The role of G0/G1 switch gene 2 in the transition from mitotic clonal expansion to terminal differentiation during adipogenesis

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The role of G0/G1 switch gene 2 in the transition from mitotic clonal expansion to terminal differentiation during adipogenesis

Directed by Professor Jae-woo Kim

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Hyeonjin Choi

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This certifies that the Doctoral Dissertation of Hyeonjin Choi is approved.

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감사합니다.

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ABSTRACT

The role of G0/G1 switch gene 2 in the transition from mitotic clonal expansion to terminal differentiation during adipogenesis

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(Directed by Professor Jae-woo Kim)

Mouse 3T3-L1 preadipocytes differentiate into adipocytes when treated with 3-isobutyl-1-methylxanthine, dexamethasone, and insulin. After hormonal induction, growth-arrested 3T3-L1 preadipocytes reenter the cell cycle, known as mitotic clonal expansion, and then express genes required for the adipocyte phenotype. Although the mechanisms of adipogenesis including transcriptional cascade have been revealed during past 40 years, it is still unclear how cells after clonal

expansion eventually enter the terminal differentiation program. This study aims to elucidate novel regulators of terminal differentiation after clonal expansion of preadipocytes. When fresh 3T3-L1 preadipocytes were exposed to the conditioned medium harvested from the cells at clonal expansion stage, cells showed acceleration of the differentiation into mature adipocytes, indicating the presence of cell-specific regulators modulating transitional events from mitotic clonal expansion to terminal differentiation. In an effort to identify novel regulators, gene expression profiles of differentiating preadipocytes were analyzed using an algorithm. As a result, I found G0/G1 switch gene 2 (G0s2) as a novel regulator of adipogenesis. G0s2 was expressed at a higher level in white and brown adipose tissues, and induced upon a hormonal cocktail in 3T3-L1 cells. Importantly, G0s2 expression was accelerated by conditioned medium, suggesting that this molecule is associated with the transition into the terminal differentiation. Knockdown of the G0s2 expression using siRNA inhibited adipocyte differentiation, whereas constitutive overexpression of G0s2 accelerated the differentiation from preadipocytes to mature adipocytes. G0s2 expression was regulated by peroxisome proliferator-activated receptor γ (PPARγ) which is the well-known regulator of adipocyte differentiation, and the absence of either PPARγ or G0s2 resulted in the activation of apoptotic pathway before undergoing terminal differentiation. To determine whether G0s2 plays a role *in vivo*, *g0s2* knockout mice were generated. *G0s2* knockout mice were normal in appearance, except reduction in adipose mass compared with wild-type

littermates, indicating an impairment of adipogenesis. These results also suggest that G0s2 plays as a contributing factor in lipid droplet formation. In conclusion, G0s2 is required for adipogenesis and accumulation of triacylglycerol, playing an important role in the transition from mitotic clonal expansion to terminal differentiation.

Key words: adipogenesis, conditioned medium, mitotic clonal expansion, terminal differentiation, G0/G1 switch gene 2

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Ⅰ. INTRODUCTION

Obesity is a serious health problem in many industrialized countries and a contributing risk factor for several diseases, such as hypertension, cancer, diabetes and atherosclerosis[1]. As the major cellular component in adipose tissue, adipocytes play a central role in normal physiology[2]. Obesity results from an excessive accumulation of white adipose tissue, accompanied by increased size and number of

adipocytes[3]. While a number of molecular mechanisms that regulate adipocyte development have been elucidated, there is a clear need to fully define the complex molecular processes that control adipose tissue formation, in an effort to manipulate its accretion. In this study, I set out to identify novel regulators expressed between mitotic clonal expansion and terminal differentiation of preadipocytes to the mature adipocytes.

Adipogenesis is the process of the formation of new adipocytes from preadipocyte precursors, thus it is important for the understanding and control of obesity[4]. *In vitro* models of adipogenesis, 3T3-L1 preadipocyte cell line represents a useful tool for our understanding of the molecular mechanisms underlying orchestrate adipocyte differentiation[5]. Adipose differentiation is a complex process which is driven by the proadipogenic hormonal cocktail. Upon reaching confluence, proliferating preadipocytes become growth arrested by contact inhibition. When post-confluent 3T3-L1 preadipocytes cells were exposed to the adipogenic agents such as dexamethasone and 3-isobutyl-1-methylxanthine, and insulin, growth-arrested preadipocytes reenter the cell cycle called mitotic clonal expansion, arrest proliferation again, and finally undergo terminal differentiation[6-8]. This process is under the control of transcriptional cascades mainly composed of CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPARγ)[9]. After hormonal induction, the cells immediately express C/EBPβ and C/EBPδ, which in turn induce the expression of the C/EBPα and PPARγ, the key

regulators for the adipose-specific phenotype[10,11]. C/EBPα and PPARγ act synergistically to govern the terminal differentiation process[12].

As they differentiate, the fibroblastic preadipocytes stop dividing and acquire a rounded shape, lipid-filled mature adipocytes[13]. These morphological changes result from changes in cytoskeletal organization and contacts with the extracellular matrix which comprise relevant growth factors and basement membrane proteins collagen IV, laminin, hyluronan, fibronectin[14,15]. During differentiation of 3T3-L1 preadipocytes to adipocytes, several proteins are secreted. Adipocyte-specific or -enriched secreted proteins have peripheral and central effects on obesity and related diseases[16,17]. At the protein level, secreted proteins are identified by one-dimensional-electrophoresis and liquid chromatography-based separation combined with tandem mass spectrometry (MS/MS)[18]. The secreted proteins during adipogenesis which include several factors involved in growth regulation and adipocyte differentiation are presented using a systemic proteomic approach, based on the selective blocking of secretion[19]. Therefore, it is likely that some factors are involved in the transition from mitotic clonal expansion to terminal differentiation. However, either a specific protein or events governing this process was not fully elucidated so far.

Mitotic clonal expansion is required for 3T3-L1 adipogenesis, because selective inhibition of each steps of this process totally blocks adipogenesis[7]. G1 arrested cells begin to express C/EBPβ, which is serially phosphorylated by MAPK

and glycogen synthase kinase β (GSK3β), obtaining a full binding capacity to DNA[20]. This activation of C/EBPβ is clearly associated with mitotic clonal expansion, as cells without C/EBPβ cannot complete clonal expansion[21]. These studies partially revealed a role of mitotic clonal expansion, explaining why C/EBPβ is needed for entering terminal differentiation process; however, it remains largely unknown which factors or events are prepared as a result of mitotic clonal expansion, thereby cells are ready to enter terminal differentiation program.

The aim of the present work is to analyze the specific event of transition from mitotic clonal expansion to terminal differentiation. I hypothesized a presence of cell-specific events during a transition period, assuming that cells can undergo the terminal differentiation program as a result of mitotic clonal expansion.

3T3-L1 preadipocytes were induced to differentiate, and after 2 days, I collected cell-exposed medium after clonal expansion, designated as conditioned medium (hereafter called CM). When fresh 3T3-L1 preadipocytes were exposed to CM, cells accelerated the differentiation into mature adipocytes, indicating a presence of cell-specific transitional events between mitotic clonal expansion and terminal differentiation.

I analyzed the gene-expression profiles of 3T3-L1 cells to identify novel genes pivotal in the adipogenic process. In this analysis, emphasis was given to genes whose expression was limited to the first 48 h after initiation of differentiation for identifying critical regulators between mitotic clonal expansion

and terminal differentiation. Candidate genes were ranked based on an algorithm modeling the complexity of each gene-expression profile. As a result, I identified the G0/G1 switch gene 2 (G0s2) with a high score.

G0s2 was originally identified by its transient induction in lymphocytes during a lectin-induced re-entry into G1 phase from G0 phase favoring cell proliferation[22]. A study showed that murine G0s2 is predominantly expressed in adipose tissue and is up-regulated by PPARγ in 3T3-L1 cells[23]. More recently, it has been suggested that G0s2 is involved in lipid metabolism as an inhibitor of adipose triglyceride lipase (ATGL)-mediated lipolysis[24]. It was also demonstrated that G0s2 and comparative gene identification (CGI)-58 can physically interact with a minimal active domain of ATGL[25]. These reports suggest that G0s2 plays a role in the control of lipolysis; however, a role of G0s2 in adipogenesis has not been investigated so far. In this report, I present results showing the G0s2 is a novel regulator of the adipogenic process. Adipogenesis was blocked in the absence of G0s2 expression, and knockout mice showed reduced fat tissues. Taken together, it is suggested that G0s2 is required for adipogenesis, playing an important role in the transition from mitotic clonal expansion to terminal differentiation.

Ⅱ. MATERIALS AND METHODS

1. Cell culture and induction of differentiation

3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 8 mg/ml biotin, supplemented with 10% heat-inactivated calf serum (Invitrogen) at 37°C, in an atmosphere of 90% air and 10% CO_2. To induce differentiation, 2-day postconfluent 3T3-L1 cells (designated day 0) were incubated in DMEM containing 10% fetal bovine serum (FBS, Invitrogen), 0.5 mM 3-isobutyl-1-methylzanthine (Sigma, St. Louis, MO, USA), 1 μM dexamethasone (Sigma), and 1 μg/ml of insulin (Roche, Penzberg, Germany) (designated as MDI) for 2 days. Cells were then cultured in DMEM containing 10% FBS and insulin for another 2 days, after which they were grown in DMEM containing 10% FBS. Cell numbers were determined on day 2. For preparation of conditioned medium, 2-day postconfluent 3T3-L1 cells were induced by hormonal agents for 48 h and harvested cell-exposed medium.

2. Oil red-O staining

After differentiation, cells were washed once in phosphate-buffered saline (PBS) and fixed with 4% formalin in phosphate-buffered saline for 5 min, rinsed with distilled water. The staining solution was prepared by dissolving 0.5 g oil red-

O (Sigma) in 100 ml of isopropanol; 6 ml of this solution was mixed with 4 ml of distilled water, and filtered. The fixed cells were stained with staining solution for 1 h. The staining solution was removed and cells were rinsed twice with distilled water.

3. RNA isolation and PCR analysis

Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA synthesis from 5 μg of total RNA was performed using SuperScript II reverse transcriptase (Invitrogen) primed by random hexamer primer. The transcripts of G0s2, PPARγ2, C/EBPα and GAPDH were evaluated by PCR analysis. PCR was performed using the forward and reverse primers; 5'-AAAGT GTGCA GGAGC TGATC-3', 5'-GGACT GCTGT TCACA CGCTT-3' (G0s2), 5'-TATGG GTGAA ACTCT GGGAG-3', 5'-GCTGG AGAAA TCAAC TGTGG-3' (PPARγ2), 5'-TGGAC AAGAA CAGCA ACGAG-3', 5'-TCACT GGTCA ACTCC AGCAC-3' (C/EBPα), 5'-ACCAC AGTCC ATGCC ATCAC-3', 5'-TCCAC CACCC TGTTG CTGTA-3' (GAPDH). Real-time qPCR was employed to detect other transcripts of Gas1, Gas2, Gas5, Gas6, G0s2, C/EBPα, PPARγ and aP2/422. Real-time qPCR was performed using SYBR Green Master mix (Applied Biosytems, Foster city, CA, USA) in a total volume of 20 μl. Transcripts were detected by real-time qPCR with a Step One instrument (Applied

Biosytems). A standard curve was used to calculate mRNA level relative to that of a control gene, ribosomal L32. Primer pairs for specific target genes were designed as listed in Table 1. All reactions were performed in triplicate. Relative expression levels and S.D. values were calculated using the comparative method.

4. Western blot analysis and antibody

For protein analysis, cells were washed with cold PBS. Cells were then lysed directly in lysis buffer containing 1% sodium dodecyl sulfate (SDS) and 60 mM Tris-HCl, pH 6.8. The cell lysate was heated at 100°C for 10 min and centrifuged for 5 min at 13,000 rpm and supernatants were then frozen until further analysis. For Western blotting, cell extracts were subjected to SDS-PAGE and then transferred to nitrocellulose membranes and the blots were blocked with blocking buffer (5% nonfat dry milk in Tris-buffered saline-tween 20, TBST) prior to incubation with antibody. Primary antibodies used for blotting were anti-C/EBPβ, anti-C/EBPα[26], anti-PPARγ (Santa Cruz, CA, USA), anti-GAPDH, anti-caspase3, anti-Bax, anti-p-Bad, anti-p21, anti-p27, anti-FAS (Cell Signaling, Danvers, MA, USA), anti-FLAG (Sigma), anti-Rb (BD Biosciences, San Jose, California, USA). After blocking filters were immunoblotted with each primary antibody for 2 h at room temperature, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Santa Cruz) for 1 h. Target proteins were visualized by enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway,

NJ, USA).

5. FACS analysis

3T3-L1 cells were trypsinized and collected by centrifugation, washed with PBS, and fixed with 90% cold methanol. Fixed cells were washed with PBS and staining with 50 μg/ml propidium iodide and 100 μg/ml RNase A in PBS for 30 min in the dark. Labeled cells were analyzed using a FACS caliber flow cytometry system (BD Biosciences) and data were analyzed using ModFit software.

6. BrdU labeling and immunofluorescence analysis

BrdU labeling was done using 5-Bromo-2'-deoxy-uridine Labeling and Detection Kit I (Roche) according to the manufacturer's instructions. Briefly, cells were plated on glass cover slips until 2-day postconfluent and induced to differentiate with differentiation medium. After 16 h, cells were incubated for 30 min with 10 μM BrdU labeling solution to label DNA synthesis. The cover slips were fixed in 70% ethanol for 20 min at -15°C to -25°C for immunofluorescence analysis. Fixed cells were incubated with anti-BrdU primary antibody for 30 min at 37°C. After washing, anti-mouse-Ig-fluorescein secondary antibody was added to cover slips for 30 min at 37°C, incubated 0.1 μg/ml DAPI (Vector Laboratories, Burlingame, CA, USA) for 5 min at room temperature, the cover slips were mounted for immunofluorescence microscope analysis.

7. RNA interference (siRNA)

3T3-L1 preadipocytes were plated into 60-mm-diameter dishes 18–24 h prior to transfection. The following double-stranded stealth siRNA oligonucleotides (Invitrogen) were used: mouse G0s2, sense 5'-AGGGA CUGCU GUUCA CACGC UUCCC-3', antisence 5'-GGGAA GCGUG UGAAC AGCAG UCCCU-3', mouse PPARγ, sense 5'-CAGAG CAAAG AGGUG GCCAU CCGAA-3', antisense 5'-UUCGG AUGGC CACCU CUUUG CUCUG-3', mouse ATGL, set of three validated siRNA oligonucleotides (cat# 1320003). Control oligonucleotides with comparable GC content were also from Invitrogen. For knockdown, cells were transfected with control or gene-specific siRNA at 50 nM in OPTI-MEM medium using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocol. The next day, the medium was replaced with fresh DMEM containing 10% calf serum and the cells were incubated for 24 h before the induction of differentiation. Total RNA and protein extracts were prepared from the cells at the indicated time points, and RT-PCR and immunoblot analyses were performed. Oil red-O staining of G0s2 knockdown was performed at day 5.

8. Staining with Annexin-V and propium iodide (PI)

The AnnexinV-FITC Apoptosis Detection Kit (BD Biosciences) was used to stain cells in Annexin binding buffer according to the manufacturer's instructions. Briefly, cells were induced differentiation, after 48h, the cells were trypsinized,

pelleted, washed in PBS, and resuspended in 1x binding buffer containing Annexin-V-FITC antibody and PI according to the manufacturer's indicated protocol. The samples were analyzed by BD FACS caliber flow cytometry system (BD Biosciences).

9. TUNEL assay

The terminal deoxynucleotidyl transferase-mediated dUTP end labeling (TUNEL) assay was used to identify cell apoptosis. Cells were seeded on glass cover slips placed in 6-well dishes, at 48 h after differentiation, fragmented DNA staining of apoptotic cells was carried out using an In Situ Cell Death Detection Kit, Fluorescein (Roche). Briefly, the cells were rinsed with PBS followed by fixation using 4% paraformaldehyde for 1 h at room temperature. The fixed cells were permeabilized by incubation with 0.5% Triton X-100 in PBS for 1 h at 37°C. The cells were rinsed again with PBS and incubated with 50 μl per sample of TUNEL reaction mixture for 1h at 37°C. Following washes with PBS, sample was mounted on glass slide with Vectashield mounting medium and examined under inverted confocal microscope.

10. Transient transfection assay

G0s2 overexpressing vector (pcDNA3.0-G0s2-FLAG) was generated by

inserting the whole open reading frame of mouse G0s2 with a N-terminal FLAG tag into pcDNA3.0 (Invitrogen). To maximize the transfection efficiency, microliter volume electrophoration of 3T3-L1 preadipocytes was performed with OneDrop MicroPorator MP-100 (Digital Bio, Seoul, Korea). The cells were trypsinized, washed with 1x PBS, and finally resuspended in 10 μl of resuspension buffer R and 0.5 μg of plasmid at a concentration of 200,000 cells per pipette. The cells were then microporated at 1,300 V, with a 20-ms pulse width, 2 pulses. Following microporation, the cells were seeded in 35-mm cell culture dishes and placed at 37℃ in a 10% CO_2-humidified atmosphere.

11. Generation of *g0s2* knockout mice and genotyping

G0s2 knockout mice were generated by homologous recombination. Exon 1 and Exon 2 of the G0s2 gene were replaced with a LacZ-neo cassette. *G0s2* knockout mice were generated by Macrogene (Seoul, Korea). ES cells were purchased from KOMP (https://www.komp.org/). Mice were maintained on a 12-h light, 12-h dark cycle. All animal procedures were approved by the Institutional Animal Case Use Committee at the Yonsei University Health System. For genotyping of mice, genomic DNAs were isolated from tails of adults or heads of 13.5 dpc embryos, by incubation in 0.5 ml 50 mM Tris-HCl (pH 6.8), 50 mM EDTA, 0.5% SDS, 0.2 mg proteinase K at 55℃ overnight, followed by precipitation with isopropanol. PCR genotyping was performed using the forward

and reverse primers; Mutant 5'-AACCG TGCAT CTGCC AGTTT-3', Wild type 5'-AAAGT GTGCA GGAGC TGATC-3', Common 5'-GCCTG TCTCC TTCTC TAATG-3'. Expected band size for G0s2 during gel analysis is 526 bp for the wild type allele and 610 bp for the knockout allele.

12. Preparation of mouse embryonic fibroblast (MEF) cells

Wild-type and *g0s2* knockout E13.5 embryos were isolated from a single heterozygous female that had been paired with a heterozygous male. Primary MEFs (passage 0 or 1) from 13.5 day embryos were grown to confluence (day 0) in DMEM containing 10% FBS. Differentiation was initiated by treating cells with 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 10 μg/ml insulin and 2 μM rosiglitazone for 6 days. Subsequently, cells were maintained in DMEM supplemented with 10% FBS, 10 μg/ml insulin, and 2 μM rosiglitazone. After 3 days, the medium was replaced with maintenance medium containing DMEM supplemented with 10% FBS.

13. BODIPY staining

MEFs were maintained at proper densities on glass cover slips placed in 6-well dishes. After differentiation, following the fixation with 4% formaldehyde for 30 min, cells were rinsed with PBS three times. To visualize lipid droplets, BODIPY 493/503 dye was added at a final concentration of 0.3 μg/ml for 20 min.

Following three washes with PBS, sample were mounted on glass slide with Vectashield mounting medium containing DAPI and examined under inverted confocal microscope.

Table 1. Sequences of oligonucleotide primers used for quantitative real time PCR (qPCR)

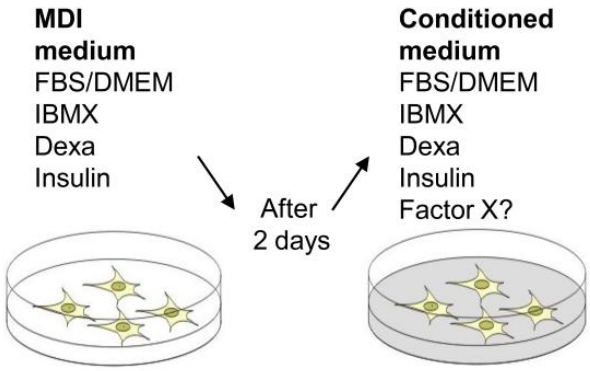
Oligonucleotide		Sequence (5'→3')
Gas1	Sense Antisense	GAATCGGTCAAAGAGAACAT GTCGTCATATTCTTCGTCGT
Gas2	Sense Antisense	CACTGAAGAGAGTTCCTTGC TTCATCAACTCCCAAATCTC
Gas5	Sense Antisense	ATGAAGGCTTACGAG TTAAAGCTATCGTCACCCCA
Gas6	Sense Antisense	CTGCCAAGATATCGATGAAT TTCTCCTTGGAGCTGTATGT
G0s2	Sense Antisense	AAAGTGTGCAGGAGCTGAT CCAGCACGTATAGCTTCACT
C/EBPα	Sense Antisense	TGGACAAGAACAGCAACGAG TCACTGGTCAACTCCAGCAC
PPARγ2	Sense Antisense	TATGGGTGAAACTCTGGGAG GCTGGAGAAATCAACTGTGG
aP2/422	Sense Antisense	TCTCCAGTGAAAACTTCGAT TTACGCTGATGATCATGTTG
GAPDH	Sense Antisense	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA

Ⅲ. RESULTS

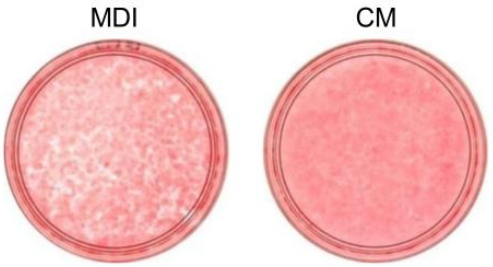
1. Conditioned medium (CM) stimulates the differentiation of 3T3-L1 preadipocytes.

In the first set of experiments, I investigated whether secreted factors during adipocytes differentiation affects adipose conversion. The preadipocytes were cultured in DMEM supplemented with 10% calf serum until the cells attained confluency. After confluency, the cells were differentiated in culture medium supplemented with 3-isobutyl-1-methylxanthine, dexamethasone, and insulin (MDI). On day 2, cell-exposed medium was harvested, designated as conditioned medium (CM) containing MDI and possible secreted factors (Fig. 1A). Growth arrested 3T3-L1 preadipocytes were induced to differentiation either by MDI or conditioned medium for 2 days. As a result, the preadipocytes treated with conditioned medium accelerated terminal differentiation evidenced by oil red-O staining (Fig. 1B). These results were confirmed by the expression of adipocyte-related genes, such as C/EBPα, PPARγ, and fatty acid synthase (FAS), showing earlier appearance in the presence of conditioned medium than MDI (Fig. 1C). But the expression of C/EBPβ was decreased after 16 h of conditioned medium induction. Because C/EBPβ is required for mitotic clonal expansion and a key regulator of C/EBPα and PPARγ, these findings indicate that conditioned medium accelerates the terminal differentiation by altered response of mitotic clonal expansion.

A


MDI
medium
FBS/DMEM
IBMX
Dexa
Insulin
After
2 days
Conditioned
medium
FBS/DMEM
IBMX
Dexa
Insulin
Factor X?

B


MDI
CM

C

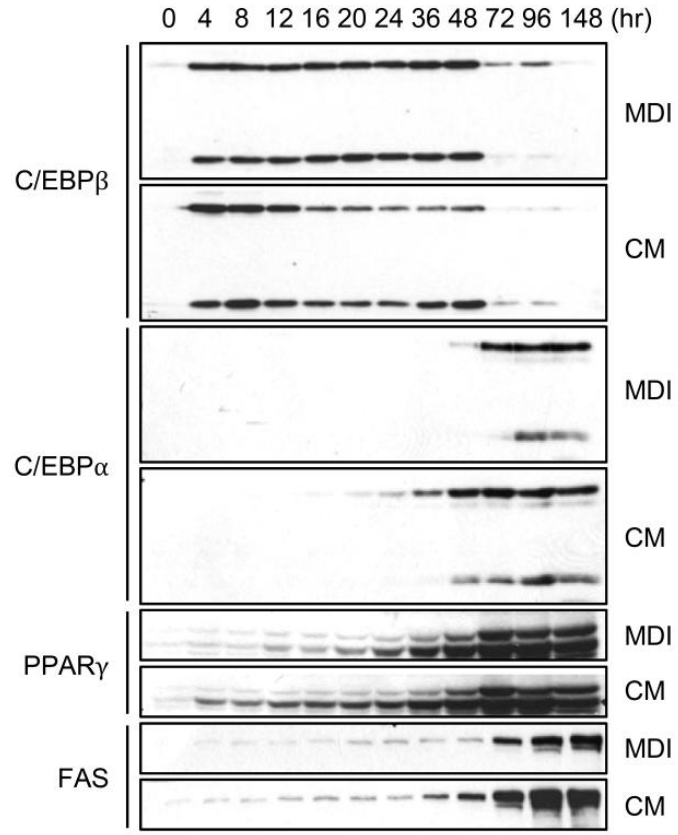
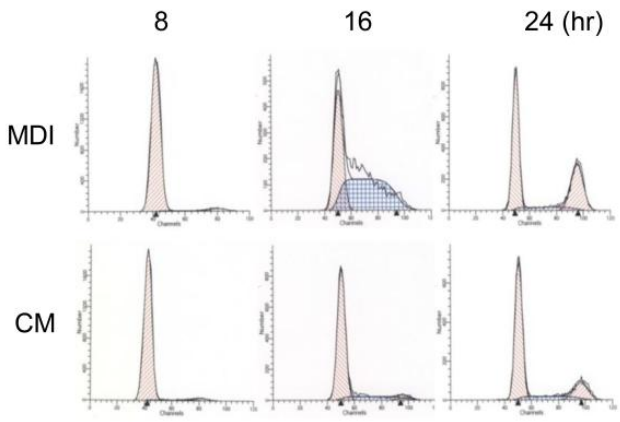

0 4 8 12 16 20 24 36 48 72 96 148 (hr)
C/EBPβ
MDI
CM
C/EBPα
MDI
CM
PPARγ
MDI
CM
FAS
MDI
CM

Figure 1. The effect of treatment with MDI or conditioned medium on differentiation of 3T3-L1 preadipocytes. (A) Schematic of conditioned medium using a preadipocyte differentiation system. 3T3-L1 preaipocytes are induced to differentiate into adipocytes with a mixture of hormonal agents, and after 2 days, cell-exposed medium was collected and designated as conditioned medium. (B) Post-confluent 3T3-L1 preadipocytes cells were treated with MDI or conditioned medium. Cells were then stained with oil red-O on day 5. (C) Cell lysates prepared at various times after adipogenic induction, subjected to SDS-PAGE and immunoblotted with antibody against C/EBPβ, C/EBPα, PPARγ, and FAS.

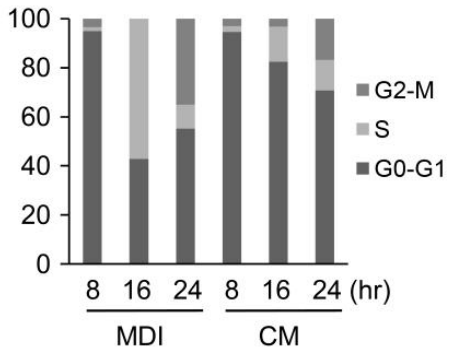
2. Conditioned medium induces the differentiation of 3T3-L1 preadipocytes without mitotic clonal expansion.

Because 3T3-L1 cells with conditioned medium promoted terminal differentiation, I examined whether mitotic clonal expansion occurred in these cells by detecting dividing cells with FACS analysis. Upon induction by MDI, 3T3-L1 cells reenter the cell cycle and DNA synthesis after 16 h of induction (S phase), resulting in the increase of G2/M population after 24 h of induction. However, preadipocytes treated with conditioned medium did not cause increased S population at 16 h (Fig. 2A, B). The results indicate that 3T3-L1 preadipocytes induced with conditioned medium differentiate into adipocytes reduced mitotic clonal expansion. To confirm these results, DNA synthesis was verified by BrdU incorporation. When preadipocytes were induced with MDI or conditioned medium, BrdU was added from the 16 h to 18 h after the stimulation. Most of the cells treated with MDI were labeled with BrdU, in contrast, only a few cells with conditioned medium were labeled with BrdU (Fig. 2C). As shown in Fig. 2D, induction of 3T3-L1 preadipocytes with MDI led to a threefold increase in cell number at day 2, whereas induction with conditioned medium caused only doubled cell number, indicating that the differentiation under conditioned medium is not dependent on clonal expansion. These findings suggest that mitotic clonal expansion is not critical for adipocyte differentiation induced by conditioned medium.

A



B



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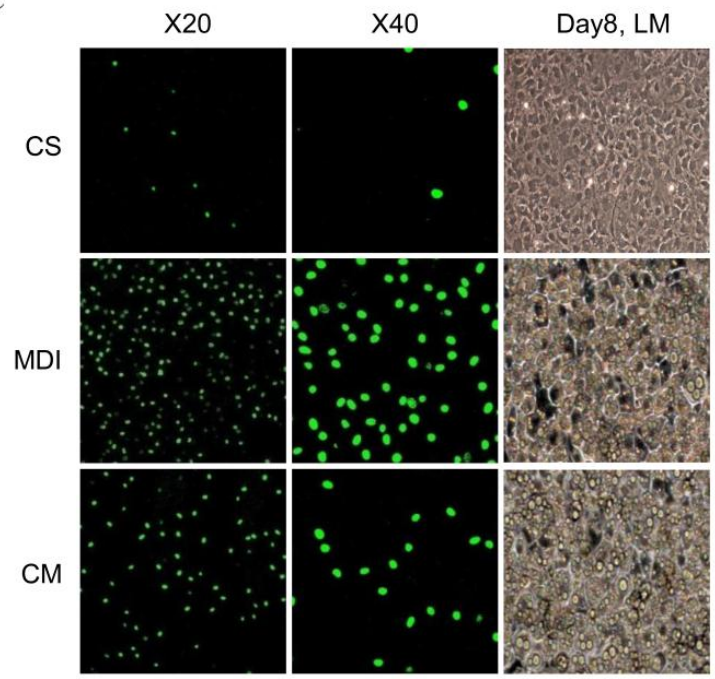

X20
X40
Day8, LM
CS
MDI
CM

D

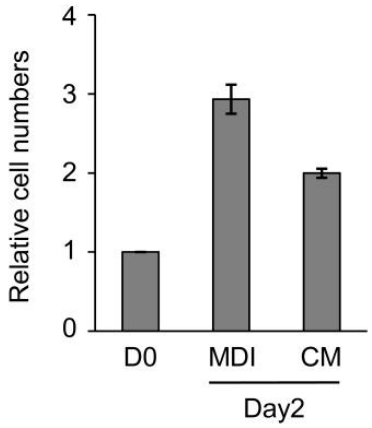

Relative cell numbers
4
3
2
1
0
D0
MDI
CM
Day2

Figure 2. Mitotic clonal expansion during differentiation of 3T3-L1 preadipocytes is induced by MDI or conditioned medium. 3T3-L1 preadipocyte cells were induced with standard hormonal cocktail or conditioned medium. (A and B) FACS analysis for DNA content of 3T3-L1 preadipocytes induced with MDI or conditioned medium. After induction, cells were harvested at 8, 16 and 24 h for FACS analysis using FACS Calibur flow cytometry system and data were analyzed by ModFit software. (C) Immunofluorescence analysis of BrdU-labeled adipocytes. 3T3-L1 cells were induced by MDI or conditioned medium and then BrdU was pulsed for 2 h (from the 16 h to the 18 h after initiation of differentiation by inducers). The time period of BrdU pulse corresponds to the S phase for standard protocol induced cells. (D) Cell number counting at day 2 after the induction. Cells were induced with MDI or conditioned medium, and the cell numbers were counted at day 2.

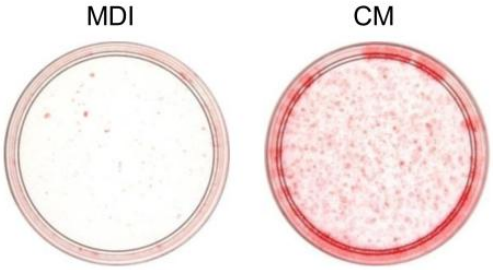
3. Conditioned medium induces the differentiation of proliferating preadipocytes.

Previous finding showed that conditioned medium did not induce the mitotic clonal expansion. This strongly suggests that conditioned medium directly causes the terminal differentiation program, probably by turning on a certain cell-specific event. Normally this cell-specific event is turned on as a result of mitotic clonal expansion of confluent cells, but not during proliferating preadipocytes in a low density. Thus, it is well-known that classic hormonal treatment does not induce the differentiation until cells reach confluent. Therefore, I next examined whether preadipocytes treated with conditioned medium differentiate into adipocytes without cell to cell contact inhibition. After 2 days of cell seeding, when the confluency was about 50%, proliferating cells were induced with MDI or conditioned medium (Fig. 3A). Surprisingly, the preadipocytes treated with conditioned medium differentiated to adipocytes, as judged by oil red-O staining (Fig. 3B). These results were confirmed by measuring the expression of C/EBPβ, C/EBPα, and PPARγ (Fig. 3C). The proliferating preadipocytes which were not growth-arrested by contact inhibition were allowed to accumulate intracellular lipid droplet (Fig. 3D). These results provide an evidence that conditioned medium can induce adipocyte differentiation of 3T3-L1 preadipocytes without cell to cell contact inhibition.

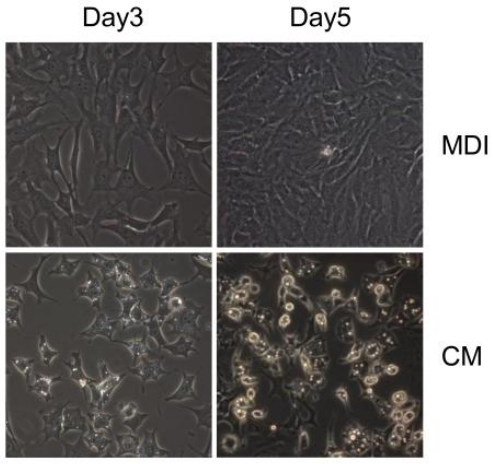
A

Day	Action	Confluency
1	Seeding	~20%
3	MDI/CM	~50%
5	Insulin	~80%
7	FBS only	~100%
10	Oil red O	

B



C



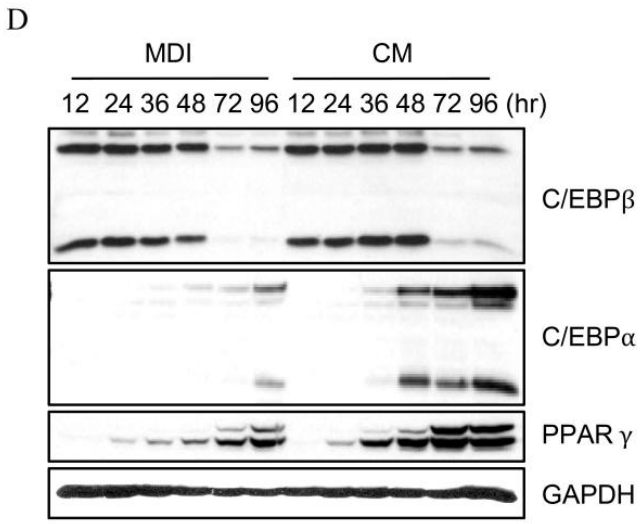


Figure 3. The effect of treatment on proliferating cells with MDI or conditioned medium on their capacity of 3T3-L1 to differentiate into adipocytes. (A) Schematic of proliferating cells induction with MDI or conditioned medium. 3T3-L1 cells were plated in a low confluency, and after 48 h, were induced with MDI or conditioned medium. The culture medium was replaced with DMEM supplemented with 10% fetal bovine serum and 1 μg/ml insulin, and the cells were then fed 2 day with DMEM containing 10% FBS. (B) Oil red-O staining of 3T3-L1 cells after the differentiation induction. 6 days after the initiation of differentiation, cells were stained with oil red-O to reveal the triglyceride droplets. (C) Cell were harvested at the indicated times, western blot analysis of C/EBPβ, C/EBPα, PPARγ during 3T3-L1 differentiation, with GAPDH as a loading control (D) Microscope images of 3T3-L1 cells induced with MDI or conditioned medium at a low confluency.

4. G0s2 is a high-scoring candidate regulator of adipogenesis.

To identify genes temporally regulated during a transition from mitotic clonal expansion to terminal differentiation in 3T3-L1 adipogenesis, I performed microarray analysis at different time points during differentiation. I compared the microarray data with gene atlas of UCSC database (http://genome.ucsc.edu) to identify important genes both *in vitro* and *in vivo*. Within the subset of genes with maximal expression at day 2 of 3T3-L1 differentiation and white adipose tissues, I identified G0/G1 switch gene 2 (G0s2) with significantly high ranked score. Then I confirmed G0s2 expression using real-time qPCR, along with other genes such as growth arrest specific genes (Gas) which might affect clonal expansion stage. As a result, conditioned medium strongly accelerated the expression of G0s2 mRNA compared to MDI medium but the expression patterns of Gas genes were not changed with conditioned medium (Fig. 4), suggesting that this molecule is associated with the transition into the terminal differentiation.

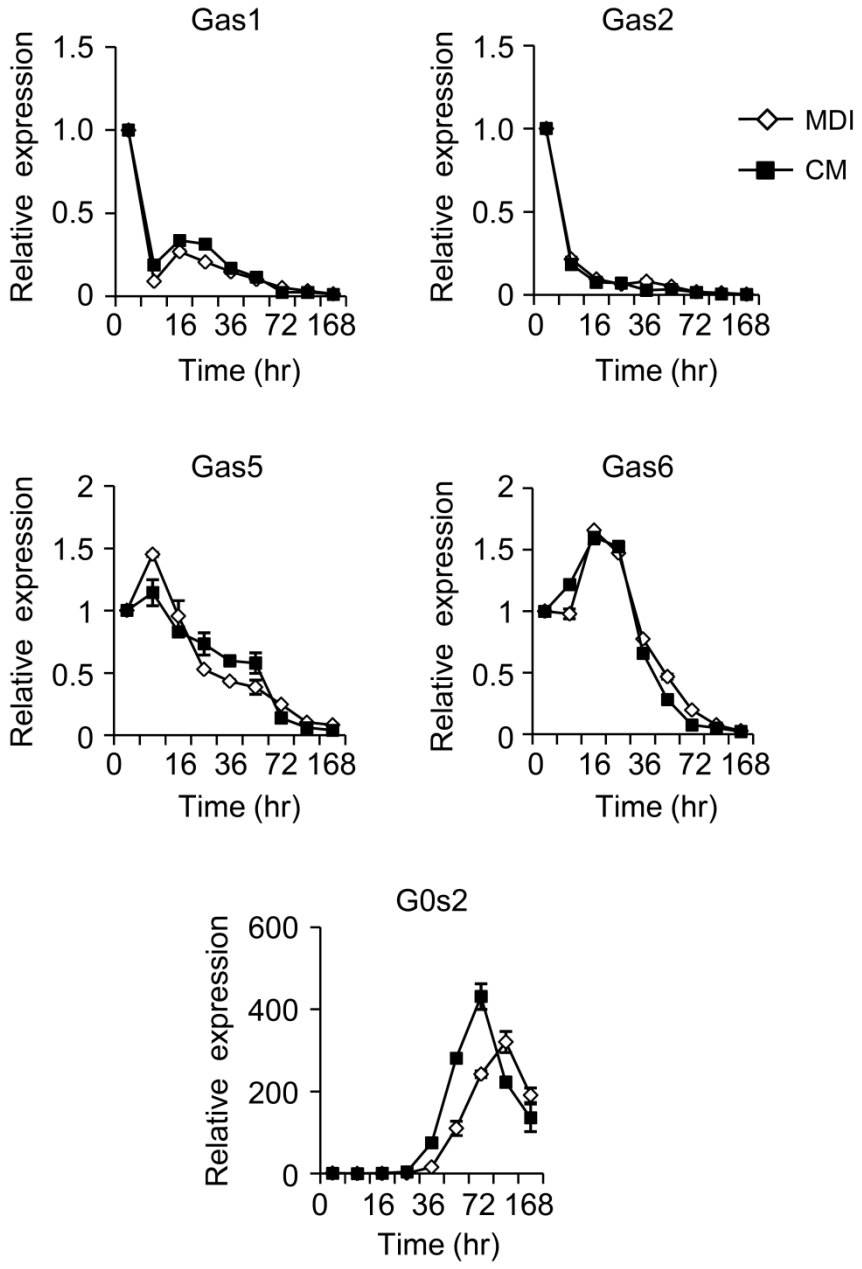
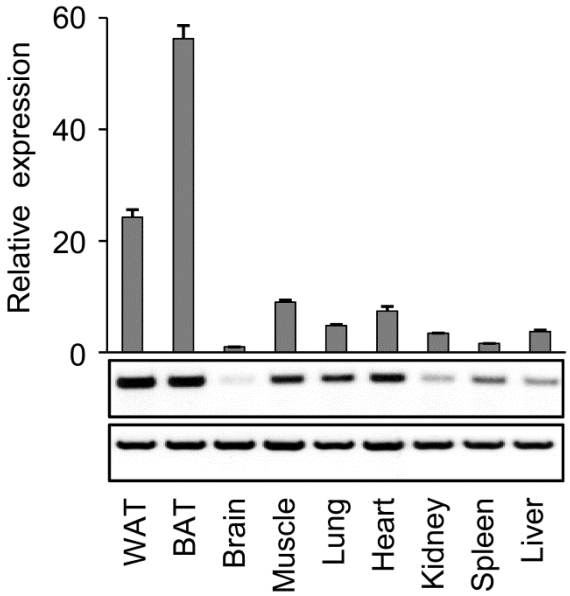

Gas1
Gas2
Gas5
Gas6
G0s2
Relative expression
Time (hr)
0
16
36
72
168
MDI
CM

Figure 4. Analysis of various genes related with cell cycle by quantitative real time PCR (qPCR). 3T3-L1 preadipocyte cells were induced with standard hormonal cocktail or conditioned medium. Cells were harvested at the indicated times. Total RNA was isolated then first-strand cDNA synthesis from 5 μg of total RNA was performed by random hexamer primer. Expression of Gas1, Gas2, Gas5, Gas6, and G0s2 during differentiation of 3T3-L1 cells was analyzed using quantitative PCR. All reactions were performed in triplicate.

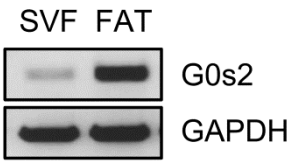
5. G0s2 is expressed in fat tissues and its overexpression resulted in enhanced adipocyte differentiation.

Tissue distribution of G0s2 mRNA by RT-PCR and quantitative real time PCR revealed that G0s2 expression was significantly high in adipose tissue and, to a lesser degree, in muscle, compared to other tissue (Fig. 5A). To examine G0s2 expression in adipose tissue further, fat cell fraction and stromal vascular fraction (SVF) of adipose tissue were separated by centrifugation. G0s2 expression was approximately 15-fold greater in FAT fraction compared to SVF, showing that G0s2 is expressed higher in mature fat cells rather than preadipocytes (Fig. 5B). In order to determine if adipogenic differentiation of preadipocytes cause increased G0s2 expression, 3T3-L1 preadipocytes were harvested in a time-dependent manner during differentiation. Using quantitative real time PCR, I observed that C/EBPα and PPARγ was induced at 24 h after differentiation. Also, aP2/422, an adipocyte marker, was significantly increased at day 2. G0s2 mRNA was induced during differentiation with similar pattern (Fig. 5C). Overall, G0s2 expression was increased both *in vitro* adipocytes and *in vivo* adipose tissue. Given these results, I sought to determine whether overexpression of G0s2 could enhance the terminal differentiation. G0s2 overexpression caused increased lipid droplet formation in 3T3-L1 adipocytes, accompanied by C/EBPα and PPARγ expression in Western blot analysis (Fig. 6A, B). This result suggests that G0s2 is involved in adipocytes differentiation and lipid accumulation.

A


Relative expression
60
40
20
0
WAT
BAT
Brain
Muscle
Lung
Heart
Kidney
Spleen
Liver

B


SVF
FAT
G0s2
GAPDH

C

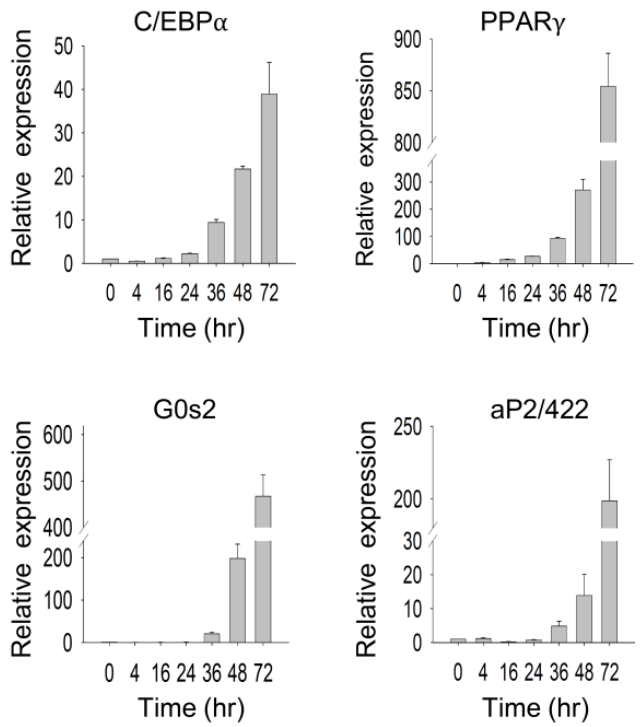


Figure 5. Expressions of G0s2 in various mouse tissues and during differentiation of 3T3-L1 preadipocytes. (A) Tissue distribution of G0s2 gene by RT-PCR and quantitative real time PCR analysis. (B) G0s2 expression in stromal vascular fraction (SVF) and fat cell fraction (FAT). Adipose tissue was separated by centrifugation. (C) G0s2 expression during adipogenesis was validated by quantitative real time PCR. C/EBPα, PPARγ and aP2/422 were used as an adipocyte differentiation marker.

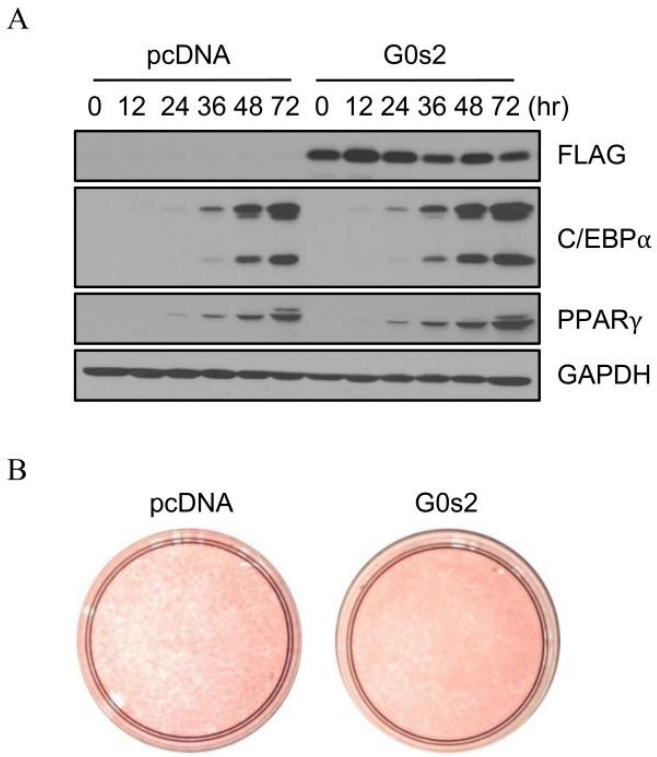


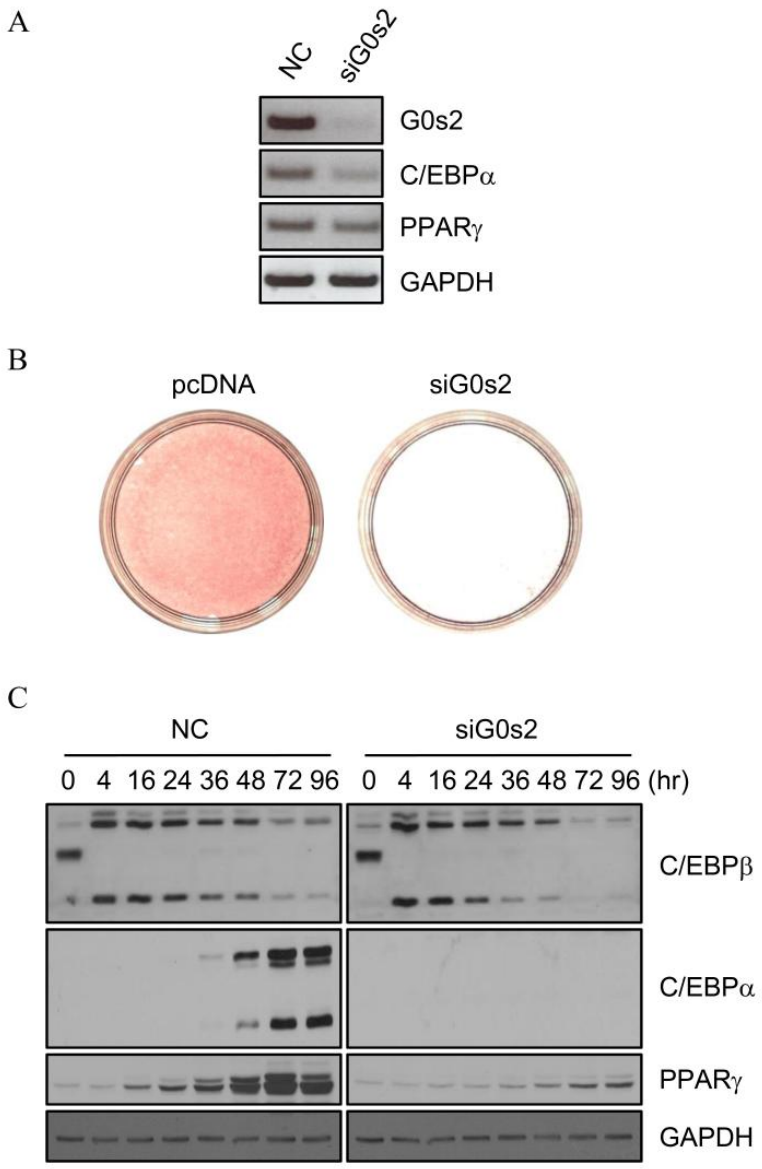
Figure 6. Overexpression of G0s2 in 3T3-L1 preadipocytes increases adipogeneis. (A) 3T3-L1 cells expressing control vector (pcDNA) or G0s2 were transfected by eletroporator and then induced to differentiation. Protein collected by indicated time after induction was used to assay the level of G0s2, C/EBPα and PPARγ, with GAPDH as a loading control. (B) Oil red-O staining after complete differentiation (day 4).

6. Knockdown of G0s2 in 3T3-L1 preadipocytes inhibits fat accumulation and stimulates apoptosis.

To examine the function of G0s2 in adipogenesis, I knocked down the expression of G0s2 in 3T3-L1 cells using siRNA. Cells were assayed for mRNA expression of C/EBPα and PPARγ, key markers of adipogenesis, showing that C/EBPα and, to a lesser degree, PPARγ were reduced in the absence of G0s2 (Fig. 7A). Knockdown of G0s2 resulted in the inhibition of differentiation, shown by oil red-O staining at day 5 (Fig. 7B). The expression of C/EBPα and PPARγ protein, which normally increase during adipogenesis, was reduced in G0s2-knockdown cells, whereas expression of C/EBPβ protein was unaffected (Fig. 7C). Interestingly, G0s2-knockdown caused decreased in cell number at day 2 (Fig. 7D). This result suggests that G0s2 affects adipogenesis at a time point between C/EBPβ expression and C/EBPα expression. Assuming that this time point is after mitotic clonal expansion, G0s2 knockdown should not affect mitotic clonal expansion, but the results of cell counting are inconsistent with this idea.

With this in mind, I wondered if inhibition of G0s2 stimulates apoptotic pathway during adipogenesis. To determine whether the reduction in cell number was caused by apoptosis, Annexin-PI staining was performed. The Annexin-PI staining combination assay detected apoptotic cell membrane phosphatidyl serine (PS) externalization and served as a measure of adipocyte viability. G0s2-knockdown cells exhibited significant increase of apoptosis (Fig. 8A). To further

investigate whether G0s2-knockdown induced apoptotic cells by undergoing DNA damage, I performed a TUNEL assay, which clearly showed increased labeling in G0s2-knockdown cells compared to negative control cells (Fig. 8B). To detect whether G0s2-knockdown mediated cell death, I analyzed apoptosis-related proteins during differentiation. Caspase 3, known as a marker of apoptosis[27], was activated at 36 ~ 48 h of induction, at which mitotic clonal expansion transit to the terminal differentiation. Previous study reported that ectopic expression of Bax induces apoptosis with an early release of cytochrome *c* preceding many apoptosis-associated morphological alterations as well as caspase activation and subsequent substrate proteolysis[28]. Also, Bax was greatly enhanced in siG0s2-transfected cells (Fig. 8C). This suggests that G0s2 depletion induces an upregulation of caspase 3 expression, which may be sufficient to activate apoptosis. Taken together these data indicate that G0s2 is required for the terminal differentiation, and without G0s2, cells undergo the apoptotic pathway upon a strong mitotic signal.


A
NC
siG0s2
G0s2
C/EBPα
PPARγ
GAPDH
B
pcDNA
siG0s2
C
NC
siG0s2
0 4 16 24 36 48 72 96 0 4 16 24 36 48 72 96 (hr)
C/EBPβ
C/EBPα
PPARγ
GAPDH

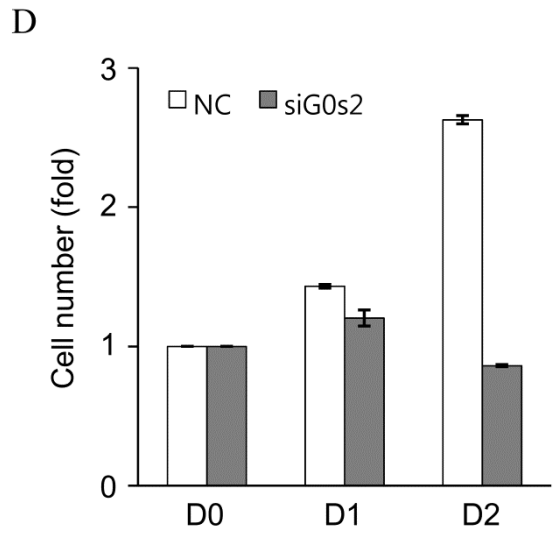
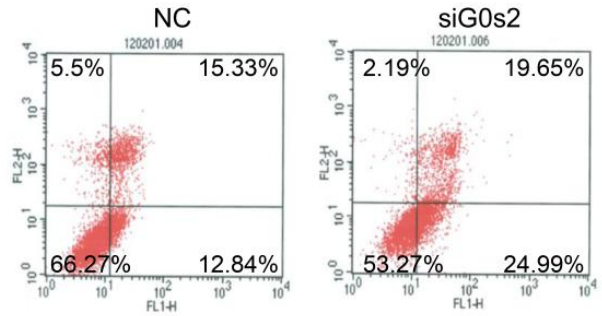
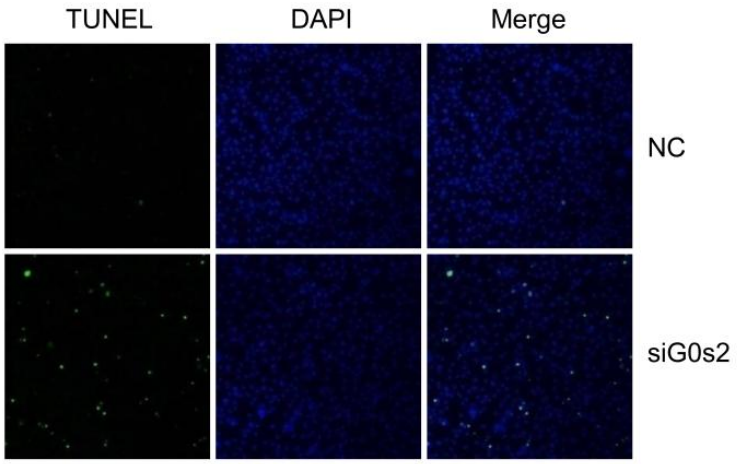


Figure 7. Knockdown of G0s2 by siRNA inhibits adipocyte differentiation. (A) Knockdown of G0s2 mRNA in 3T3-L1 adipocytes by siRNA was assessed by RT-PCR at day 2 after differentiation, along with C/EBPα and PPARγ. (B) Oil red-O staining of adipocytes with negative control (NC) and G0s2-knockdown (siG0s2) at day 5. (C) C/EBPβ, C/EBPα and PPARγ protein levels were measured in the NC and siG0s2 cells by western blotting, with GAPDH as a loading control. (D) Cell number counting at day 1, 2 after the induction. Cells were induced with MDI after transfeciton of siRNA, the cell numbers were counted.

A


NC
siG0s2
120201.004
120201.006
5.5%
15.33%
66.27%
12.84%
2.19%
19.65%
53.27%
24.99%
FL2-H
FL1-H

B


TUNEL
DAPI
Merge
NC
siG0s2

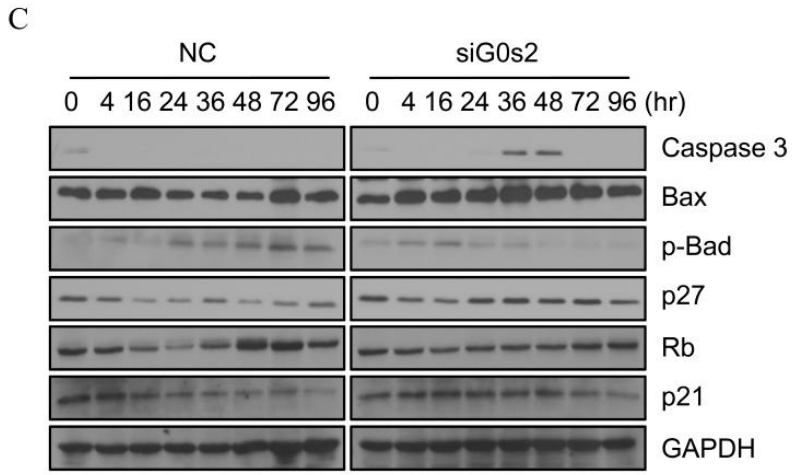


Figure 8. Knockdown of G0s2 induces apoptosis during adipogenesis. (A) Knockdown of G0s2 induced apoptosis during adipogenesis evaluated by Annexin-V/PI-FACS analysis. Annexin: FL1-H; PI: FL2-H. (B) Cells with negative control (NC) or knockdown of G0s2 (siG0s2) at 48 h after induction were fixed and stained with DAPI and analyzed by fluorescence microscopy, or analyzed for DNA fragmentation by TUNEL. (C) Cells were harvested at the indicated times, western blot analysis of Caspase 3, Bax, p-Bad, p27, RB and p21 during 3T3-L1 differentiation, with GAPDH as a loading control.

7. G0s2 expression is regulated by PPARγ.

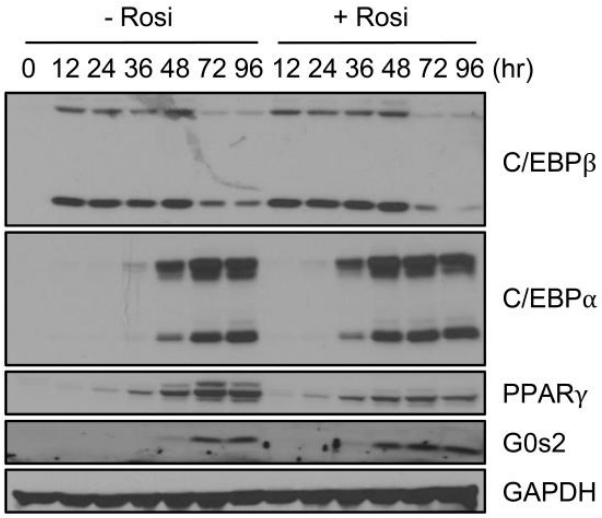
Previous reports indicate that direct up-regulation of G0s2 by PPARγ is associated with cell-cycle withdrawal in 3T3-L1 cells[23]. Several reports have suggested that rosiglitazone, a powerful ligand of PPARγ, increases G0s2 expression[24]. Therefore, I tested whether rosiglitazone enhances terminal differentiation by up-regulation of G0s2. In cells treated with 2 μM rosiglitazone for a maximum of 2 days, C/EBPα expression was increased 24 h after differentiation and G0s2 was also increased (Fig. 9A, B). To substantiate further this notion, G0s2-knockdown cells were treated with the 2 μM rosiglitazone. While rosiglitazone enhanced the differentiation in control cells, deletion of G0s2 induced apoptosis with the expression of caspase 3, even in the presence of rosiglitazone (Fig. 9C). This indicates that G0s2 is a target gene of PPARγ, and that G0s2 is required for the maintenance of terminal differentiation program.

To examine the function of PPARγ in apoptosis related with G0s2, I depleted PPARγ by RNA interference (Fig. 10A). As expected, PPARγ-knockdown cells were not able to differentiate into adipocytes (Fig. 10B). PPARγ-knockdown caused decreased cell number at day 2 after differentiation (Fig. 10C). Moreover, PPARγ knockdown resulted in apoptosis after induction of differentiation with similar pattern observed with G0s2 knockdown cells (Fig. 10D). Expression of C/EBPα was also markedly reduced and caspase 3, Bax and p27 were increased in PPARγ knockdown cells (Fig. 10E).

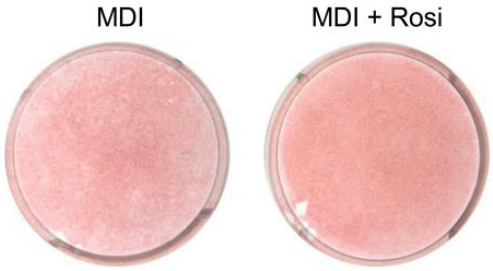
To demonstrate that these apoptosis effects were mainly mediated by G0s2, next experiment was performed. A retroviral construct encoding G0s2 cDNA was used to produce G0s2-overexpression 3T3-L1 cells (Fig. 10F). Using this cell line, I knocked down the expression of PPARγ using siRNA. Whereas siPPARγ resulted in a significantly increased expression of caspase 3 compared to control cells, G0s2-overexpressing cells did not express caspase 3 even in the absence of PPARγ. Moveover, G0s2-overexpressing cells with siPPARγ led to the reactivation of the differentiation program slightly, shown by oil red-O staining at day 5 (Fig. 10G). These indicate that PPARγ is required not only for the adipose-specific gene expression, but also for protecting clonal expanded cells from apoptotic pathway. In this regard, G0s2 is thought to play a major role for this protection because G0s2 expression without PPARγ showed to rescue the differentiation program.

Taken together, these results indicate that PPARγ is absolutely required for the terminal differentiation and based upon previous observations, it appears that G0s2 is downstream of PPARγ and regulates apoptosis and terminal differentiation.

A


- Rosi
+ Rosi
0 12 24 36 48 72 96 12 24 36 48 72 96 (hr)
C/EBPβ
C/EBPα
PPARγ
G0s2
GAPDH

B


MDI
MDI + Rosi

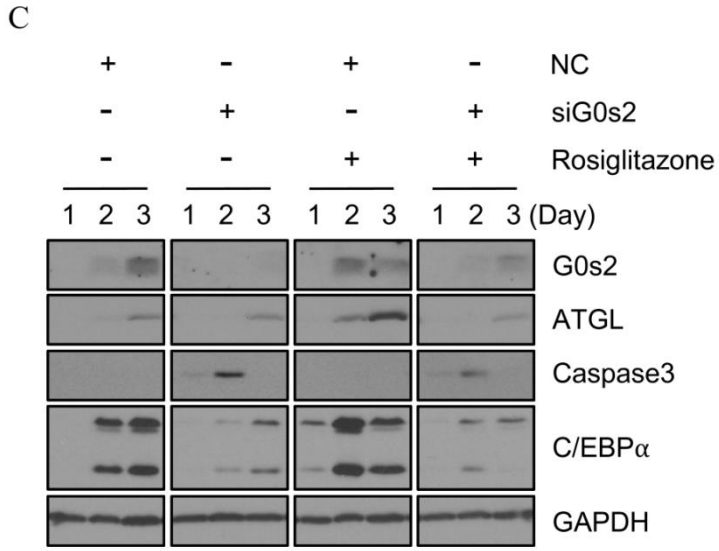
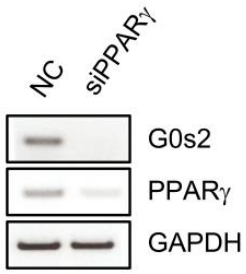
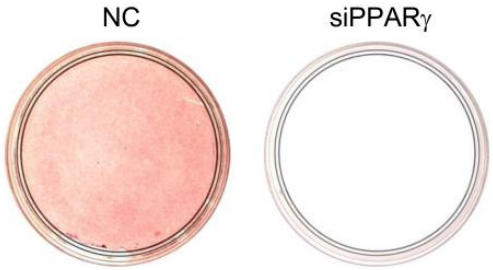


Figure 9. Rosiglitazone enhances G0s2 expression and promotes fat accumulation. (A) Cells were harvested at the indicated times after treatment of rosiglitazone, and western blot analysis of C/EBPβ, C/EBPα, PPARγ and G0s2 was carried out during 3T3-L1 differentiation, with GAPDH as a loading control. (B) Oil red-O staining after complete differentiation (day 4). (C) Western blot analysis showing the expression of G0s2, ATGL, Caspase 3, C/EBPα, and GAPDH in 3T3-L1 transfected negative control or G0s2 in the presence or absence of 2 μM rosiglitazone.

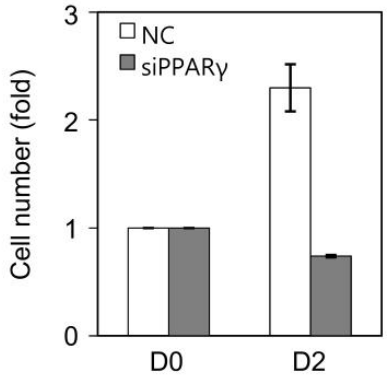
A


NC
siPPARγ
G0s2
PPARγ
GAPDH

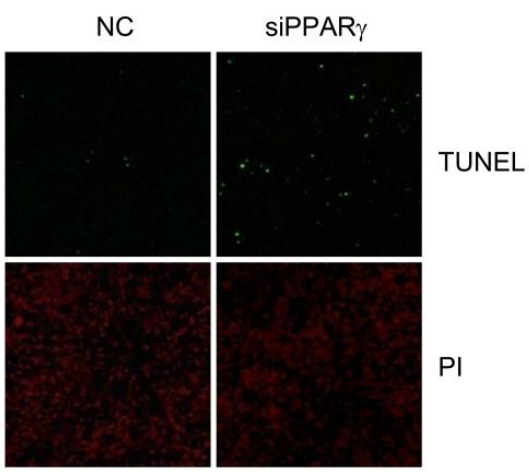
B


NC
siPPARγ

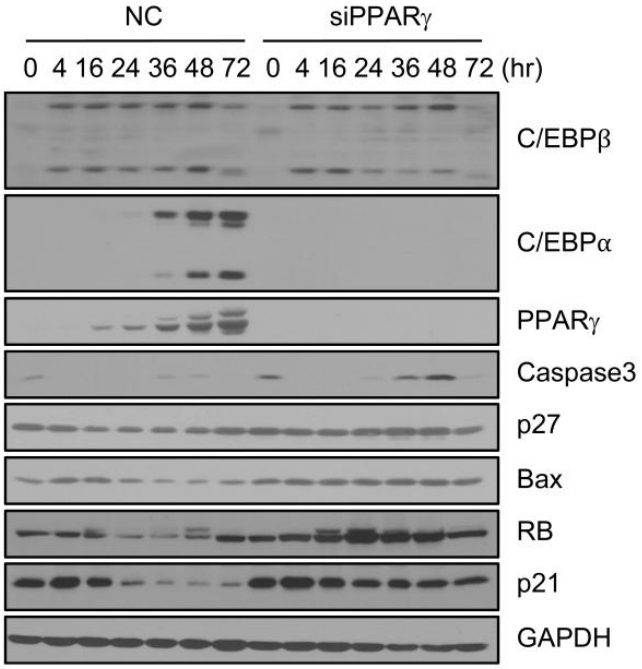
C


3
2
1
0
Cell number (fold)
NC
siPPARγ
D0
D2

D


NC
siPPARγ
TUNEL
PI

E


NC
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C/EBPα
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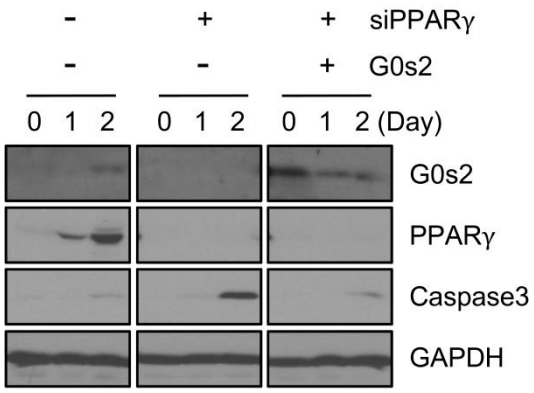
GAPDH

F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- - + G0s2
0 1 2 0 1 2 0 1 2 (Day)
G0s2
PPARγ
Caspase3
GAPDH

G

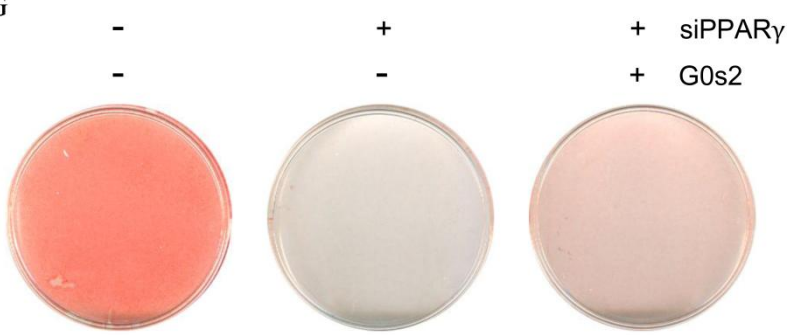

- + + siPPARγ
- - + G0s2

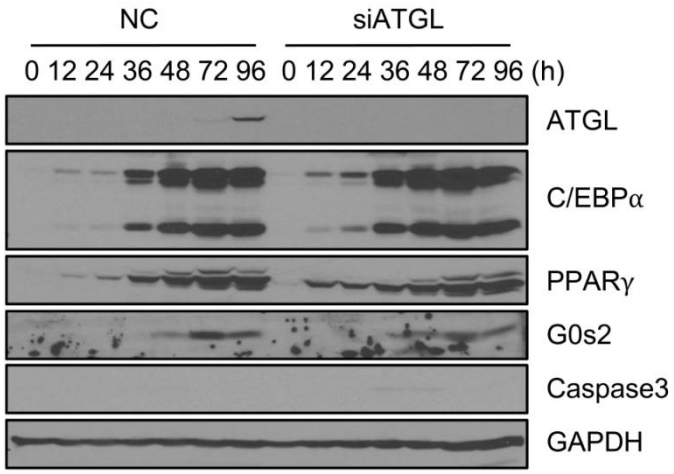
Figure 10. Knockdown of PPARγ by siRNA inhibits differentiation of 3T3-L1 preadipocytes and induces apoptosis during adipogenesis. (A) Knockdown of PPARγ mRNA in 3T3-L1 adipocytes by siRNA was assessed by RT-PCR. (B) Oil red-O staining of adipocytes expressing negative control (NC) and PPARγ-knockdown (siPPARγ). (C) Cell numbers were counted on day 0 and day 2 after differentiation. (D) Cells with negative control (NC) and knockdown of PPARγ (siPPARγ) at 48 h after differentiation were either fixed and stained with DAPI and analyzed by fluorescence microscopy, or analyzed for DNA fragmentation by TUNEL. (E) Protein expression of the adipogenesis-associated target C/EBPβ, C/EBPα and PPARγ and apoptosis-related genes caspase 3, Bax, p27, p21. (F) 3T3-L1 cells stably expressing control vector or G0s2 were transfected siPPARγ. Efficacy of PPARγ knockdown was tested after induction of differentiation. (G) Lipid droplets were visualized by oil red-O staining, at day 7, in the G0s2 overexpressing cells.

8. Absence of G0s2 induces apoptosis independent on ATGL.

Previous reports indicate that G0s2 specifically interacts with ATGL, and G0s2 functions to attenuate ATGL action. To examine whether the previous findings of G0s2 in terminal differentiation program is dependent on ATGL, I knockdown the expression of ATGL in 3T3-L1 cells using siRNA. Cells expressing negative control differentiated normally, and ATGL expression was induced at day 3. ATGL expression was significantly reduced in ATGL knockdown cells. The knockdown cell lines were then induced to differentiate, and lipids were stained on day 5 using oil red-O. As shown in Fig. 11A and B, knockdown of ATGL resulted in slightly enhanced lipid accumulation shown by oil red-O staining without significant affect on the expressions of C/EBPα, PPARγ, G0s2, and caspase 3. This data suggests that a deficiency of ATGL prevents from lipolysis, which causes the acceleration of lipid accumulation in 3T3-L1 cells.

To determine whether the induced apoptosis in G0s2 knockdown cells is dependent on ATGL, I knocked down both G0s2 and ATGL in 3T3-L1 cells. Deletion of G0s2 and ATGL was confirmed by western blotting at indicated times (Fig. 11C). As G0s2 depletion caused the expression of caspase 3, double knockdown cells also led to induce caspase 3 expression at day 2. These results indicated that the G0s2-depleted cells undergo the apoptotic pathway regardless of ATGL. In other word, the G0s2 function in the adipocyte differentiation is thought to be independent on ATGL.

A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siATGL
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G0s2
Caspase3
GAPDH

B


NC
siATGL

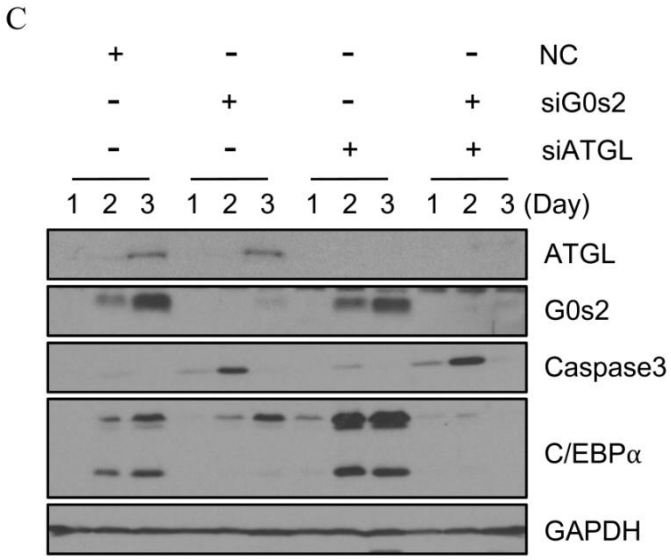
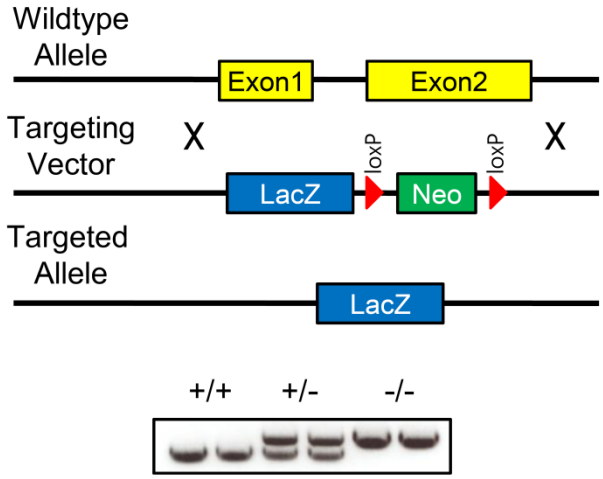


Figure 11. ATGL gene knockdown does not induce apoptosis in 3T3-L1 preadipocytes. (A) G0s2 expression was suppressed in 3T3-L1 preadipocytes by siRNA and cells were differentiated into adipocytes. Cells were harvested at the indicated times, western blot analysis of ATGL, G0s2, C/EBPα, PPARγ, caspase 3, and GAPDH was performed. (B) Oil red-O staining after differentiation (day 5). (C) 3T3-L1 cells transfected with siRNA constructs targeting individually G0s2 or ATGL and cells were differentiated into adipocytes. The level of ATGL, G0s2, caspase 3, C/EBPα, and GAPDH proteins were analyzed at the indicated days by immunoblot analysis.

9. *G0s2* knockout mice have decreased adiposity.

To elucidate the physiological role of G0s2, I generated *g0s2* knockout mice. Gene trap targeted *g0s2* knockout male mice were made on the original 129Sv founder background and the C57BL/6J background (Fig. 12A). I confirmed successful ablation by the virtual absence of G0s2 gene expression (Fig. 12B). Analysis of adipose stores from 12-week-old wild type and *g0s2* knockout mice showed a 15% reduction in body weight in *g0s2* -/- versus +/+ mice (Fig. 12C), due to a 30% reduction in adipose content (Fig. 12D, E). There was no difference in liver (Fig. 10F). A decrease in adipose tissue mass could result from a reduction in adipocyte size as well as adipocyte number with impaired differentiation. Histological analyses of epididymal fat pad and brown adipose tissue after fixation revealed that adipocytes from *g0s2* knockout mice smaller than wild type mice (Fig. 12G). These data suggest that G0s2 plays a role in promoting *in vivo* fat tissue formation.

A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Allele
Exon1
Exon2
Targeting
Vector
X
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loxP
Neo
loxP
X
Targeted
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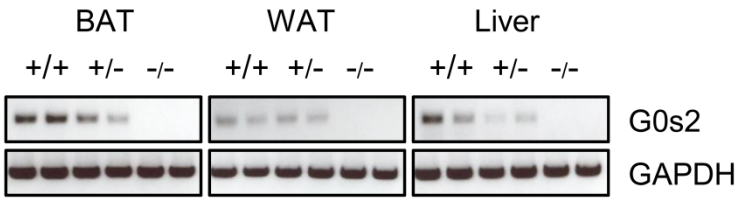
+/-
-/-

B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WAT
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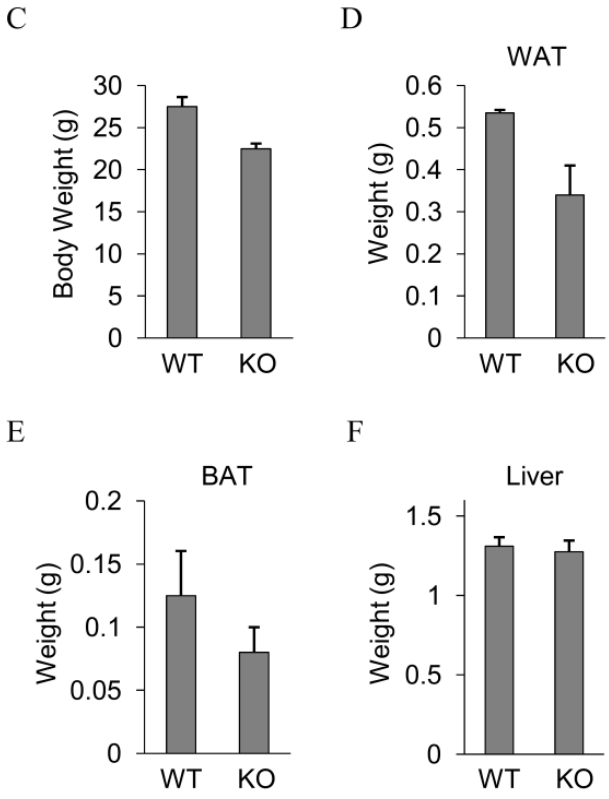
+/+ +/- -/-
+/+ +/- -/-
G0s2
GAPDH


C
Body Weight (g)
30
25
20
15
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0
WT
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D
WAT
Weight (g)
0.6
0.5
0.4
0.3
0.2
0.1
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BAT
Weight (g)
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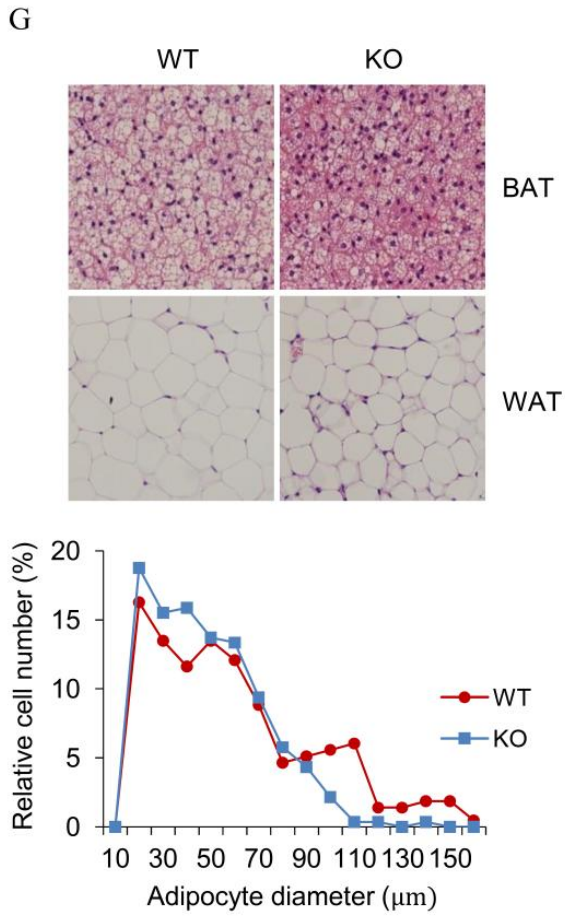


Figure 12. Generation and characterization of *g0s2* knockout mice. (A) A G0s2 gene was replaced with LacZ-neo cassette by homologous recombination. For genotyping of mice, genomic DNAs were isolated from mice and PCR was performed. Expected band size for G0s2 during gel analysis were 526 bp for the wild type allele and 610 bp for the knockout allele, showing wild type (+/+),

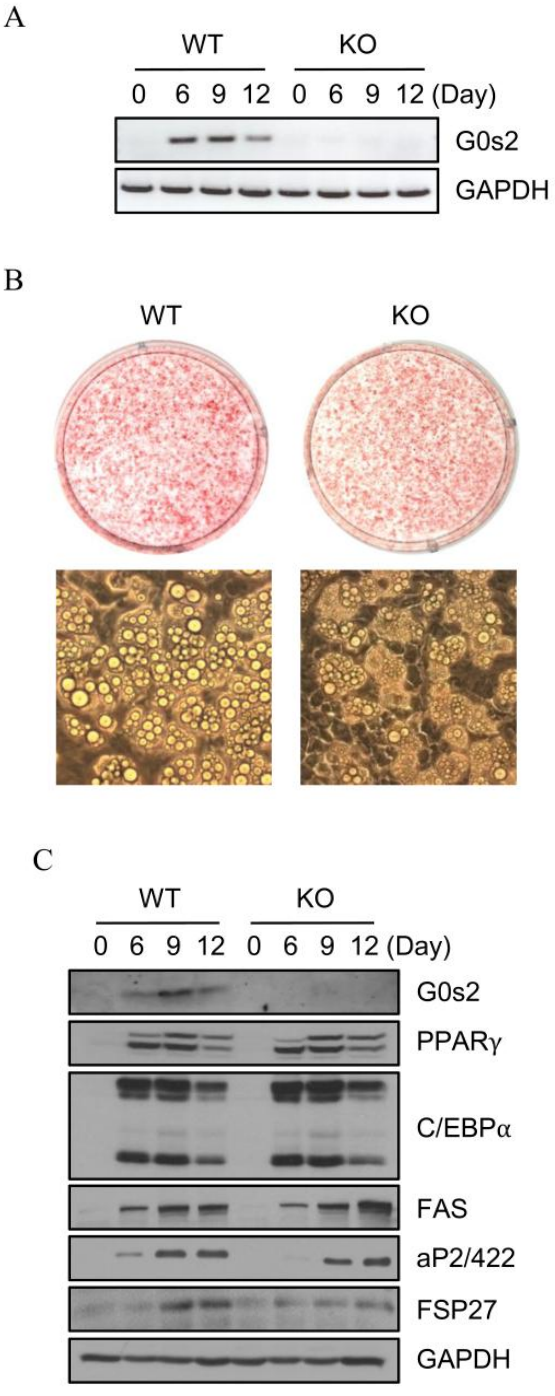
heterozygote (+/-) or homozygote of knockout mice. (B) RT-PCR analyses of genes of BAT, WAT and liver from control and *g0s2* knockout mice. (C-F) Body weight, WAT, BAT and liver weight were evaluated in *g0s2* knockout mice and wild-type control mice at the age of 12 weeks. (G) Hematoxylin/eosin-stained BAT and WAT sections in WT and KO mice. Mean relative frequency distribution of epididymal adipocyte cell size; n = 3 per genotype.

10. *G0s2* -/- MEF shows impaired adipogenesis.

I explored the specific action of G0s2 on the adipocyte differentiation process per se by using mouse embryonic fibroblasts (MEFs), isolated from *g0s2* -/- or *g0s2* +/+ littermate mouse embryos (Fig. 13A). When an adipogenic mixture was added, the accumulation of lipid droplets was reduced in *g0s2* -/- compared with *g0s2* +/+ MEFs (Fig. 13B). As G0s2 mRNA is induced substantially at day 2 of adipogenesis (Fig. 5C), it is not surprising that the effect of G0s2 deficiency in MEF cells became apparent only after cells were induced to differentiate. *G0s2* -/- MEF cells accumulated many small lipid droplets during adipogenesis, but these did not mature into the larger lipid droplets observed in wild type MEFs. Thus, I hypothesized that knockout of G0s2 not only alters adipocyte differentiation but also affects lipid droplet formation. Immunoblot analysis of day 6, 9 and 12 adipocytes revealed no changes in PPARγ and C/EBPα expression between wild type and *g0s2* -/- MEFs, suggesting that in MEF cells, in contrast to 3T3-L1 cells, G0s2 does not affect PPARγ or C/EBPα expression (Fig. 13C). This might be from a difference between MEFs and 3T3-L1 cells, because mitotic clonal expansion has not been observed in MEF cells[8]. Nevertheless, *g0s2* -/- MEFs showed impaired adipogenesis compared to wild type MEF, suggesting that G0s2 plays a major role in normal development of fat pad *in vivo*.

I found that protein levels of aP2/422, fat-specfic protein 27 (FSP27) were decreased in *g0s2* -/- MEFs (Fig. 13C). Previous reports indicate that FSP27

deficiency inhibits development of large lipid droplets and increases mitochondrial number but no effects on adipogenesis[29]. Next, I rescued G0s2 gene expression in *g0s2* -/- MEF by transfection a vector expressing G0s2 (Fig. 13D). Restoring G0s2 expression in *g0s2* -/- MEF cells led to the reactivation of the adipocyte differentiation program as shown by the appearance of lipid droplets revealed by oil red-O staining (Fig. 13E). Consistently, aP2/422 and FSP27 were induced after addition of the adipogenic mixture in the rescued MEF cells as checked by immunoblotting (Fig. 13D). The G0s2-overexpressing MEFs accumulated mature lipid droplets during adipogenesis, *g0s2* -/- MEF cells did not into the larger lipid droplets observed in *g0s2* +/+ MEF cells (Fig. 13E). Taken together, G0s2 is required for expansion of lipid droplets and related to regulation of adipogenesis.


A
WT
KO
0 6 9 12 0 6 9 12 (Day)
G0s2
GAPDH
B
WT
KO
C
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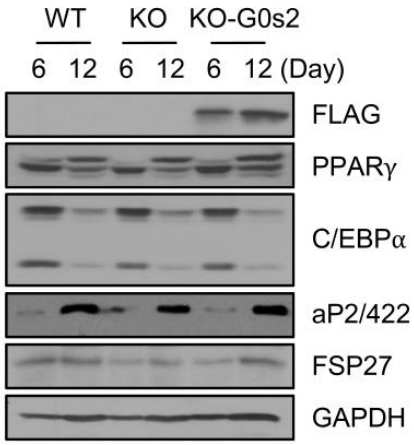
G0s2
PPARγ
C/EBPα
FAS
aP2/422
FSP27
GAPDH

D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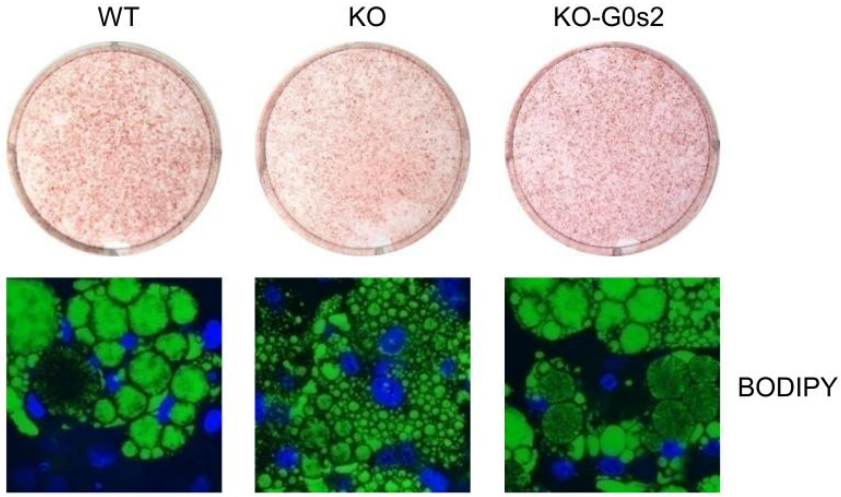
WT
KO
KO-G0s2
BODIPY

Figure 13. Adipocyte differentiation is inhibited in *g0s2* knockout MEF cells and ectopic expression of G0s2 in *g0s2* knockout MEF restores adipogenesis. (A) Expression of G0s2 analyzed by RT-PCR in *g0s2* -/- and WT MEFs. (B) Oil red-O staining of WT and *g0s2* -/- MEFs after complete differentiation (day 12). (C) Western blot analysis of G0s2 protein and adipocyte specific factors levels in WT and *g0s2* -/- MEFs. (D) Western blot analysis of G0s2 protein and adipocyte specific factors level in WT containing the empty vector (WT), *g0s2* -/- containing the empty vector (KO) or rescued G0s2 -/- (KO + G0s2). (E) Oil red-O stained WT, *g0s2* -/- containing the empty vector (KO), rescued *g0s2* -/- (KO-G0s2) MEF cells. The fluorescent dye BODIPY and DAPI were used to stain the lipids contained in the droplets of differentiated fat cells and nuclei of existing cells.

Ⅳ. DISCUSSION

3T3-L1 adipocytes have been used as a primary model for the study of adipogenesis, the process wherein fibroblastic cells differentiate into fat cells. It is thought that several proteins are secreted from differentiating 3T3-L1 cells, which are involved in the regulation of growth and differentiation. In this regard, some candidates of secreted proteins from 3T3-L1 cells have been presented using a systemic proteomic approach, based on the selective blocking of secretion[19]. Although the secreted proteins during the adipogenesis have been suggested to be important, the identity of their true roles remains elusive. Other studies revealed that an endogenous PPARγ ligand is generated in a time- and cAMP-dependent fashion during an early phase of the adipogenic process in 3T3-L1 cells[30]. These indicate a possibility that some factors are released during adipogenesis, particularly as a result of mitotic clonal expansion, which are essential for the transition into the terminal differentiation of the cells. In this study, I showed that conditioned medium accelerated the differentiation into mature adipocytes, suggesting that additional events are required for the transition into the terminal differentiation. More importantly, the conditioned medium was able to induce adipogenesis in proliferating preadipocytes without mitotic clonal expansion. Mitotic clonal expansion is reported to be necessary for the progression through subsequent steps in the differentiation program[31]. However, its role in the adipocyte differentiation

process has not been fully understood. In this point of view, it is surprising that conditioned medium directly led to the terminal differentiation program without mitotic clonal expansion as proven by FACS and BrdU experiment. This indicates that some cell specific events for the entry of terminal differentiation are truly associated with a result of mitotic clonal expansion, by secreting some factors. It remains to be elusive what proteins are secreted during mitotic clonal expansion and how they act on the cells for the entry of terminal differentiation.

This study defines the events that are necessary and sufficient for 3T3-L1 cell differentiation. To identify genes temporally regulated during a transition from mitotic clonal expansion to terminal differentiation in 3T3-L1 adipogenesis, I found G0/G1 switch gene 2 (G0s2) as a novel regulator of adipogenesis and fat accretion. My results suggest that G0s2 is essentially involved in adipocyte differentiation and lipid accumulation. Functionally, overexpression of G0s2 stimulated lipid droplet formation in 3T3-L1 adipocytes, and knockdown of endogenous G0s2, on the other hand, inhibited fat accumulation and stimulated apoptosis.

G0s2 is a small basic protein with 78% identity between mouse and human isoforms. Results from a limited number of studies have implied that G0s2 is a multifaced protein involved in proliferation, apoptosis, inflammation, metabolism, and carcinogenesis. In cultured human Simpson-Golabi-Behmel syndrome (SGBS) and mouse 3T3-L1 preadipocyte cell lines, both G0s2 mRNA and protein are steadily increased as the cells enter the growth arrest stage preceding the terminal

differentiation[23]. However, despite its postulated role as a cell cycle regulator, whether G0s2 plays any active role in the cell cycle withdrawal during adipogenic differentiation remains unclear. The current study advanced prior work by identifying a previously unrecognized G0s2 function. In this study, I demonstrated that expression of G0s2 is mainly found in adipose tissue and markedly induced during adipogenesis. Because C/EBPβ expression was not altered by G0s2 knockdown, it is suggested that G0s2 affects adipogenesis at a time point between C/EBPβ expression and C/EBPα expression.

Recent studies have provided compelling evidence that G0s2 complexes with ATGL and inhibits ATGL lipolytic activity[24]. Immunofluorescence staining of G0s2 protein expressed in Hela cells revealed the localization of G0s2 at the surface of lipid droplets[24]. This study suggests a unique role of G0s2 for ATGL-catalyzed lipolysis; however, it is unclear whether G0s2 plays a diverse role during adipocyte differentiation. The involvement of G0s2 in the cell proliferation or apoptosis is not clear yet. In the study using a different cell type, G0s2 has been shown to localize to mitochondria and promote apoptosis by interacting with the anti-apoptotic protein Bcl-2[32]. This interaction, and other possible roles at the mitochondria, would also likely function in an ATGL-independent manner. My study showed that without G0s2, differentiating 3T3-L1 cells underwent apoptotic program as evident by the expression of caspase 3. Therefore, it is likely that G0s2 has a dual mechanistic function, one being ATGL-dependent and the other ATGL-independent[33].

Previous studies have shown that G0s2 mRNA level is upregulated by PPARγ agonist in adipocytes[23]. In my experiment, G0s2 depletion induced an upregulation of caspase 3 expression, which may be sufficient to activate apoptosis. Although PPARγ is suggested as having an anti-proliferative function, little is known about the molecular mechanisms by which PPARγ ligands control cell proliferation and apoptosis. In this study, PPARγ knockdown also resulted in apoptosis after induction of differentiation with similar pattern observed with G0s2 knockdown cells. Neither this mean that cells undergo apoptotic pathway in the absence of PPARγ, nor that PPARγ is an apoptotic regulator. Rather, my result suggests that G0s2 or PPARγ is essential for a transition from the end of cell cycle to the terminal differentiation. In other word, cells expressing G0s2 and PPARγ can steadily enter and maintain the terminal differentiation process, but without G0s2, cells cannot stand for the strong mitotic signal, leading to apoptosis. Similarly, C/EBPα is well known to have anti-proliferative capacity. Because C/EBPα and PPARγ reciprocally regulate each other in adipogenesis, I suggest that the anti-proliferative functions of C/EBPα or PPARγ might be essentially mediated by G0s2.

In the present study, I demonstrated that the G0s2 has a strong impact on the adipocyte formation. To show this, I used fibroblasts from normal and *g0s2* knockout mouse embryos. In fact, the differentiation of the adipocyte was reduced in cultured MEF cells in the absence of G0s2. In MEF cells, in contrast to 3T3-L1

cells, G0s2 does not affect PPARγ or C/EBPα expression. This might be from a difference between MEFs and 3T3-L1 cells. Among the cell models of adipogenesis, 3T3-L1 and 3T3-F442A cells clearly undergo one or two rounds of cell division called mitotic clonal expansion[34]. However, this post-confluence mitosis was not observed in mouse C3H10T1/2 or human preadipocytes[8,35]. From this point of view, it is controversial whether mitotic clonal expansion is required for differentiation *in vivo*; however, it is clear that some of the checkpoint proteins for mitosis are involved in the regulation of adipogenesis[8]. Nevertheless, my data showed that WAT mass in *g0s2* knockout mice was significantly reduced *in vivo*. Given the phenotype of many small droplets exhibited by *g0s2*-knockout adipocytes, it might be a result from defects in droplets enlargement. But I cannot exclude a possibility that, like 3T3-L1 cells, a deficiency of G0s2 in the mice affected the integrity and maintenance of existing committed stem cells, which resulted in small fat pads in living mice. It would interest to measure the active proliferating cells in white fat pad using BrdU in living mice.

Ⅴ. CONCLUSION

In summary, the data presented in this report support that conditioned medium accelerated the differentiation into mature adipocytes, indicating a presence of cell-specific transitional events between mitotic clonal expansion and terminal differentiation.

I also found that G0s2 is required for adipogenesis, playing an important role in the transition from mitotic clonal expansion to terminal differentiation. In addition, G0s2 is required for accumulation of triacylglycerol, playing as a contributing factor in lipid droplet formation.

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ABSTRACT (IN KOREAN)

지방세포 분화 중 mitotic clonal expansion에서 terminal differentiation으로 이행에서 G0s2의 역할

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최 현 진

3T3-L1 세포는 지방세포의 분화 연구에 가장 많이 사용해 온 세포이며, 3-isobutyl-1-methylxanthine, dexamethasone, insulin을 처리 할 경우 성숙한 지방세포로 분화된다. 성장 정체되었던 3T3-L1 지방전구세포는 호르몬 유도 후 mitotic clonal expansion라고 부르는 세포 주기가 다시 돌아간 이후에 terminal differentiation 프로그램에 들어가게 된다. 지난 40년에 걸쳐 전사과정을 포함한 지방세포 분화 메커니즘이 밝혀져 있으나 clonal expansion에서 terminal differentiation으로 이행 과정은

잘 알려져 있지 않다. 본 연구자는 지방세포 분화에서 clonal expansion과 terminal differentiation사이의 관계에 대해 밝히고자 이 연구를 수행하였다. 먼저 mitotic clonal expansion 이후 세포에 노출되었던 배지를 수거하여 conditioned medium이라고 명명하였다. 이 배지를 새로운 3T3-L1 세포에 처리한 경우 성숙한 지방세포로 분화가 촉진되었으며, 이는 mitotic clonal expansion과 terminal differentiation 사이에 세포 특이적인 이행 과정이 존재함을 시사한다. 이러한 이행 과정에서 중요한 지방분화를 조절하는 조절 인자를 찾고자, 분화중인 지방전구세포의 유전자 발현 profile을 알고리즘을 이용하여 분석하였으며, 그 결과로 G0s2를 발견하였다. G0s2는 백색지방과 갈색지방에서 많이 발현하고 3T3-L1 세포의 분화과정에서 증가하였다. 특히, G0s2의 발현은 conditioned medium에서 촉진되었으며, 이는 terminal differentiation으로 이행과 관련이 있다는 것을 말해준다. 3T3-L1 세포 분화 중 siRNA로 G0s2 발현을 저해하면 지방분화가 억제되었으며, G0s2를 과발현하면 지방전구세포에서 성숙한 지방세포로 분화가 증가되었다. G0s2는 PPARγ에 의해 조절되며, PPARγ나 G0s2가 없을 경우 terminal differentiation으로 가기 전 apoptotic pathway가 활성화 되었다. 생체 내에서 G0s2의 역할을 확인하고자 G0s2가 없는 쥐를 제작하였다. G0s2가 없는 쥐는 전반적으로 정상적 소견을 보이나 정상 쥐에 비해 지방조직이 감소되었으며, 이는 지방

분화 과정이 손상되었음을 시사한다. 결과적으로 G0s2는 지방형성에서 중요한 인자로 작용함을 시사한다. G0s2는 mitotic clonal expansion에서 terminal differentiation으로의 이행에 중요한 역할을 하며, 지방분화와 중성지방 축적에 요구되어짐을 발견하였다.

핵심되는 말: 지방분화, conditioned medium, mitotic conal expansion, terminal differentiation, G0/G1 switch gene 2