent of de-methylation of six CpG sites was evaluated. LEP mRNA transcript and protein could not be detected on either day 0PID or 2PID. In contrast, both were detected on day 4PID (P<0.05) and 6PID (P<0.001) and both were inhibited by COS (P<0.001). Of the six CpG sites analyzed, CpG_52, CpG_62 and CpG_95 became 11.5, 5.0 and 5.0% de-methylated between day 2PID and 6PID, respectively. COS blocked this de-methylation event at CpG_52 (P<0.001), CpG_62 (P<0.01) and CpG_95 (P<0.01) on day 6PID. These data suggest that COS can have an epigenetic effect on differentiating adipocytes, a novel biological function of COS which has potential applications for the manipulation of leptin gene expression, adipogenesis, and conditions within the metabolic syndrome spectrum.

Introduction
Obesity is rapidly becoming an epidemic in both developed and developing nations of the world [1], [2]. It is characterized by a disruption in energy homeostasis, leading to the excessive accumulation of fat in adipocytes [3]. Excessive body fat is associated with a spectrum of diseases in the metabolic syndrome spectrum [4] including obesity and type-2-diabetes. The molecular mechanisms underlying the maturation of adipocytes involve the activation of biochemical pathways mediated through signaling molecules including cytokines, chemokines and adipokines [5]. Mouse 3T3-L1 cells are a widely used model of adipocytes [6] and are frequently used to elucidate various underlying cellular and molecular events involved in the process of adipogenesis [7], [8]. This cell line was previously used to identify the anti-adipogenic potential of a range of compounds including herbal drugs [9], [10] and nutraceuticals [11], [12], [13].

The LEP gene is switched on by epigenetic modulation when pre-adipocytes are stimulated to begin the process of adipogenesis [14], [15], [16]. The proximal promoter region of the LEP gene has a number of conserved methylation sites (CpG) which remain highly methylated in the pre-adipocyte and thereby prevent expression of the LEP gene at this stage of development [16], [17]. However, de-methylation of the LEP promoter occurs during the differentiation process, resulting in a loss of methyl groups from CpG sites of the LEP promoter in mature adipocytes [17]. This epigenetic modulation of the LEP promoter is a vital mechanism underlying absence of any leptin secretion in pre-adipocytes while driving abundant synthesis and secretion of leptin by mature adipocytes [18].

Differential of pre-adipocytes into mature adipocytes can be inhibited in vitro following exposure to chito-oligosaccharide (COS) [11], [19]. COS is a polymer of glucosamine with a number of bioactive properties: recent studies have suggested that COS inhibits adipogenesis through altered expression of a number of key regulators of lipid metabolism, including leptin [11], [20]. However, this seems to be cell cycle stage dependent, as COS inhibits the expression of the LEP gene in the differentiating adipocyte [11], while glucosamine, a constituent of COS, can stimulate LEP gene expression in mature adipocytes [21]. Recently, we have demonstrated in the porcine model that dietary inclusion of chitosan, a parent compound of COS, suppressed body weight gain which was associated with elevated serum leptin concentrations [22]. Based on the facts that COS can inhibit the differentiation of pre-adipocyte to mature adipocyte, and that de-methylation of the LEP gene promoter is necessary for secretion of...
leptin by mature adipocytes, we hypothesized that COS interferes with the epigenetic modulation of the *LEP* gene promoter during adipocyte differentiation. Therefore, the first objective of this experiment was to compare *LEP* gene expression and leptin secretion during the different stages of adipogenesis and to investigate the effect of COS on these processes. The second aim was to investigate the methylation dynamics of a ‘CpG’ island in the proximal region of the *LEP* promoter during adipogenesis and to determine the effect of COS on this process.

**Materials and Methods**

**Chito-oligosaccharide (COS)**

Low molecular weight COS (5–10 kDa, >70% chitoooligosaccharide content, >70% deacetylation) was purchased from Kittolife Co. Ltd (Kyunghi-do, Seoul, Korea). This source of COS inhibited the differentiation of mouse 3T3-L1 pre-adipocytes into mature adipocytes [19].

**Cell Culture**

Mouse 3T3-L1 pre-adipocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in a Dulbecco modified eagle’s medium (DMEM, Gibco, Invitrogen Corp., San Diego, CA, USA) containing 10% fetal calf serum (Gibco) and 1% penistrep solution (Sigma-Aldrich Corp., St. Louis, MO, USA) in a 37°C humidified incubator with 5% CO₂. During the multiplication phase of pre-adipocytes, the media was changed every alternate day and cells were trypsinized before reaching full confluence and plated afresh.

Pre-adipocytes were differentiated as described previously [19]. Supernantant and cells were collected relative to post induction of differentiation (PID). Pre-adipocytes were collected on day 0PID. Differentiating adipocytes were collected on days 2, 4 and 6PID.

COS was added to the media on day 0, 2, and 4 PID at final concentrations of 0, 600, 1200, 2400 and 4800 mg/ml.

**Measurement of Leptin**

Leptin was measured in the supernatant on day 0, 2, 4 and 6PID in absence or presence of COS. Leptin was quantified using a mouse leptin sandwich ELISA (R&D Systems Europe, Ltd. Abingdon, UK) according to the manufacturer’s instructions. Signal detection was performed in a microtiter plate reader at an absorbance of 450 nm against 570 nm. Each measurement was performed in triplicate on three independent occasions.

**RNA Extraction**

Pre- and differentiating adipocytes were harvested in TRI reagent (Applied Biosystems, Foster City, CA, USA) and total RNA was extracted using the Trizol method according to the manufacturer’s instructions. RNA was dissolved in 20 μl of nuclease-free water and then subjected to Deoxyribonuclease I (Sigma-Aldrich Corp., St. Louis, MO, USA) in a 37°C humidified incubator with 5% CO₂. Before reaching full confluence and plated afresh.

Pre-adipocytes were differentiated as described previously [19]. Supernantant and cells were collected relative to post induction of differentiation (PID). Pre-adipocytes were collected on day 0PID. Differentiating adipocytes were collected on days 2, 4 and 6PID.

COS was added to the media on day 0, 2, and 4 PID at final concentrations of 0, 600, 1200, 2400 and 4800 μg/ml.

**Bisulfite Modification of DNA and Pyrosequencing**

Based on observations that COS can inhibit the differentiation of pre-adipocytes to mature adipocytes and de-methylation of the *LEP* promoter is necessary for production of leptin by mature adipocytes, we investigated the methylation pattern at the CpG sites present in a 134 bp region of the mouse *LEP* promoter. For this, the pre-adipocyte was allowed to differentiate as described previously [19] and differentiating pre-adipocytes were treated with 0, 600, 1200, 2400 and 4800 mg/ml COS and harvested on day 2, 4 and 6PID. Genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega Corp. Madison, WI, USA) following manufacturer’s instructions.

Genomic DNA was bisulfite modified, according to the manufacturer’s guidelines, using the EZ DNA Methylation-Direct™ Kit (Zymo Research, Irvine, CA, USA). About 200 ng of DNA (in a 20 μl volume) was modified directly by mixing the sample with 130 μl CT reagent (sodium bisulfite conversion solution) and incubating at 98°C for 8 min, followed by 64°C for 8 hr. Modified DNA samples were transferred directly to ZymoSpin IC columns pre-loaded with 600 μl M-Binding Buffer and mixed with gentle pipetting. Columns were centrifuged at 12,000 rpm for 45 sec, the flow-through was discarded and the column was washed with 100 μl M-Wash Buffer. M-desulphonation buffer (200 μl) was added to the column and left to stand at room temperature for 30 min. Two final wash steps with 200 μl wash buffer were carried out before eluting the DNA in 40 μl M-Elution buffer.

Murine *LEP* pyrosequencing primers were designed using Pyromark assay design software (Qiagen) in accordance to the genomic DNA sequence of a 216 bp CpG island located upstream of the transcription start site, obtained using the UCSC genome browser (http://genome.ucsc.edu/). PCR primers were supplied HPLC purified by MWG Eurofins, the forward primer was 5’-biotinylated. A 134 bp amplicon, containing six CpG dinucleotides, was evaluated for methylation analysis.

**Bisulfite PCR** was carried out in 25 μl reactions containing 1 mM MgCl₂, 0.2 μM each of the forward and reverse primers, 0.2 mM dNTPs, 1X PCR Buffer (minus Mg), Platinum Taq DNA polymerase and 3 μl bisulfite modified DNA. Cycling conditions were as follows: 95°C for 5 mins followed by 40 cycles of 95°C, 30 sec; 54°C for 30 sec; 72°C, 30 sec and a final elongation step of 5 min at 72°C. PCR products were verified by electrophoresis on a 2% (w/v) agarose gel. Verified PCR products (20 μl) were bound to 2 μl streptavidin coated sepharose beads (GE Healthcare, Waukesha, WI, USA) in a reaction containing 40 μl Binding buffer (Qiagen) and 20 μl nuclease free H₂O (Promega). Pyrosequencing was performed as outlined before [23]. Complete bisulfite conversion of DNA samples was verified using an internal control at a ‘non-CpG’ cytosine during pyrosequencing.

**LEP Gene Promoter Assay**

**Promoter construct.** A 1 kb region (~991 nt to +8 nt relative to the transcription start site) of the human *LEP* promoter containing the proximal promoter region was amplified by PCR. The forward primer (5’ CTTTAGATCTACCAGAA-TAGGCTGGTTGC 3’) was designed to include a Bgl II restriction digest site (underlined). The reverse primer (5’ TAGTAAAGCTTATCCGCGGCCTCCGATGC 3’) incorpo-
rated a Hind III restriction digest site (underlined). Both forward and reverse primers have a four nucleotide anchor at the 5' end. PCR amplification was performed in a PTC-225 DNA Engine Tetrad (MJ Research, Inc. Massachusetts, USA) using 1× Platinum PCR SuperMix High Fidelity (Invitrogen Corp., San Diego, CA, USA), 0.2 μM of each of the forward and reverse primers, and 50 ng of human genomic DNA (Promega Corp.) in a final volume of 100 μL. The PCR cycle condition included an initial denaturation step of 94°C for 2 min followed by 39 cycles of 94°C for 45 sec, 60°C for 45 sec and 70°C for 1 min and a final extension of 72°C for 10 min. The PCR product (999 bp) was assessed on a 1.2% (w/v) agarose gel stained with ethidium bromide. The PCR product was cloned into firefly luciferase expression vector pGL4.17 (Promega Corp.) as described previously [19]. Transfection grade endotoxin free plasmid construct containing the human LEP promoter insert was prepared using EndoFree Plasmid Maxi Kit (Qiagen, Chatsworth, CA, USA).

**Transfection assay.** The day before the transfection assay, pre-adipocytes (6×10^5 cells/ml) were initially cultured in DMEM containing 10% fetal calf serum in a 24 well cell culture plate. The transfection cocktail (for each well) contained 25 μL DMEM basal media, 0.3 μL FuGENE HD transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) and 100 ng of LEP promoter construct DNA. Following incubation at room temperature for 15 min, this cocktail was introduced drop wise onto the cells. Cells were grown on a DMEM containing 10% fetal calf serum for 48 hrs. Transiently transfected cells were treated with COS in a serum and antibiotic free basal medium.

Transiently transfected cells were allowed to grow for 24 hrs before harvest and luciferase assay was performed using the Luciferase Reporter Assay system (Promega Corp.). In brief, the media was removed and the cells washed with 500 μL of phosphate buffer saline. Lysis of cells was performed by adding 250 μL of passive lysis buffer (Promega Corp.) followed by incubation at 37°C in a shaking incubator for 30 min at 700 rpm. The firefly relative luciferase unit activity was measured in 20 μL of the cell lysate in a 20/20 n Single Tube Luminometer (Turner Systems, Inc. Sunnyvale, CA, USA).

**In-vitro methylation of LEP gene: In-vitro methylation of the LEP promoter construct was performed using CpG Methyltransferase (M.SssI) enzyme (New England Biolabs Inc. Herts, United Kingdom).** The methylation reaction contained 3 μg of the promoter construct DNA, 12U of SssI methylase and 1X NE Buffer 2 supplemented with 640 μM S-adenosylmethionine (SAM) in a final volume of 20 μL. The content was incubated at 37°C for 2 hr followed by stopping of the reaction by treating at 65°C for 20 min. The promoter construct was cleaned using Gen Elute PCR clean-up Kit (Sigma-Aldrich Corp.) following the manufacturer's instructions.

**Statistical Analysis**

Data for all the variables were checked for a normal distribution. Data on leptin protein abundance, LEP gene expression (fold change), % methylation were analysed using one-way ANOVA and means were compared by Tukey’s test. The promoter assay data were compared by student- t test.

**Results**

**Quantification of LEP Gene Expression and Leptin Protein Secretion during Adipogenesis**

LEP gene expression: The expression of the LEP gene was at the minimum limit of detection in pre-adipocytes (day 0PID) and early differentiation stage adipocytes (day 2PID) (Figure 1A). LEP mRNA transcript was detected on day 4PID (P<0.05), with a significant increase in gene expression evident on day 6PID (P<0.001).

Leptin protein secretion: Leptin was not detected in the supernatant on days 0PID or 2PID; Figure 1B). As adipocyte differentiation progressed, an increase in leptin secretion was evident: with leptin concentrations increasing from 193.7±14.33 pg/ml on day 4PID (P<0.05) to 1269.0±122.3 pg/ml on day 6PID (P<0.001) (Figure 1B).

**Effect of COS on Leptin Protein Secretion and LEP Gene Expression during Adipogenesis**

LEP gene expression: As day 6PID was determined to be the day of maximum leptin secretion in the control sample, the effect of COS on LEP gene expression was evaluated on day 6PID. The relative abundance of the LEP mRNA transcript was 1.12±0.13 fold in the control (COS 0 μg/ml) sample (Figure 2). A significant decrease in the abundance of the LEP mRNA transcript was evident with exposure to 2400 (0.54±0.12 fold, P<0.05) and 4800 (0.11±0.02 fold, P<0.001) μg/ml COS.

Leptin protein secretion: As leptin was not detectable on day 0PID or 2PID, the effect of COS was not evaluated at these time points. COS inhibited leptin secretion on day 4PID and 6PID (P<0.001; Figure 3A and B). On day 6PID, when leptin concentrations were at its maximum in the control sample (COS 0 μg/ml: 1269.0±122.3 pg/ml), COS inhibited leptin secretion (P<0.001) to 121.5, 122.3 pg/ml on day 6PID (P<0.05); Figure 1B). As adipocyte differentiation progressed, an increase in leptin secretion was evident: with leptin concentrations increasing from 193.7±14.33 pg/ml on day 4PID (P<0.05) to 1269.0±122.3 pg/ml on day 6PID (P<0.001) (Figure 1B).

**De-methylation of the LEP Promoter during Adipogenesis**

To determine the methylation status of the LEP gene promoter during adipogenesis, the extent of methylation of a ‘CpG’ island was evaluated on days 2, 4 and 6PID. Of the six CpG sites analyzed, de-methylation was observed at four CpG sites (CpG_52, CpG_62, CpG_81 and CpG_95) during differentiation. The most conspicuous CpG site de-methylated during the differentiation process was CpG_52, where 11.5% de-methylation was evident between day 2 and day 6PID. During this period, de-methylation was also observed at CpG_62 (5%), CpG_95 (5%) and CpG_81 (3%). No alteration in methylation status was observed at two CpG sites (CpG_1 and CpG_85).

**Effect of COS on the De-methylation of the LEP Promoter during Adipogenesis**

The ability of COS to inhibit the de-methylation of CpG_52, CpG_62, CpG_95 of the LEP promoter on days 2, 4 and 6PID was evaluated. COS had no effect on the de-methylation of any of these three CpG sites on days 2 and 4PID. However, on day 6PID, COS inhibited the de-methylation of CpG_52 (Figure 4A), CpG_62 (Figure 4B) and CpG_95 (Figure 4C).

**Comparison of the Expression of a Non-methylated and a Methylated LEP Promoter Construct**

To investigate whether the methylation of the LEP promoter is an underlying cause behind the absence of expression of leptin in pre-adipocytes (day 0PID), the expression of non-methylated and an in-vitro methylated LEP promoter constructs was evaluated in 3T3-L1 pre-adipocytes. The relative promoter transcriptional activity data (Figure 5) revealed that the non-methylated LEP promoter had a transcriptional activity of 8210.0±1462.0 RLU,
while the in-vitro methylated LEP promoter had only limited transcriptional activity (247.5±21.7 RLU) in the pre-adipocytes.

Discussion

We have previously determined that chitooligosaccharide (COS) suppresses the expression of a number of lipid metabolic pathway regulatory genes, including the LEP gene, and inhibits the accumulation of lipid in differentiating adipocytes [19]. Epigenetic regulation of the LEP gene was investigated in this study as an underlying mechanism of COS mediated inhibition of adipogenesis. Our results suggest that pre-adipocytes do not express leptin due to the presence of a highly methylated LEP promoter at that stage of cellular differentiation. The de-methylation of the LEP promoter occurs during the differentiation of pre-adipocytes to mature adipocytes, resulting in the expression of the LEP gene and subsequent production of leptin by the differentiated adipocytes. The process of de-methylation of selective CpG sites of the LEP promoter can be blocked by COS. This novel finding of inhibition of LEP gene expression by COS at an epigenetic level in adipocytes can be explored for manipulation of leptin gene expression, adipogenesis, energy homeostasis and obesity.

In this study, COS inhibited the de-methylation of three CpG sites (at −52, −62 and −95) in the LEP promoter. Of these three CpG sites, the most conspicuous de-methylation event was evident at CpG_52, which is present in a binding motif for the adipogenic transcription factor C/EBPα in the proximal promoter region of the LEP gene. This proximal promoter region (−55 to −47 bp), containing the C/EBPα site, is required for the trans-activation of LEP gene transcription [24] and subsequently leptin secretion [25], [26]. The fact that de-methylation of CpG_52 occurs during adipogenesis and de-methylated CpG_52 is required for binding of C/EBPα transcription factor and subsequent activation of the LEP gene expression [24], blocking of de-methylation of CpG_52 by COS most likely inhibited leptin expression. This might be an important mechanism by which COS can block the binding of C/EBPα, a key adipogenic transcription factor, and thus inhibits LEP gene expression in differentiating pre-adipocytes.

The mouse 3T3-L1 pre-adipocytes lack expression of LEP mRNA and protein, which indicated that the LEP gene as such is not expressed in the pre-adipocyte. However, as the differentiation of pre-adipocyte to mature adipocyte progresses, the abundance of LEP mRNA and protein increases. This is in agreement with previous reports on mouse 3T3-L1 adipocytes [17], [27] and human pre-adipocytes [16] which suggested that leptin is not produced by pre-adipocytes. We have compared the expression of...
an un-methylated and a chemically methylated LEP promoter in the pre-adipocyte and demonstrated that the presence of a highly methylated LEP promoter contributed to the lack of expression of leptin in pre-adipocytes.

In this experiment, a reduction of 4–12% methylation was recorded between the cells treated with the carrier only (COS 0 μg/ml) and highest concentration of COS (4800 μg/ml). These small but significant differences in the de-methylation could be due to: a) lack of complete differentiation of the cells at harvest (day 6PID), b) failure of every cell coming in contact to COS homogenously [28] and c) an inherent PCR bias associated with bisulfite PCR amplification of the genomic DNA [29]. Nevertheless, methylation differences as little as 7% was previously reported to cause significant difference in gene expression [30]. Therefore, a reduction of 4–12% methylation recorded in this study due to the action of COS in three CpG sites is considered adequate for alteration in LEP gene expression.

COS being an anti-adipogenic bioactive compound, inhibits leptin expression at mRNA and protein levels in the differentiating pre-adipocyte. This inhibition of leptin secretion by adipocytes may alter the cellular fat reserve, which potentially has implication for conditions within the metabolic syndrome spectrum including obesity and type-2-diabetes. In both healthy and lean individuals, leptin works as a satiety signal reflecting the body energy reserve [31], [32]. In contrast, obese individuals generally have a high level of circulating leptin termed as ‘leptin resistance’ in which an excessive circulating leptin fails to signal for satiety and body energy reserve [32], [33]. Leptin interferes with glucose homeo-

Figure 3. Effect of chitooligosaccharide (COS) on the production of leptin by 3T3-L1 pre-adipocytes during adipogenesis. Mouse 33-L1 pre-adipocytes were induced to differentiate in the absence or presence of chitooligosaccharide and cell culture media was harvested for quantification of leptin production on days 2–4 post-induction of differentiation (A) and days 4–6 post-induction of differentiation (B) Data represents mean ± standard error from three independent replicate experiments. Note the differences in the scale of Y-axis representing the leptin concentration (pg/ml) in the media for different figures.
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Figure 4. Effect of chitooligosaccharide on the de-methylation three CpG sites in the LEP promoter. Extent of methylation at CpG_52 (A), CpG_62 (B) and CpG_95 (C) of the mouse LEP promoter at days 6 post-induction of differentiation are presented as mean ± standard error from two independent replicate experiments.
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stasis and is reported to inhibit insulin gene expression and insulin secretion by pancreatic β-cells [33], [34], [35]. Therefore, COS mediated inhibition of leptin secretion may lead to an anti-diabetic effect in type-2-diabetes which is associated with secretion of excessive leptin leading to hypoinsulinaemia. Recent reports on anti-diabetic effect of chitosan in mice suggested that feeding of COS lead to an increase in glucose inducible insulin expression [36] and glucose uptake in streptozotocin induced diabetic mice [37].

The in-vitro data presented in this manuscript were obtained by using a homogeneous pre-adipocyte cell population and it was evident that COS can alter epigenetic modification of LEP gene expression due to chemically induced adipogenesis. However, adipocyte development and differentiation in live animal is a complex and often an unsynchronized process where different populations of pre-adipocyte differentiate at various stages of development [38]. Therefore, the regulation of leptin promoter de-methylation by COS may be a complex phenomenon in the live animal which warrants further research.

Leptin also induces a pro-inflammatory response [31], [39], [40]. It induces the secretion of pro-inflammatory cytokines TNFα, IL-6 and IL-12 in macrophages [41] and causes a Th1 pro-inflammatory cytokine response [42]. If these in-vitro results translate in-vivo, COS mediated inhibition of leptin expression by adipocytes may be potentially helpful in reducing the chronic pro-inflammatory response in obese individuals. Interestingly, a number of anti-diabetic compounds such as thiglizatone [43], pioglitazone [44], rosiglitazone [45] and metformin [46] used to treat type-2-diabetes work through inhibition of leptin expression.

In conclusion, we have demonstrated that pre-adipocytes do not express the LEP gene due to the presence of a highly methylated LEP promoter. However, de-methylation of selective CpG sites of the LEP promoter, which occur during the differentiation process, can be inhibited by treatment with COS. This epigenetic inhibition of the LEP gene by COS in differentiating adipocytes could have implications for leptin regulation and for associated metabolic syndrome diseases such as obesity and type-2-diabetes.

**Author Contributions**

Conceived and designed the experiments: BB JO TS. Performed the experiments: BB AO. Analyzed the data: BB AO JO. Contributed reagents/materials/analysis tools: TS AO. Wrote the paper: BB TS JO.

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Chito-Oligosaccharide Affects Methylation of LEP

![Figure 5. Transcriptional activities of an un-methylated and chemically methylated human LEP promoter in mouse 3T3-L1 pre-adipocytes.](https://example.com/figure5)

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Chito-Oligosaccharide Affects Methylation of LEP

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