Inhibition of adipogenesis by transcription factor Snail

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Inhibition of adipogenesis by transcription factor Snail

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ABSTRACT

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Snail belongs to the superfamily of zinc-finger transcription factors and plays a crucial role in processes regulating cell fate, such as the formation of mesoderm and initiation of epithelial-mesenchymal transition. We have previously discovered that Snail modulates adiponectin expression in 3T3-L1 cells during adipogenesis. In the present study, we elucidated the functional role of Snail in adipocyte differentiation and its underlying molecular mechanism. Snail expression was dramatically decreased during adipogenesis in 3T3-L1 cells. Overexpression of Snail blocked adipocyte differentiation by suppressing the expression of peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein alpha. Chromatin immunoprecipitation assay and luciferase assay showed that Snail inhibits the transcriptional activity of the PPARγ gene by directly binding to the E-box motifs in the PPARγ promoter. Wnt10b induced phosphorylation of glycogen synthase kinase 3 beta (GSK3β), leading to inhibition of adipogenesis in 3T3-L1 cells in accordance with increased expression of Snail, whereas

adipogenic capacity was restored in Snail siRNA-transfected preadipocytes. LiCl (a GSK3β inhibitor)-treated cells also showed increased expression of Snail, with a reduced adipogenic potential. Snail-overexpressing 3T3-F442A cells did not differentiate into mature adipocytes in immunodeficient nude mice. Taken together, Snail is a novel regulator of adipocyte differentiation, which acts by direct suppression of PPARγ expression. Our data also indicate that the expression of Snail is mediated by the Wnt-GSK3β signaling pathway.

Key words : adipogenesis, Snail, Wnt signaling, PPARγ

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I. INTRODUCTION

Obesity is a worldwide epidemic[1] and has immense impact on the development of various diseases including cardiovascular diseases, diabetes, and cancer.[2] From a cell-biological aspect, obesity is defined as the expansion of adipose tissue in the body, which is caused by an increased size of adipocytes (hypertrophy) as well as an increased number of adipocytes (hyperplasia). Hyperplasia of the adipocytes is initiated by adipogenesis of the mesenchymal stem cells or preadipocytes. Therefore, understanding the molecular mechanism that regulates the development and growth of adipose tissues is essential to treat and prevent obesity.

Adipocyte differentiation involves a complex network of transcription factors that modulate the expression of various genes responsible for the development of mature adipocytes.[3] The members of the Wnt signaling pathway are known to be an important component of adipogenesis inhibition.[4] Although both canonical pathways (including β-catenin) and non-canonical pathways play

an essential role in adipocyte differentiation, the downstream signaling cascades have not been fully elucidated.

The Snail superfamily of zinc-finger transcription factors plays a crucial role in processes regulating cell fate, such as the formation of mesoderm and initiation of epithelial-mesenchymal transition (EMT) during embryonic development and tumor metastasis or progression.[5] The Snail family of proteins is involved in neural differentiation, cell survival, and left–right asymmetry. [5] Snail (SNAI1) usually acts as a transcription repressor and triggers EMT by directly downregulating the expression of E-cadherin in the mesoderm.[6] In addition, Snail regulates osteoblast differentiation by repressing the expression of Runx2 and the vitamin D receptor in osteoblasts.[7] As osteogenesis is closely related to adipogenesis, Snail may play a certain role in adipocyte differentiation. The function of Slug (SNAI2), which is the second member of the Snail family, has been identified in transgenic mice, and the overexpression of Slug in these mice increased the amount of white adipose tissue by stimulating adipogenesis.[8] Recently, we have found that Snail inhibits the expression of adiponectin by directly binding to the E-box of the adiponectin promoter in 3T3-L1 adipocytes.[9] In this study, overexpression of Snail in 3T3-L1 adipocytes resulted in decreased levels of peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT-enhancer-binding protein alpha (C/EBPα), which are two major elements of adipogenic transcription factors, suggesting a plausible link between Snail and adipogenesis.

However, to date, there has been no evidence regarding the functional role of Snail in adipocyte differentiation.

Furthermore, diverse signaling pathways such as fibroblast growth factor, transforming growth factor-β, and Wnt have been linked with the activation of Snail in the processes of EMT.[5] The role of the Wnt pathway in Snail activation is demonstrated by the finding that Snail expression is modulated by Wnt-GSK3β-dependent phosphorylation in breast carcinoma cells 10. Therefore, the aim of the present study was to investigate the functional role of the transcription factor Snail in adipocyte differentiation using 3T3-L1 preadipocytes and a xenograft animal model, and to demonstrate that the Wnt-GSK3β signaling cascades regulate the expression of Snail.

II. MATERIALS AND METHODS

1. Cell culture and differentiation of preadipocytes into adipocytes

The 3T3-L1 and 3T3-F442A mouse preadipocyte cell lines were cultured according to the method described previously.[9] Two-day post-confluent cells (designated as day 0) were cultured in DMEM (WelGENE Inc., Daegu, Korea) supplemented with 10% fetal bovine serum (FBS, WelGENE Inc.), 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX, Sigma-Aldrich, MO, USA), 2 μg/ml dexamethasone (Sigma-Aldrich), and 1 μg/ml insulin (Roche, Mannheim, Germany) for 2 days. Every 2 days thereafter, the cells were incubated with fresh DMEM supplemented with 10% FBS and 1 μg/ml insulin. Fully differentiated adipocytes were used for experiments after 8 days. Lipid droplets were visualized by Oil Red O staining on day 8. To measure the quantification of lipid accumulation, Oil Red O was eluted by adding 100% isopropanol and optical density was detected using a spectrophotometer at 520 nm.

2. Plasmid transfection and Snail silencing by lentiviral-delivered RNA interference

3T3-F442A and 3T3-L1 preadipocytes were grown in 12-well culture plates until confluence. To induce overexpression of Snail, C/EBPα or Wnt10b, the preadipocytes were transfected with the expression vector pcDNA3 that encoded Snail or C/EBPα and pCMV6-Entry vector with Wnt10b (Origene, MD, USA), using Tfx-50 (Promega, WI, USA) and electroporation (model No. 640: BTX Harvard apparatus; MA, USA) in accordance with the manufacturer's instructions. Following electroporation, the cells were replated on tissue culture plates and allowed to recover for 48 hours. Snail and control small interfering RNAs (siRNAs, Santa Cruz, CA, USA) were used for knockdown experiments. A mouse shRNA kit for silencing Snail expression was purchased from Santa Cruz and 3T3-L1 cells were infected with either control or Snail shRNA lentiviral particles according to the manufacturer's instructions.

3. Site-directed mutagenesis on PPARγ promoter

To assess whether each E-box motif in the mouse PPARγ promoter is functional, we performed site-directed mutagenesis to destruct putative E-boxes in the -707 and -154 of the mouse PPARγ promoter. E-boxes in the -472 of the PPARγ promoter were excluded by the evidence that consensus sequence of E-box-472 was not found in rat or human. The plasmids containing the mouse PPARγ promoter followed by luciferase constructs were mutated with the following oligonucleotide primers: sense 5'- ATT GGA ATA CTA CTG **TGGTAC** CTA TTG ATA GAT AAA-3' and antisense 5'-TTT ATC TAT CAA TAG **GTACCA** CAG TAG TAT TCC AAT-3 (mutant E-box-707), sense 5'-CAC ACC ATT TTG TCA **TTACGC** GCT CTC AGT CAG GAC-3' and antisense 5'-GTC CTG ACT GAG AGC **GCGTAA** TGA CAA AAT GGT GTG-3' (mutant E-box-154). PCR was conducted with using 2X EF-Taq DNA Premix (SolGent, Seoul, Korea) according to the manufacturer's instruction. Cycle conditions were: 2 min hot start at 95 °C, 40 cycles of 20 s at 95 °C, 40 s at 60 °C, 7 min at 72 °C, and extension at 72 °C for 20 min. Digestion with DpnI was then proceeded in 26 μl of the reaction at 37 °C for 2 h, followed by transformation into DH5α Escherichia coli to select and amplify the mutated plasmids. All constructs were confirmed by sequencing (SolGent).

4. Luciferase reporter assay

Plasmids containing the mouse PPARγ promoter (–2,000 bp to +27 bp from transcription start site)-luciferase constructs were transfected using Lipofectamine^{TM} 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. Plasmids containing mouse C/EBPα and Snail were simultaneously co-transfected in 3T3-L1 preadipocytes. Assays were performed according to the method described previously[11] using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was measured using a microplate luminometer (Centro XS3 LB960; Berthold Technologies, Bad Wildbad, Germany). The *Renilla* luciferase signals were normalized to the internal firefly luciferase transfection control. Transfections were performed in triplicate for each independent experiment.

5. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cultured 3T3-L1 pre-adipocytes and adipocytes using TRIzol® reagent (Invitrogen) as described by the manufacturer's instructions. Twenty micrograms of RNA were treated with RNase-free DNase I (QIAGEN Korea Ltd., Seoul, Korea) for 10 min at 25°C. Random hexamers were used to synthesize single-stranded complementary DNA (cDNA) using 2 μg of DNase I treated-RNA in a 20-μL reaction volume containing 50 mM of Tris-HCl (pH 8.3), 8 mM of $MgCl_2$, 50 mM of NaCl, 1 mM of dithiothreitol, 1 mM of each dNTP (Bioneer, Seoul, Korea), 22 U of RNase inhibitor (Bioneer), and 10 U of Moloney murine leukemia virus RT (Promega) for 60 min at 37°C. A portion (1/40) of the cDNA solution was treated with 2.5 U of Taq DNA polymerase (SolGent) for amplification of Snail and β-actin (used as an internal control). The PCR was performed under the following conditions: 15 min hot start at 95°C, followed by 35 cycles, with each cycle programmed to 20 sec at 95°C, 40 sec at 58°C, followed by 30 sec at 72°C, and a final extension step at 72°C for 5 min. An aliquot (20%) of each PCR product was resolved by electrophoresis on a 1.5% agarose gel, and the DNA product was visualized using ethidium bromide. Oligonucleotide primers of mouse used for PCR amplification were as follows: Snail, forward 5′- TTC CAG CAG CCC TAC GAC CAG -3′ and reverse 5′- CGG ACT CTT GGT GCT TGT GGA -3′; PPARγ, forward 5′-CTC CGT GAT GGA AGA CCA

CT-3′ and reverse 5′-AAC CAT TGG GTC AGC TCT TG-3′; C/EBPα, forward 5′-TGG ACA AGA ACA GCA ACG AG-3′ and reverse 5′-TCA CTG GTC AAC TCC AGC AC-3′; β-actin, forward 5′-CCA GGG TGT GAT GGT GGG AAT G-3′ and reverse 5′-CGC ACG ATT TCC CTC TCA GCT G-3′.

6. Western blotting

Preadipocytes and adipocytes in 10-cm plates were lysed in RIPA buffer (Cell Signaling, MA, USA), and the protein content was measured using the Coomassie (Bradford) Protein Assay Kit (Pierce, IL, USA). Equal amounts of protein (60 μg) were heat-denatured in 4× sample buffer (2% sodium dodecyl sulfate, 62.5 mM Tris, pH 6.8, 0.01% bromophenol blue, 1.43 mM mercaptoethanol, and 0.1% glycerol), separated on 10% or 12% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Roth, Karlsruhe, Germany). The membranes were treated with the appropriate antibodies against the following proteins: Snail (Abcam, MA, USA), C/EBPα, PPARγ, histone deacetylase (HDAC) 1, HDAC2 (Santa Cruz), adiponectin (Affinity Bioreagents, IL, USA), GSK3β (Cell Signaling, MA, USA), β-actin (Sigma-Aldrich).

7. Preparation of nuclear extracts

Cells cultured to confluence in 10 cm culture dishes were rinsed 2 times using PBS and harvested 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. 15 μL 10% NP-40 was added followed by centrifugation at 12,000 g for 5 min. The supernatant was removed and the pellet was resuspended in 200 μL buffer C (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and protease inhibitor cocktail (Roche applied science, IN, USA)) by vortexing for 5 s, followed by shaking at 4 °C for 30 min. After centrifugation at 12,000 g for 10 min at 4 °C, the supernatant was collected and protein quantified with the Bradford reagent (Sigma-Aldrich).

8. Immunofluorescence

To detect the expression of Snail in cultured 3T3-L1 preadipocytes and adipocytes, cells were plated on fibronectin-coated cover slips and washed with PBS twice and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 5 minutes. Cells were blocked in PBS containing 5% bovine serum albumin for 2 hours at room temperature and incubated with the primary rabbit anti-Snail antibody (1:200, Abcam) overnight at 4°C, followed by incubation with the secondary goat anti-rabbit IgG-FITC (1:400, Invitrogen) for 2 hours at room temperature. Propidium iodide (PI, 1:1,000, Invitrogen) was used as a nuclear counterstain. Images were obtained using a Zeiss microscope (Axioskop, Carl Zeiss Inc., Oberkochen, Germany).

9. Cell proliferation and survival assay

Cell survival was assessed using the MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide) assay (Sigma-Aldrich). Cells were plated on 24-well plates (5×10^4 cells/well) and transfected at 70% confluence with the pcDNA3 and pcDNA3-Snail plasmid using Lipofectamin^{TM} 2000 reagent (Invitrogen). MTT was added at a final concentration of 0.5 mg/ml at 48 hours after transfection, after which the cells were incubated for 4 hours at 37 °C and suspended in 250 μl of dimethylsulfoxide (Sigma-Aldrich). Optical density values were read at 540 nm using a microplate reader (Molecular Devices, CA, USA). Each experiment was carried out in triplicate and three measurements were taken for each experiment. To confirm the total cell number, 1×10^5 cells were seeded into 6-well plates. Forty-eight hours after transfection, cells were collected by trypsin-EDTA treatment and suspended in PBS containing 10% FBS. Cell viability was determined by direct counting of cells on a hemocytometer in the presence of 0.5% trypan blue.

10. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was carried out using the online protocol resource (http://mescaline.igh.cnrs.fr/EpiGeneSys/images/stories/protocols/pdf/20111025152730_p10.pdf; accessed February 4 2009) provided by the Epigenome Network of Excellence (Institute of Human Genetics, CNRS UPR 1142, Montpellier, France) as follows: (A) cells were cultured to 90% confluence in 150-mm culture dishes, (B) cells were collected by scraping in 20 ml ice-cold PBS, (C) after sonication, the supernatant was centrifuged at 16,000 × g for 5 minutes, and (D) the supernatant was precleared by incubation with G-Sepharose (GE Healthcare, WI, USA). Antibodies against acetylated-H3, and histone H3 were purchased from Santa Cruz. The precipitated DNA was analyzed by 30 cycles of PCR and primers used for PCR amplification were as follows: ChIP primer 1, forward 5′-CAC TTA AAC ATC AAC CAT TGG -3′ and reverse 5′-GGA GTT TCA ACC AAA GAT AA-3′; ChIP primer 2, forward 5′- TTC ACG CCC CTC ACA GAA CA-3′ and reverse 5′- GTG CCA GCC AAT TCA GGC CTG-3′. The primers spanned the upstream region of the PPARγ promoter from –738 bp to –439 bp (ChIP primer 1) and –277 bp to –112 bp (ChIP primer 2).

11. Animals, histological analysis and immunohistochemistry

Animal experiments were performed using 8-wk-old male athymic immunodeficient nude mice (Charles River Japan, Inc., Yokohama, Japan). All animal studies were approved by the Animal Care and Use Committee of the Yonsei University College of Medicine (No. 2011-0234). Each group had four animals and was housed in an animal room maintained at a temperature of 23 ± 2 °C and a humidity of 55 ± 5%. The mice were exposed to a 12-h light, 12-h dark cycle and fed a standard unrestricted diet. Transfected 3T3-F442A preadipocytes were collected, washed twice with PBS, resuspended in DMEM, and injected (3×10^7 cells) into the flanks of mice at day 0. After 5 weeks from injection, the mice were euthanized, and tumor masses were removed. The collected masses were fixed using 10% neutral-buffered formalin and processed into paraffin blocks. Sections (4 μm) were stained with hematoxylin and eosin and reviewed by a pathologist. Immunohistochemical study for the expression of Snail was performed as previously described.[10] After antigen retrieval process with citrate buffer (pH 6.0 at 90°C), specimens were incubated with anti-Snail antibody (1:400; Abcam), specific biotinylated secondary antibodies (1:100; Vector Laboratories, Burlingame, CA, USA) and streptavidin-peroxidase (DAKO, Kyoto, Japan), sequentially. Diaminobenzidine (Vector Laboratories) was used as a chromogen, and counterstaining was conducted using hematoxylin.

12. Statistical analyses

The Mann-Whitney *U* test and Kruskal-Wallis test were applied to determine significant differences between the groups. The R software (version 2.15.0) was used for all statistical analyses. A *P* value less than 0.05 was considered statistically significant. All results are presented as representative data from three experiments.

III. RESULTS

1. Expression of the transcription factor Snail during adipocyte differentiation

Immunofluorescent staining with anti-Snail antibody showed that Snail (stained green) is localized in the nucleus of 3T3-L1 preadipocytes (stained red), whereas Snail is not detected in differentiated adipocytes (Fig. 1). To examine the time course of Snail expression during differentiation, 3T3-L1 cells were cultured for the indicated times from 1 to 8 days after initiating the treatment with adipogenic hormonal cocktails. RT-PCR and immunoblot analyses showed that the amount of Snail mRNA and protein were high in preadipocytes, whereas Snail expression dramatically decreased during adipogenesis after induction with adipogenic cocktails (Fig. 2 and 3). Consistent with previous findings, the expression of PPARγ and C/EBPα, which are the two major transcription factors required for adipocyte differentiation, was observed within approximately 2 days of induction, followed by the expression of adiponectin.

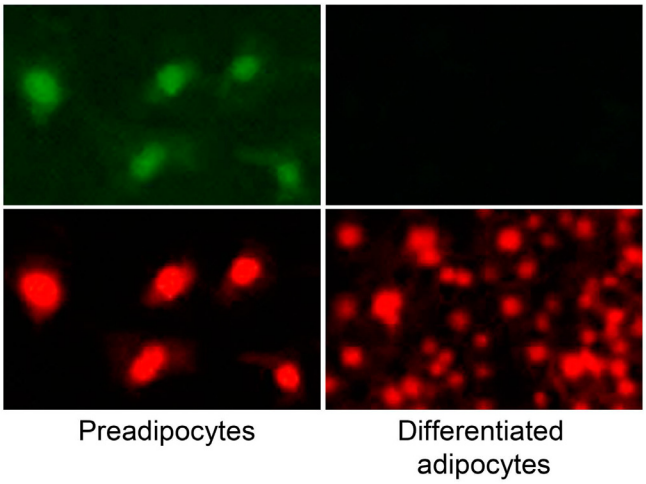

Figure 1. Immunofluorescent assay using anti-Snail antibody in preadipocytes and differentiated adipocytes. The expression of Snail was evaluated by immunofluorescence with anti-Snail antibody (stained green) in 3T3-L1 preadipocytes and fully differentiated adipocytes. Propidium iodide (stained red) was used for nuclear stain. Magnification, ×400.

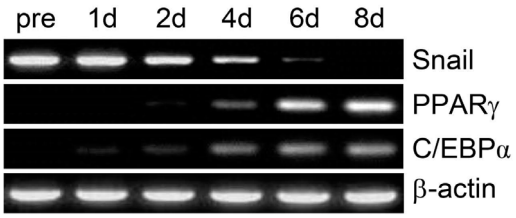

Figure 2. RT-PCR analysis during adipogenesis. To examine the time course of the expression of Snail mRNA during differentiation of 3T3-L1 preadipocytes, 3T3-L1 cells were cultured for the indicated times after initiating the treatment of adipogenic hormonal cocktail. All results are representative of three independent experiments.

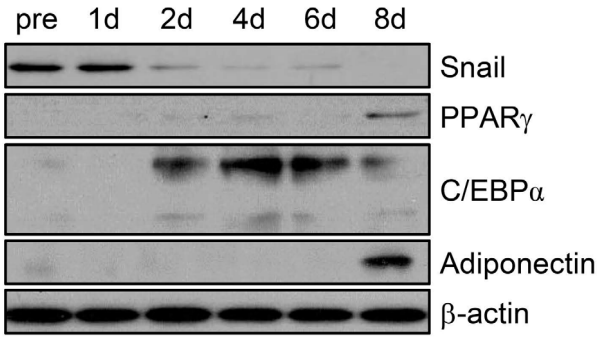

Figure 3. Immunoblot analysis during adipogenesis. To examine the time course of the expression of Snail protein during differentiation of 3T3-L1 preadipocytes, 3T3-L1 cells were cultured for the indicated times after initiating the treatment of adipogenic hormonal cocktail. All results are representative of three independent experiments.

2. Snail inhibits adipogenesis in vitro

To investigate whether the constitutive expression of Snail affects adipocyte differentiation in vitro, the expression construct pcDNA3 Snail was transfected into preadipocytes. Oil Red O staining at 8 days after induction with adipogenic cocktails demonstrated that Snail overexpression significantly suppressed adipogenesis in the transfected 3T3-L1 and 3T3-F442A cells (Fig. 4). This finding was further supported by the results from RT-PCR analysis, showing that cells transfected with a plasmid encoding Snail did not express mRNA for PPARγ and C/EBPα after 8-day treatment with adipogenic cocktails (Fig. 5).

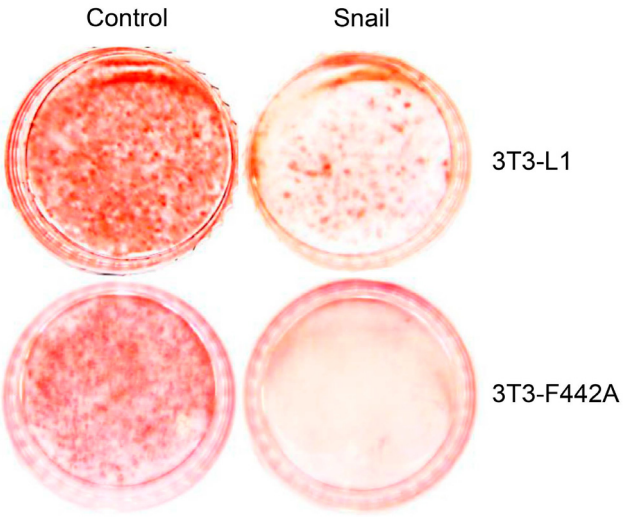

Figure 4. Oil Red O staining of murine adipocytes after overexpression of Snail. On day 8 after treatment with adipogenic cocktails, 3T3-L1 and 3T3-F442A cells transfected with plasmids harboring Snail-encoding DNA or backbone control plasmids were stained with Oil Red O. The data are representative of three independent experiments.

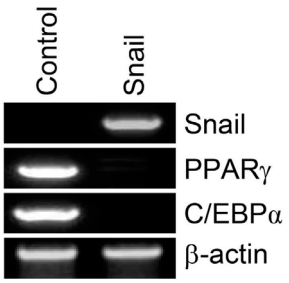

Figure 5. RT-PCR analysis of 3T3-L1 adipocytes after overexpression of Snail. The expression of Snail, C/EBPα, and PPARγ expression were examined in 3T3-L1 cells on day 8 after transfection with either a control plasmid or a plasmid expressing Snail (pcDNA3-Snail). The data are representative of three independent experiments.

During adipocyte differentiation, impairment in the process of mitogenic clonal expansion can result in the failure of preadipocytes to effectively differentiate into mature adipocytes.[12] To explore whether Snail regulates the proliferation or viability of the preadipocytes in vitro, the 3T3-L1 preadipocytes were transfected with pcDNA3 Snail or control backbone plasmids. Forty-eight hours after transfection, MTT assay and direct cell counting were performed using the transfected preadipocytes (Fig. 6). The proliferation of preadipocytes in the presence of the pcDNA3 Snail or the control plasmid was not significantly different, and the amount of cell death was similar in both the groups. We explored the capacity of adipogenic potentials in 3T3-L1 preadipocytes after knocked-down the expression of Snail using shRNA. Under the condition of FBS, IBMX + dexamethasone or IBMX + insulin, the proportion of differentiated adipocytes was significantly increased in cells with Snail shRNA comparing to those with control shRNA (Fig. 7 and 8), which was further confirmed by immunoblots showing that the expression of PPARγ, C/EBPα and adiponectin were significantly increased in Snail shRNA-treated cells (Fig. 9). These findings indicate that Snail blocks adipogenesis in 3T3-L1 preadipocytes without affecting cell proliferation or viability.

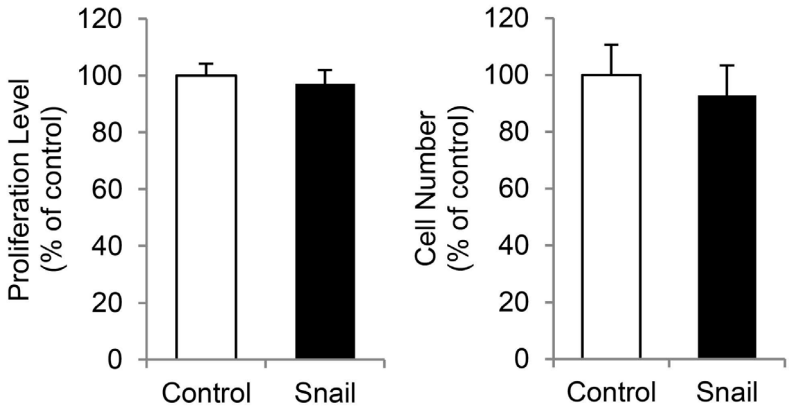

Figure 6. Effect of Snail on the proliferation and survival of 3T3-L1 preadipocytes. Cells were transfected with either a control plasmid or a plasmid expressing Snail. After 48 h of transfection, proliferation of the 3T3-L1 cells was assessed by MTT assay (Left panel), and cell viability was examined using tryptophan assay (Right panel). The proliferation levels of cells harboring the Snail plasmids or the control plasmids were not significantly different. The viability of the Snail-overexpressing cells was similar to that of the control cells. The number of Snail-expressing cells is represented as a percentage of those in the control group. Results of six independent experiments were averaged and plotted as the mean ± SD.

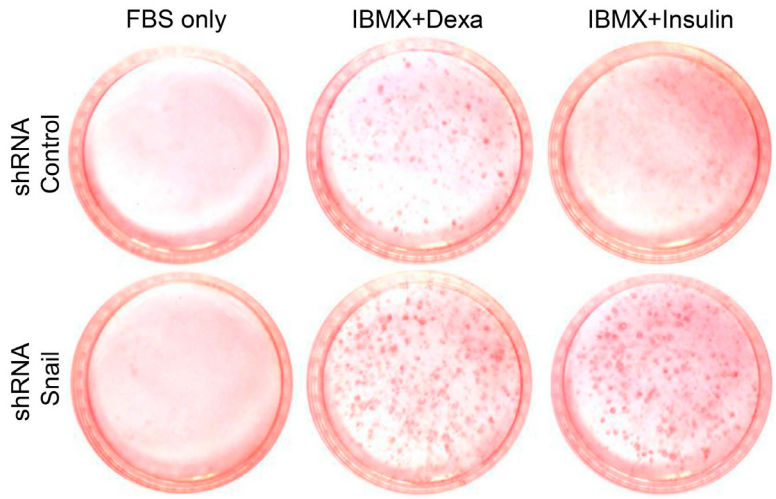

Figure 7. Oil Red O staining of murine adipocytes after knockdown of Snail. Snail or control shRNA were introduced into 3T3-L1 cells followed by treatment with indicated adipogenic inducers or FBS only. Knockdown of Snail stimulates the in vitro adipogenesis of murine preadipocytes. The data are representative of three independent experiments.

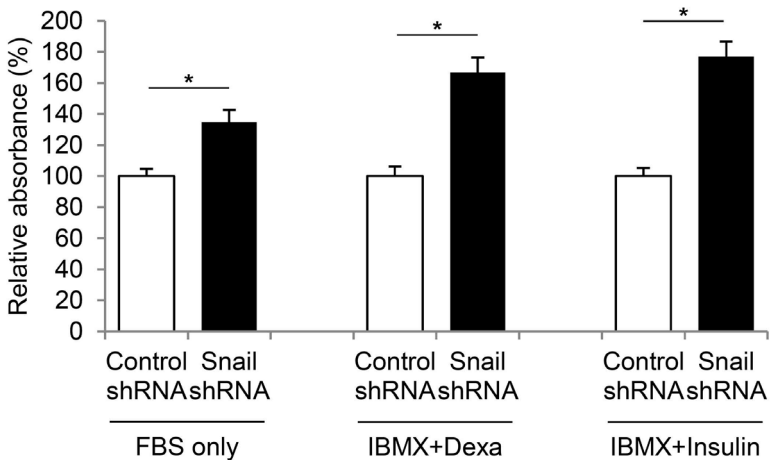

Figure 8. Spectrophotometer analysis of murine adipocytes after knockdown of Snail. Snail or control shRNA were introduced into 3T3-L1 cells followed by treatment with indicated adipogenic inducers or FBS only. Spectrophotometer analysis was performed for quantification of lipid accumulation. Knockdown of Snail stimulates the in vitro adipogenesis of murine preadipocytes. Results of three independent experiments were averaged and plotted as the mean ± SD.

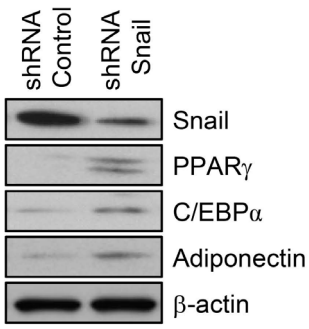

Figure 9. Immunoblot analyses in murine adipocytes after knockdown of Snail. Snail or control shRNA were introduced into 3T3-L1 cells followed by treatment with indicated adipogenic inducers or FBS only. Immunoblot analyses of Snail, C/EBPα, PPARγ and adiponectin expression were conducted in 3T3-L1 cells on day 8 after treated with either Snail or control shRNA. The data are representative of three independent experiments.

3. Snail suppresses the expression of PPARγ by directly binding to the E-box in the PPARγ gene promoter

Overexpression of Snail significantly suppressed the expression of PPARγ, which is one of the most crucial transcription factors for induction of adipogenic differentiation. It is known that transcription factor Snail represses E-cadherin expression by directly binding to the E-box motifs in the E-cadherin promoter.[6] In a similar manner, we hypothesized that Snail might regulate the expression of PPARγ by directly binding to the E-box motifs in the PPARγ promoter region. A schematic diagram of the mouse PPARγ2 promoter sequence is depicted in Figure 10. Three putative E-box motifs are located in the 1-kb PPARγ2 promoter sequence. Each motif contains a slightly different DNA sequence, which is consistent with the consensus sequence of the E-box (CANNTG). Among three putative E-box motifs in the mouse PPARγ2 gene promoter, two E-box sequences (E-box-707 and -154) are conserved in rat and human PPARγ2 gene promoters (Fig. 11). The presence of these conserved motifs suggests the possibility that E-boxes in the PPARγ2 gene promoter might be involved in regulation of PPARγ2 expression.

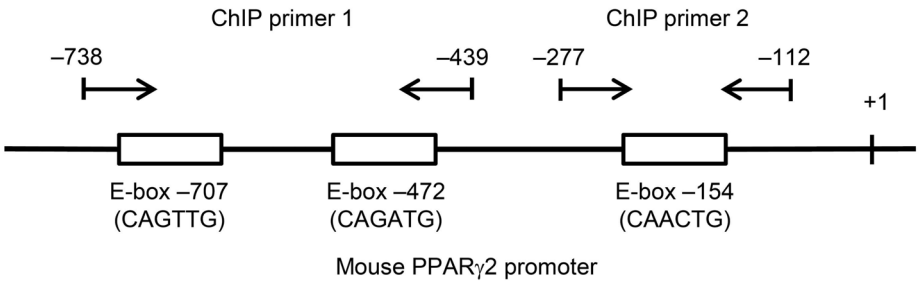

Figure 10. A schematic diagram of the mouse PPARγ2 promoter sequence. A schematic diagram of the mouse PPARγ2 promoter sequence spanning from –1021 bp to +1 bp (GenBank: NT_039353.7), with arrows indicating the forward and reverse primers used to amplify ChIP products. Pairs of ChIP primer 1 are located around the two distal E-boxes, which are the possible binding sites for Snail, and one proximal E-box is surrounded by pairs of ChIP primer 2.

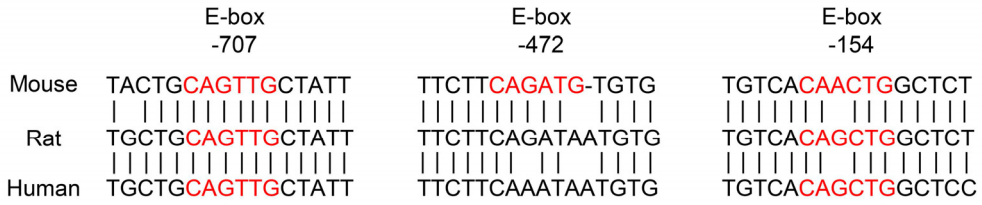

Figure 11. E-box motifs in the PPARγ2 gene promoter. E-box motifs in the mouse PPARγ2 gene promoter are conserved in rat and human PPARγ2 gene promoters. Mouse has three putative E-box regions, while rat and human have two E-box motifs (E-box-707 and -154). Predicted E-box motifs in each group are presented in red.

To further confirm that Snail is recruited to the putative E-box motifs in the PPARγ2 promoter, a ChIP assay was performed using two primers that covered the region of the three putative E-boxes (Fig. 12). Chromatin samples were prepared from 3T3-L1 preadipocytes and from differentiated adipocytes and immunoprecipitated with specific antibodies against Snail, HDAC1, HDAC2, acetylated histone H3 and total histone H3. In preadipocytes, both Snail and HDAC1/2 were recruited at the PPARγ2 promoter region containing the E-box motifs. However, the binding of Snail and HDAC1/2 was significantly decreased after adipogenic differentiation, whereas acetylation levels at histone H3 were reciprocally increased.

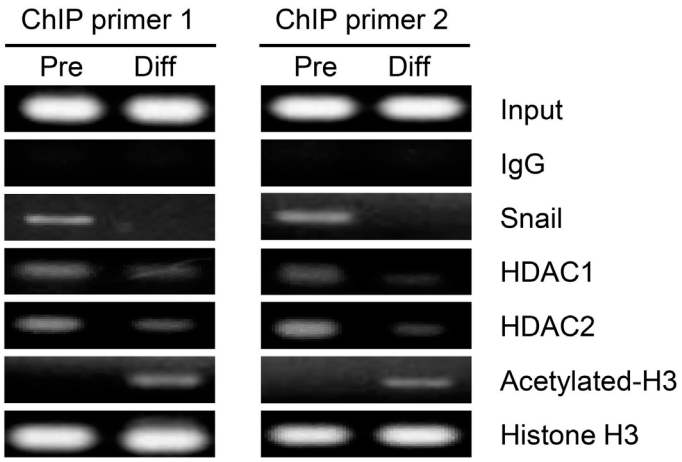

Figure 12. Chromatin immunoprecipitation assay for the upstream region of the PPARγ2 promoter in 3T3-L1 cells. ChIP assay was performed using antibodies against Snail, HDAC1, HDAC2, acetyl histone H3, or total histone H3 for the upstream region of the PPARγ2 promoter in 3T3-L1 cells. Presence of the promoter sequence prior to immunoprecipitation was confirmed by PCR (input sample), and immunoglobulin G antibody was used as a negative control. Snail is recruited to the E-box motif of the PPARγ2 promoter in 3T3-L1 preadipocytes, whereas the recruitment of Snail to the PPARγ2 promoter is decreased after differentiation of the preadipocytes into adipocytes. PCR products were separated by electrophoresis on 2% agarose gels containing ethidium bromide. The results are representative of three independent experiments.

To determine whether Snail regulates PPARγ expression, the activity of the PPARγ2 promoter in 3T3-L1 preadipocytes was measured by a luciferase assay using reporter plasmids that contained a 2-kb proximal fragment of murine PPARγ2 promoter (pGL-PPARγ2, Fig. 13).[13] Cells were transfected with either C/EBPα or Snail expression vectors, or both. The expression of PPARγ2 was dramatically increased under the presence of C/EBPα expression, whereas the co-expression of Snail was able to inhibit the luciferase activity stimulated by C/EBPα (Fig. 14). Similarly, the transfection of Snail significantly suppressed the luciferase activity when compared with luciferase activity in presence of the empty vector, implying an inhibitory effect of Snail on PPARγ2 transcription.

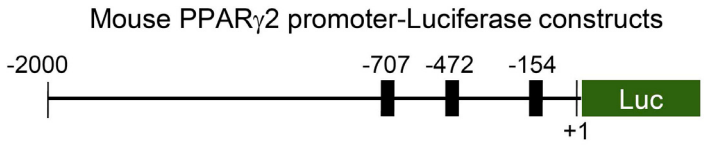

Figure 13. A schematic diagram of the luciferase reporter assay plasmid containing the mouse PPARγ2 promoter. The number indicates the 5′ end of the PPARγ2 promoter gene.

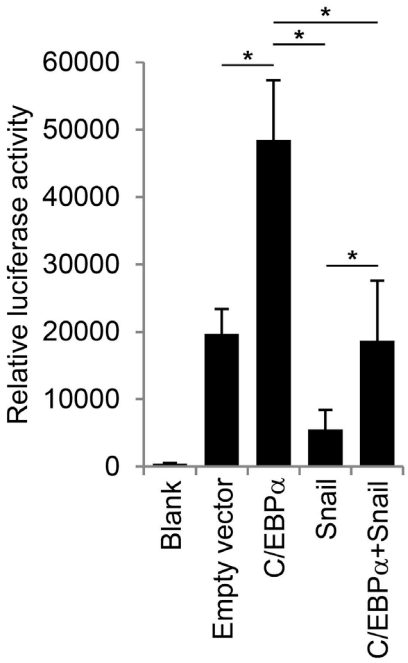

Figure 14. Luciferase reporter assay in 3T3-L1 preadipocytes transfected with indicated plasmids. To confirm whether Snail acts as a transcriptional repressor of PPARγ2 expression by binding to the PPARγ2 promoter, plasmids containing Snail or C/EBPα were cotransfected into 3T3-L1 preadipocytes along with the reporter vector containing the PPARγ2 promoter. Reporter assay showed that Snail suppresses C/EBPα-induced transactivation of the PPARγ2 promoter as well as the basal activity of the PPARγ2 promoter. The pGL3-basic vector and the blank well were used as negative controls, whereas C/EBPα-expressing plasmid was used as a positive control. Luciferase activity was normalized using Renilla expression activity. The data are presented as the mean ± SD of four independent experiments. *P<0.001.

To further confirm whether each putative E-box motif is functionally acting as a negative cis element to suppress the promoter activity of PPARγ, mutation of putative E-boxes was generated by site-directed mutagenesis. After cotransfection with reporter plasmids bearing mutant E-box-707 or -154 and either Snail plasmid or empty pcDNA3 vector, mutation in either E-box-707 or -154 significantly restored the luciferase activity suppressed by Snail expression in 3T3-L1 cells (Fig. 15). These findings indicate that Snail suppresses the expression of PPARγ2 by directly binding to the E-box in the PPARγ gene promoter.

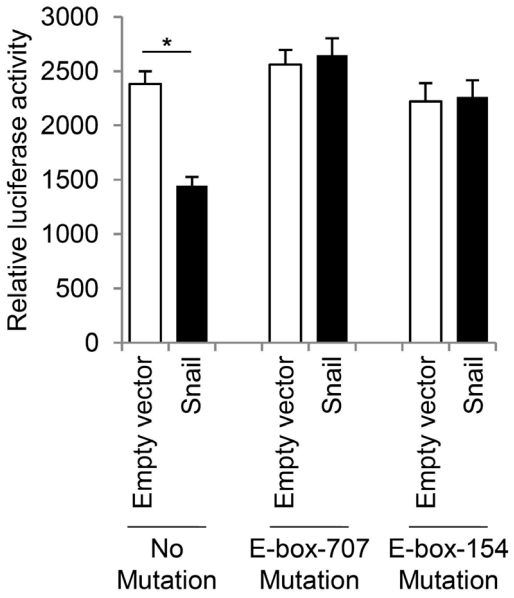

Figure 15. Luciferase reporter assay in 3T3-L1 preadipocytes after site-directed mutagenesis in the mouse PPARγ2 promoter. To assess whether each putative E-box is functional in vitro, sequences of E-boxes in the mouse PPARγ2 promoter were replaced by site-directed mutagenesis. 3T3-L1 preadipocytes were cotransfected with reporter plasmids bearing mutant E-box-707 or -154 and either Snail plasmid or empty pcDNA3 vector (control). Luciferase activity was normalized using Renilla expression activity. The data are presented as the mean ± SD of three independent experiments. *P<0.001.

We also evaluated the association between Snail and HDAC in the nuclear extract of 3T3-L1 cells. As shown in Fig. 16, the immunoprecipitate with anti-Snail antibodies showed a possible interaction of Snail with HDAC1 or HDAC2 in preadipocytes, whereas this association was diminished after differentiation due to decreased expression of Snail. The expression of Snail and adipogenic capacity were examined in 3T3-L1 cells after co-treated with adipogenic cocktails and trichostatin A (TSA, 100 nM), a potent HDAC inhibitor which blocks adipogenesis in vitro. As expected, cells cultured with TSA did not differentiate into mature adipocytes (Fig. 17) and they showed significantly increased expression of Snail in both preadipocytes and cells treated in adipogenic media for 8 days (Fig. 18).

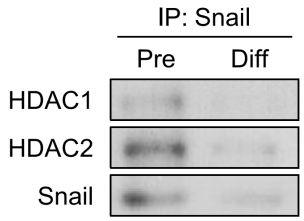

Figure 16. Interaction between Snail and HDAC1/2 in 3T3-L1 preadipocytes and differentiated adipocytes. Nuclear extracts of 3T3-L1 cells were immunoprecipitated with anti-Snail antibody and analyzed by immunoblot blotting with antibodies against HDAC1/2 and Snail.

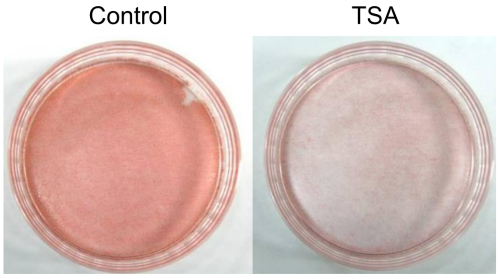

Figure 17. Oil Red O staining of 3T3-L1 adipocytes after treatment with trichostatin A. On day 8 after treatment with adipogenic cocktails, control and cells treated with TSA (100 nM) were stained with Oil Red O.

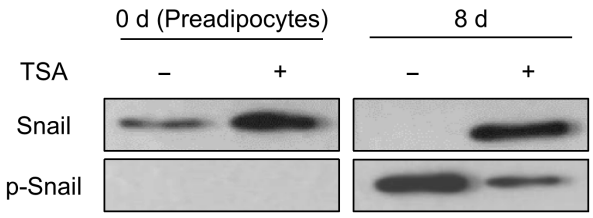

Figure 18. Immunoblot analysis of Snail in 3T3-L1 cells after treatment with trichostatin A. Immunoblot analysis of pre- and differentiated adipocytes treated with the indicated reagents on day 8. The expression levels of total Snail was significantly increased in cells treated with TSA, while phosphorylated Snail was induced after adipocyte differentiation.

4. Snail is involved in Wnt-GSK3β signaling-mediated inhibition of adipogenesis

Wnt signaling is one of the most essential pathways that blocks adipocyte differentiation through inhibition of adipogenic transcription factors, such as C/EBPα and PPARγ.[4] Moreover, it has been established that Wnt regulates its downstream enzyme GSK3β, which mediates the phosphorylation of Snail to modulate the expression of E-cadherin.[10] Based on these findings, we investigated whether Snail expression is affected by the Wnt-GSK3β axis. Wnt10b-expressing cells were not able to differentiate into adipocytes, whereas control preadipocytes showed a normal adipogenic potential (Fig. 19). Immunoblotting revealed that the expression of Snail was more than 2-fold higher in both Wnt10b-transfected preadipocytes and differentiated adipocytes when compared with control cells not transfected with Wnt10b (Fig. 20 and 21).

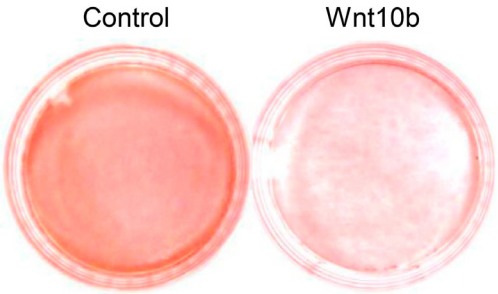

Figure 19. Oil Red O staining of 3T3-L1 adipocytes after treatment with Wnt10b. On day 8 after treatment with adipogenic cocktail, the control cells and cells transfected with plasmids harboring Wnt10b were stained with Oil Red O. These data are representative of three independent experiments.

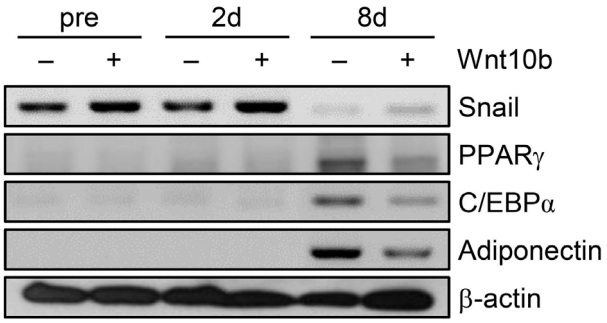

Figure 20. Immunoblot analysis of 3T3-L1 adipocytes after treatment with Wnt10b. Immunoblot analysis shows that Wnt10b increases the expression of Snail in preadipocytes and during adipogenic differentiation. The transfected 3T3-L1 cells were cultured for the indicated times before and after initiating the treatment with adipogenic hormonal cocktail. Actin was used as a loading control.

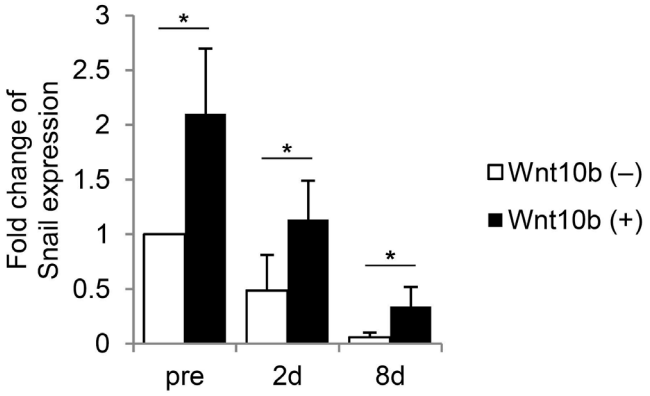

Figure 21. Densitometric graph of immunoblotting in 3T3-L1 adipocytes after treatment with Wnt10b. The graph shows densitometric analysis of the optical density-based data of the immunoblots shown in Fig. 20. It can be seen that Wnt10b increases the expression of Snail in 3T3-L1 cells, regardless of differentiation. *P<0.001 vs. compared to the cells without Wnt10b treatment (control). Data are presented as the mean ± SD of three independent experiments.

To examine whether Snail is involved in the pathway of Wnt-mediated suppression of adipogenesis, Snail expression was knocked down by using siRNA against Snail, followed by the cultivation of the preadipocytes in adipogenic media for 8 days. The capacity of adipogenic differentiation in Wnt10b-expressing preadipocytes was restored after treatment with siRNA against Snail, whereas control siRNA had no effect on the Wnt-mediated suppression of adipogenesis (Fig. 22). This finding was further supported by the results from immunoblots, showing that cells treated with Snail siRNA had significantly higher levels of PPARγ, C/EBPα and adiponectin (Fig. 23 and 24).

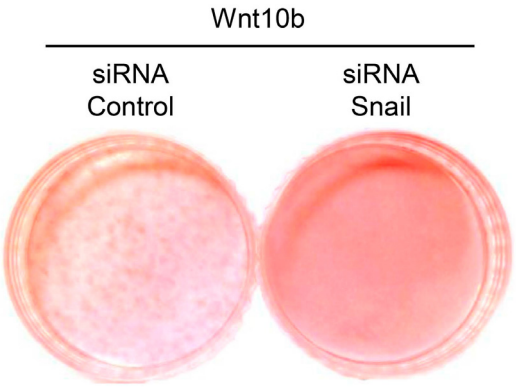

Figure 22. Oil Red O staining of Wnt10b-treated 3T3-L1 adipocytes after knockdown of Snail. To assess the role of Snail in the Wnt-mediated suppression of adipogenesis, 3T3-L1 preadipocytes transfected with Wnt10b-expressing plasmids were treated with Snail siRNA or scrambled control siRNA. On day 8, after the treatment with adipogenic cocktails, Snail siRNA-transfected cells show full differentiation into adipocytes.

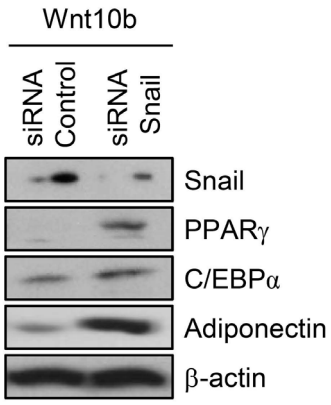

Figure 23. Immunoblot analysis of Wnt10b-treated 3T3-L1 adipocytes after knockdown of Snail. Immunoblot analysis of Snail, C/EBPα, PPARγ and adiponectin expression in Wnt10b-treated 3T3-L1 cells on day 8 after treated with either Snail or control siRNA. The data are representative of three independent experiments.

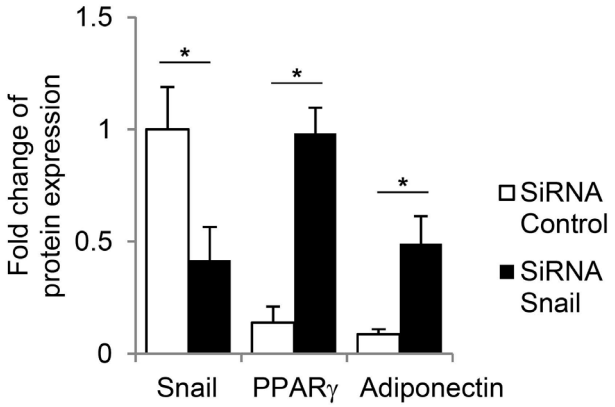

Figure 24. Densitometric graph of immunoblotting in Wnt10b-treated 3T3-L1 adipocytes after knockdown of Snail. Densitometric graph of the optical density-based data of the immunoblots. *P<0.001 vs. compared to the cells with control siRNA treatment (control). Data are presented as the mean ± SD of three independent experiments.

GSK3β mediates posttranslational regulation of Snail via phosphorylation; phosphorylated Snail is then targeted for degradation by the ubiquitin system. The kinase activity of GSK3β is abolished when Wnt signaling induces phosphorylation at Ser 9 in GSK3β. To test whether GSK3β regulates adipocyte differentiation via modulating the expression of Snail, immunoblotting was performed using antibodies against the phosphorylated form of GSK3β and total GSK3β protein after treatment with Wnt10b (Fig. 25). Compared to the control group, Wnt10b-treated preadipocytes and adipocytes showed increased expression levels of phosphorylated GSK3β and Snail. To manipulate the activity of GSK3β, 3T3-L1 cells were cultured in the presence of either LiCl, which is a well-known inhibitor of GSK3, or NaCl (as a control), followed by Oil Red O staining. As expected, inhibition of GSK3β suppressed adipocyte differentiation (Fig. 26), and the expression level of Snail significantly increased in a dose-dependent manner in LiCl-treated cells (Fig. 27). During the treatment with LiCl, Snail expression was knocked down by using siRNA against Snail, followed by the cultivation of the preadipocytes in adipogenic media for 8 days. The capacity of adipogenic differentiation in LiCl-treated preadipocytes was restored after treatment with Snail siRNA (Fig. 28), which was further confirmed by immunoblots showing that the expression of PPARγ, C/EBPα and adiponectin were significantly increased in Snail siRNA-treated cells (Fig. 29 and 30). These results indicate that the Wnt-GSK3β pathway blocks adipogenesis via induction of Snail in 3T3-L1

cells.

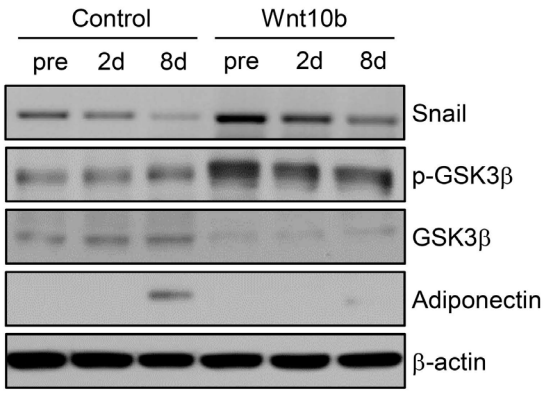

Figure 25. Immunoblot analysis of GSK3β in 3T3-L1 cells after treatment with Wnt10b. Immunoblot analysis shows that Wnt10b increases the expression of phosphorylated GSK3β and Snail in preadipocytes during adipogenic differentiation. Transfected 3T3-L1 cells were cultured for the indicated times before and after initiating the treatment with adipogenic hormonal cocktail. Actin was used as a loading control.

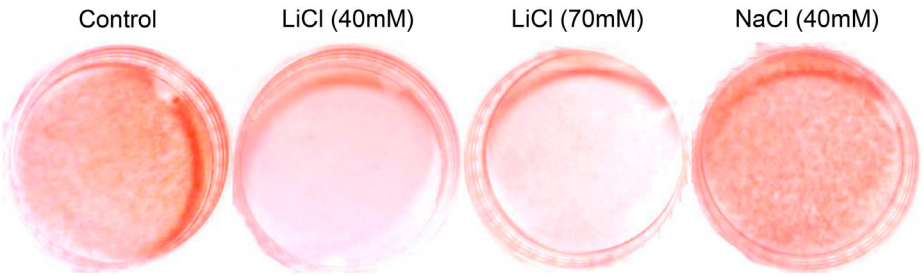

Figure 26. Oil Red O staining of 3T3-L1 adipocytes after treatment with LiCl, an inhibitor of GSK3. On day 8 after treatment with adipogenic cocktail, control and cells treated with LiCl (40 and 70 mM) or NaCl (40 mM) were stained with Oil Red O. Cells with no reagent and NaCl-treated cells were used as controls. The figures are representative of three independent experiments.

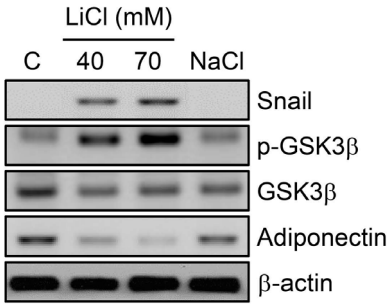

Figure 27. Immunoblot analysis of GSK3β in 3T3-L1 cells after treatment with LiCl. Immunoblot analysis of differentiated adipocytes treated with the indicated reagents on day 8. The expression levels of phosphorylated GSK3β and Snail are significantly increased in LiCl-treated cells. Cells with no reagent and NaCl-treated cells were used as controls. Actin was used as a loading control. The figures are representative of three independent experiments.

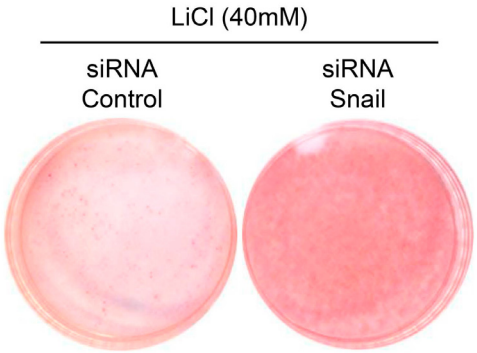

Figure 28. Oil Red O staining of LiCl-treated 3T3-L1 adipocytes after knockdown of Snail. To assess the role of Snail in the GSK3β-mediated suppression of adipogenesis, 3T3-L1 preadipocytes transfected with Snail siRNA or scrambled control siRNA were treated with LiCl (40 mM). On day 8, after the treatment with adipogenic cocktails, Snail siRNA-transfected cells differentiated into adipocytes, confirmed by Oil Red O staining. The figures are representative of three independent experiments.

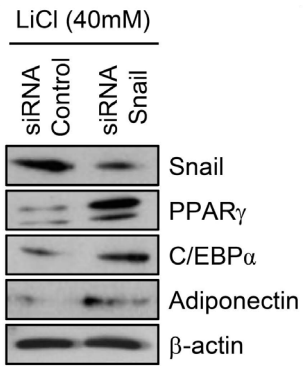

Figure 29. Immunoblot analysis of LiCl-treated 3T3-L1 adipocytes after knockdown of Snail. To assess the role of Snail in the GSK3β-mediated suppression of adipogenesis, 3T3-L1 preadipocytes transfected with Snail siRNA or scrambled control siRNA were treated with LiCl (40 mM). On day 8, after the treatment with adipogenic cocktails, immunoblotting was conducted. The figures are representative of three independent experiments.

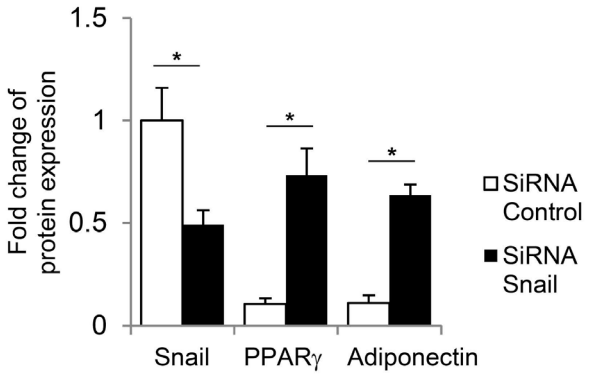

Figure 30. Densitometric graph of immunoblotting in LiCl-treated 3T3-L1 cells after knockdown of Snail. Densitometric graph of the optical density-based data of the immunoblots. *P<0.001 vs. compared to the cells with control siRNA treatment (control). Data are presented as the mean ± SD of three independent experiments.

5. Snail inhibits adipogenesis in vivo

To support the *in vitro* evidence that Snail inhibits adipogenesis, we adopted an *in vivo* model of adipocyte differentiation.[14] The 3T3-F442A preadipocytes were transfected with either Snail-expressing or control backbone plasmids (Fig. 31) and harvested with trypsin, after which 3×10^7 cells were injected subcutaneously into the flank area of athymic nude mice. Injected sites were initially swollen; however, the swelling disappeared shortly over a few days. After 7 days, a flat thickened mass began to appear in the injected flank area, and discrete fat pads were formed, which increased in size over the next several weeks (Fig. 32). The mice were sacrificed at 5 weeks after implantation of the transfected preadipocytes. A well-defined mass resembling fat tissue (indicative of normal adipocyte differentiation) was observed in the mice injected with cells containing a backbone control plasmid. Histology of the isolated tissues showed that the control plasmid-transfected 3T3-F442A cells were normally differentiated into adipocytes, whereas Snail plasmid-transfected cells were not differentiated into adipocytes, presenting as undifferentiated fibroblast-like cells (Fig. 33). These findings suggest that Snail also blocks adipocyte differentiation in a physiological *in vivo* system.

Figure 31. Overexpression of Snail in 3T3-F442A cells. The 3T3-F442A cells were transfected with plasmids harboring Snail or backbone control plasmids, confirmed by immunoblots.

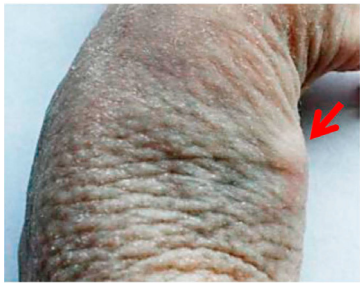

Figure 32. Fat mass formation in athymic mice after subcutaneous injection of 3T3-F442A cells. After 2 days of transfection with Snail plasmids, preadipocytes (3×10^7 cells) were harvested with trypsin and were injected subcutaneously into the flank area of athymic mice. After 5 weeks of implantation, a mass was developed at the site of injection. Red arrows indicate mass formation in the flank.

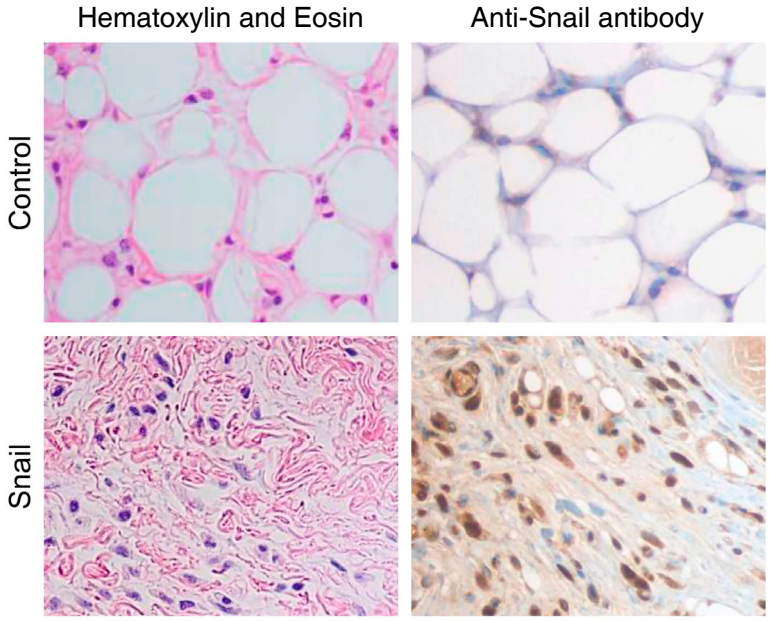

Figure 33. Histology and immunohistochemistry of the tissues isolated from athymic mice xenografts. The dissected mass was fixed in formalin, followed by staining with hematoxylin and eosin (left column). Immunohistochemistry with anti-Snail antibody was also performed in the background of hematoxylin (right column). The left upper figure shows normal adipogenesis in 3T3-F442A cells of the control group, whereas the left bottom figure indicates that Snail-overexpressing cells were not differentiated into adipocytes in vivo. Immunohistochemistry shows Snail positive brown nuclei in the tissue of mice injected with Snail-overexpressing cells (right bottom), while differentiated adipocytes in control mice have no expression of Snail. Figures are representative of four tissues. Magnification, ×400.

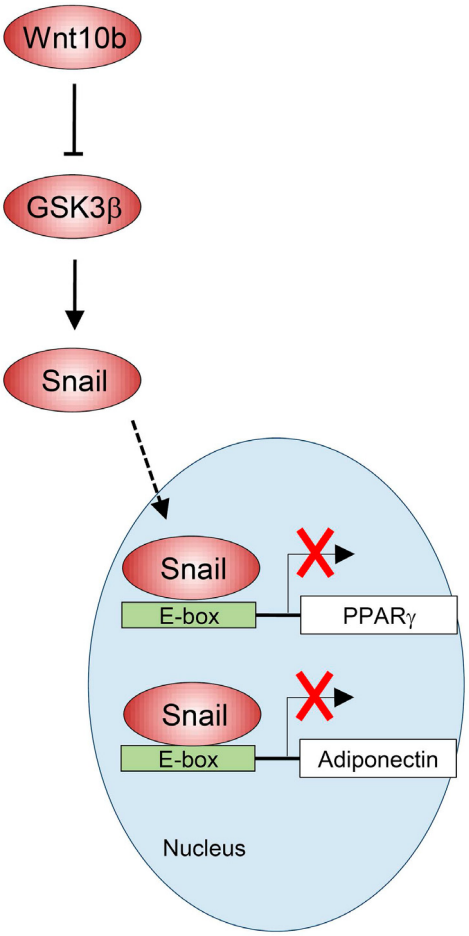

Figure 34. A schematic illustration showing the putative mechanism by which Snail inhibits adipocyte differentiation.

IV. DISCUSSION

Recently, the obesity epidemic has drawn much attention and persuaded research in the area of adipose cell biology, particularly the development and formation of white adipose tissue. Adipocyte differentiation is a complex process induced by external stimuli such as hormones and chemokines, followed by the precise coordination and control of transcriptional factors, cell-cycle regulators, and other cofactors.[3] During adipogenesis, the transcription factors PPARγ and C/EBPα are major determinants of cell differentiation.[15] PPARγ, a member of the nuclear receptor superfamily, is a well-established target of thiazolidinedione, which is an insulin-sensitizing antidiabetic drug.[16] A number of studies have shown that PPARγ is essential for adipogenesis both in vitro and in vivo, and that its expression is tightly regulated by various factors.[3] However, factors/effectors that control the expression of PPARγ have not been fully elucidated.

Our study is the first to identify the functional role of Snail in adipogenesis. The principal findings in the current study are: (1) the nuclear expression of Snail gradually decreases during adipocyte differentiation after exposure to an adipogenic cocktail, (2) Snail inhibits adipocyte differentiation by directly binding to the E-box motifs of the PPARγ promoter, without affecting the proliferation and survival of preadipocytes, and (3) Snail is involved in the Wnt-GSK3β signaling pathway-mediated suppression of adipogenesis.

It has been previously reported that Snail is expressed in NIH-3T3 fibroblasts,[17] which lack the potential to differentiate in the presence of conventional adipogenic hormones. However, no research has been conducted to show the expression pattern of Snail in 3T3-L1 cells during adipocyte differentiation. Time-course analysis of the expression of Slug, which is the second member of the Snail superfamily, showed that its expression significantly increased in preadipocytes,[8] which is consistent with our results. However, contrary to Snail, Slug expression was slightly diminished, but still observed in differentiated adipocytes, implying that their functions in the adipocytes might be different.

Consistent with our findings, Park et al. also showed that Snail did not affect proliferation or viability of mesenchymal stem cells and preosteoblasts.[18] This suggests that Snail does not affect cell proliferation or apoptosis in preadipocytes, however, this finding does not guarantee that Snail is not involved in the process of mitotic clonal expansion during adipocyte differentiation. It is well established that the PPARγ promoter contains C/EBP binding sites, where C/EBPδ, C/EBPβ, and C/EBPα are able to bind and transactivate PPARγ gene expression during adipogenesis.[19,20] Similar to Snail, GATA-2 and GATA-3, which belong to the zinc-finger family of transcription factors, directly suppress the expression of PPARγ by binding to the promoter region of the PPARγ gene.[21] These GATA transcription factors are initially found in preadipocytes, but dissipate after induction with adipogenic media,[21]

which is consistent with the expression pattern of Snail.

There are few published reports on the regulation of PPARγ expression by E-boxes, which are present in the proximal region of the PPARγ promoter. Fajas et al. reported that PPARγ expression is regulated by ADD-1/SREBP-1 and SREBP-2 through the PPARγ1 and PPARγ3 promoters that contain a consensus E-box motif.[22] Interestingly, genetic studies showed that polymorphism at the putative E-box in the PPARγ2 promoter region determined the promoter activity and was associated with obesity and type 2 diabetes in humans.[23] This suggests the clinical significance of E-box in the PPARγ gene promoter. Slug has been shown to bind to the PPARγ promoter and regulate PPARγ expression by modulating HDAC recruitment.[8] Similarly, our ChIP assay showed that the recruitment of HDAC and the status of the acetylated histone H3 were changed after differentiation of preadipocytes into adipocytes, indicating that Snail may be involved in HDAC-mediated transcriptional regulation process. This is a plausible hypothesis, particularly in the light of previous reports that Snail suppresses E-cadherin expression by recruitment of HDAC and mSin3A complex in mammalian keratinocytes.[24] Therefore, the identification of putative Snail-binding sites in the PPARγ promoter provides further understanding of the complex regulation of PPARγ expression during adipogenesis.

Wnt10b, which is secreted from preadipocytes, inhibits the kinase activity of GSK3β. This is followed by the activation and accumulation of

β-catenin, which translocates into the nucleus and stimulates the TCF/LEF transcription factors.[15] These events eventually inhibit the expression of PPARγ. However, further downstream effectors of this pathway have not been fully elucidated. It is known that Snail (SNAI1) usually acts as a transcription repressor and triggers EMT by directly downregulating the expression of E-cadherin in the mesoderm.[6] The Wnt-GSK3β-Snail axis has been identified in breast carcinoma cell lines by Yook et al., indicating that Snail contains β-catenin-like canonical motifs that are regulated by Wnt-GSK3β-dependent phosphorylation.[10] Based on this speculation and our current findings, it is reasonable for us to propose that Snail can also act as a mediator in the process of adipogenic differentiation under the control of the Wnt and GSK3β pathways (Fig. 34).

We have previously demonstrated that Snail not only suppresses the expression of PPARγ, but also inhibits adiponectin expression via directly binding to the E-box of the proximal region of the PPARγ.[9] Taken together, our data shows that Snail plays a crucial role during both early and late stages of adipocyte differentiation. Although we demonstrated the inhibitory effect of Snail on adipogenesis using an in vivo murine model, a previous study of transgenic animals expressing Snail reported that no abnormal phenotypes were observed.[25] This model showed only a mild increase (~20% above normal) in Snail expression and was not examined for the purpose of adipose tissue research, such as effects of high fat diet. Unfortunately, the effect of Snail on

PPARγ expression in adipose tissue cannot be assessed in Snail knockout mice, because these mice show a lethal phenotype during gastrulation.[26]

Recently, it was shown that human adipose tissue progenitor cells are not able to differentiate into normal fat cells through the action of TGFβ family members produced from macrophages within adipose tissues.[27] Progenitor cells affected by these macrophages were altered into myofibroblast-like cells, which expressed high levels of Snail. This decrease in the adipogenic potential of progenitor cells indicates a reduction in normal adipocyte hyperplasia, with a reciprocal increase in myofibroblasts. In addition, myofibroblasts can cause fibrosis in adipose tissue, which may inhibit adipocyte hypertrophy in mice.[28] Therefore, both decreased adipocyte hyperplasia and hypertrophy may reduce expandability of the subcutaneous fat tissue, leading to ectopic fat deposition.[27] Taken together, dysregulation of Snail in progenitor cells of adipose tissue by external factors, such as TGFβ, might affect the lipid-storing capacity of adipose tissues and promote a lipodystrophy-like condition.

The present study has some limitations, which should be addressed by further experiments. Further research is necessary to determine the association between Snail and β-catenin, which are both downstream mediator molecules of Wnt signaling and have inhibitory effects on adipogenesis. Moreover, experiments with genetically engineered animal models such as adipose tissue-specific Snail knockout mice or transgenic animals expressing Snail should be conducted in the future to confirm the putative role of Snail in

adipocyte differentiation.

V. CONCLUSION

The present study demonstrated for the first time that Snail regulates adipocyte differentiation by binding to the E-box motif in the PPARγ promoter. Elucidation of the underlying mechanisms of Wnt/GSK3β-mediated suppression of adipogenesis may broaden the knowledge of the complex network of developmental processes in the adipose tissue and support the idea that Snail may be a promising candidate for the treatment of obesity and obesity-related diseases. Furthermore, identification of molecules that modulate the expression of Snail could be a rewarding approach for future obesity research.

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ABSTRACT(IN KOREAN)

전사 인자 Snail의 지방 세포 분화 억제 기전 규명

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이 용 호

전사인자 Snail은 중배엽 형성, epithelial-mesenchymal transition의 유도 등 세포의 운명 결정을 조절하는 과정에서 중요한 역할을 하는 것으로 알려져 있다. 최근 본 연구진은 3T3-L1 세포의 지방 분화 과정에서 Snail이 아디포넥틴(adiponectin)의 발현을 조절한다는 사실을 발견하였다. 본 연구에서는 새로운 지방 분화 조절 매개체를 규명하기 위해 Wnt, GSK3β (Glycogen synthase kinase-3β) 등에 의해 조절되며, 주로 발달 과정에서 전사 억제 인자로 작용하는 Snail의 지방 세포 분화 억제

작용을 검증하고 그 기전을 밝히고자 하였다. 3T3-L1 세포의 지방 분화시, Snail 발현은 급격히 감소하였으며, Snail을 과발현시키면 PPARγ, C/EBPα의 발현이 감소하면서 지방 분화가 억제됨을 관찰하였다. 크로마틴 면역침강법과 luciferase 분석을 통해 Snail이 PPARγ 프로모터의 E-box 부위에 직접 결합하여 PPARγ의 전사 활성도를 억제함을 확인하였다. Wnt10b는 GSK3β를 인산화시켜 Snail 발현이 증가되고, 그로 인하여 3T3-L1 세포에서 지방 분화가 억제되었다. 반면, Snail siRNA를 발현시킨 지방전구세포에서는 이러한 과정이 억제되어, 지방 분화능이 회복되었다. GSK3β 억제제인 LiCl을 처리한 세포에서도 Snail 발현이 증가되고, 지방 분화가 억제됨을 관찰하였다. Snail을 과발현시킨 3T3-F442A 세포는 면역결핍 쥐 모델에서 지방세포로 분화되지 않았다. 이러한 결과를 토대로, Snail은 지방 분화의 새로운 조절자임을 확인하였고, 이는 PPARγ의 발현을 직접 억제함으로써 이루어진다. 또한 Snail의 발현은 Wnt-GSK3β 신호 전달을 통해 조절됨을 확인하였다.

핵심되는 말 : 지방 분화, Snail, PPARγ, Wnt 신호

PUBLICATION LIST

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