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Full Length Research Paper

Growth arrest induction of 3T3-L1 preadipocytes by serum starvation and their differentiation by the hormonal adipogenic cocktail

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The current methods for differentiation of 3T3-L1 preadipocytes start with growth arrest by contact inhibition. However, on day 6, 3T3-L1 preadipocytes reliably detach and differentiation could no longer proceed. In the present study, we used serum starvation to induce growth arrest in 3T3-L1 preadipocytes and investigated their differentiation by the modified hormonal adipogenic cocktail. About 85% of 3T3-L1 cells were in G0/G1 stage under serum deprivation. There were no significant difference between the percentage of cells in G0/G1 phase under these serum deprivation condition and that of post-confluent cells. Growth assays indicated that cells under serum starvation condition grew slow, two-days later than growing and post-confluent cells. We determined that 0.5% FBS for 48 h was the optimal condition to arrest 3T3-L1 cells at the G0/G1 phase. The grow-arrested 3T3-L1 preadipocytes were induced to differentiation by hormonal adipogenic cocktail. After 19 days of differentiation, over 90% 3T3-L1 cells exhibited adipocyte morphology with ring-like lipid droplet in cytoplasm. The mRNA levels of critical transcriptional factor CCAAT/enhancer binding proteins-α and peroxisome proliferator-activated receptor-γ were determined to validate the differentiation. These results indicated that serum starvation effectively arrested the growth of 3T3-L1 preadipocytes and prevented cells from detachemnt caused by contact inhibition at confluence. Furthermore, growth arrested 3T3-L1 preadipocytes by serum starvation successfully differentiated into adipocytes by the hormonal adipogenic cocktail.

Key words: Cell cycle, detachment, differentiation, growth arrest, preadipocytes, serum starvation,

INTRODUCTION

Obesity is a risk factor for arteriosclerosis, diabetes, hyperlipidemia, and is a major public health problem (Mokdad et al., 2003). The development of obesity is characterized by increased number of fat cells and their lipids due to the process of so-called mitogenesis and differentiation, which are regulated by genetic, endocrine, and nutritional factors (Unger and Zhou, 2001; Farmer and Auwerx, 2003; Fu et al., 2005).

The 3T3-L1 preadipocytes are commonly used as an adipocyte differentiation model system to investigate the molecular mechanisms for adipogenesis and pharmacology of agents preventing obesity. The current methods for differentiation of 3T3-L1 preadipocytes employ the adipogenic cocktail to treat postconfluent cultures of 3T3-

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L1 cells with a combination of insulin (Ins), 3-isobutyl-1-methylxanthine (MIX), dexamethasone (Dex). During this differentiation process, the first stage of 3T3-L1 is growth arrest, which is achieved by contact inhibition at post confluence. Immediately after induction by the hormonal cocktail, growth-arrested postconfluent 3T3-L1 preadipocytes re-enter the cell cycle (called mitotic clonal expansion) and start the adipocyte differentiation program (Rosen and Spiegelman, 2000). During the clonal expansion, usually on day 4, 3T3-L1 preadipocytes exhibit overlapped growth and netting. After 6 d, when preadipocytes are continuously stimulated by Ins, cells reliably detached like a paper from culture plates and lost. Some studies have reported the deatachment during differentiation process of adipocytes and other types of animal cells (Moxley et al., 2002; Griffin et al., 2004; Kamishina et al., 2008). It is possible that a higher cell density and more heterogeneously differentiated cells provided various growth factors that maintained cells in an “indefinitive” adherent status (Moxley et al., 2002).

Serum starvation can restrict cell growth into an out of cycle state referred to as G0/G1 phase (Aaronson, 1991), which is commonly used to synchronize cells (Kues et al., 2000). The recent study has shown that reduced serum concentration increases endocrine differentiation from murine embryonic stem cells (Vincent and Odorico, 2009). Besides, cells usually have grown to 60-80% confluency for serum deprivation. Because growth-arrested 3T3-L1 preadipocytes achieved by contact inhibition undergo at least one round of DNA replication and cell doubling, excessive 3T3-L1 preadipocytes plus contraction may lead to cell detachment. As addressed above, we therefore hypothesized that serum deprivation may be used to arrest the growth of subconfluent 3T3-L1 preadipocytes in G0/G1 phase, which may prevent them from detachment initialed by excessive growth of cells in the following process of clonal expansion.

In order to test this hypothesis, we examined the cell cycle of 3T3-L1 preadipocytes under serum conditions at various time points. The results have shown 3T3-L1 preadipocytes by serum starvation effectively were arrested growth in G0/G1 phase. During the following differentiation process, compared with growth-arrested cells by contact inhibition, growth-arrested 3T3-L1 pre-adipocytes under serum deprivation in culture remained attached to the culture plates after induction by the adipogenic cocktail and afterwards they finally differentiated the mature adipocytes using the differentiation protocols.

MATERIALS AND METHODS

Chemical reagents

Dulbecco's Modified Eagle's Medium (DMEM) cell culture medium was purchased from GIBCO-BRL Life Technologies (NY, USA). Fetal bovine serum (FBS) was obtained from HYCLONE (Logan, UT). All other chemicals and reagents were purchased from Sigma-Aldrich (MO, USA) unless otherwise specified.

Cell culture

The mouse 3T3-L1 preadipocytes (ATCC CL-173) were maintained in DMEM complete medium containing 100 units/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated FBS at 37°C in the presence of 5% CO_2. Because serum contained the factors for facilitating 3T3-L1 differentiation from preadipocytes to adipocytes when they were confluent, these cells were subcultured before reaching confluency.

Flow cytometric analysis

The cell cycles of 3T3-L1 cells in the eight experimental groups were determined: Subconfluent (60 to 80% confluent cells), Post-confluent (confluent cells were cultured in DMEM supplemented with 10% FBS for two days), 0.5% FBS 24 h (subconfluent cells were cultured in DMEM supplemented with 0.5% FBS for 24 h), 0.5% FBS 48 h (subconfluent cells were cultured in DMEM supplemented with 0.5% FBS for 48 h), 0.5% FBS 72 h (subconfluent cells were cultured in DMEM supplemented with 0.5% FBS for 72 h), 0.2% FBS 24 h (subconfluent cells were cultured in DMEM supplemented with 0.2% FBS for 24 h), 0.2% FBS 48 h (subconfluent cells were cultured in DMEM supplemented with 0.2% FBS for 48 h), 0.2% FBS 72 h (subconfluent cells were cultured in DMEM supplemented with 0.2% FBS for 72 h). Cellular DNA content was determined by staining cells with propidium iodide and measuring fluorescence in a Becton Dickinson FACScan (Rutherford, NJ) (Kues et al., 2000).

Growth curve assays

The growth curve of serum-starved 3T3-L1 preadipocytes was assessed by 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT). For the MTT assay, cells were plated at 1000 cells/well, in 96-multiwell culture plates with flat bottoms (Grenier, Germany) and cultured in DMEM supplemented 10% FBS. Cell proliferation assays were conducted every day for seven days. Each time point was run in triplicate.

Oil Red-O staining

After the induction of differentiation, the cells were stained with Oil Red O. Briefly, cells were washed with phosphate-buffered saline and fixed with 10% formalin in phosphate buffered saline for 1 h, and then washed with distilled water. Cells were stained with Oil Red O (Ward Hill, MA) (six parts of 0.6% Oil Red O dye in isopropanol and four parts of water) for 1 h. Excess stain was removed by washing with distilled water, and the stained cells were dried.

Quantitative reverse-transcription polymerase chain reaction (q-RT-PCR)

Total RNA was extracted from 3T3-L1 cells with the TransZol reagent (Transgen Biotech, Beijing, China) following the supplier’s protocol. RNA was subsequently reverse transcribed to cDNA with EasyScript First-strand cDNA Synthesis SuperMix (Transgen Biotech, China) according to the manufacturer’s directions. Generated cDNA was subjected to real-time RCR undertaken using SYBR Green Ⅰ type (TaKaRa SYBR® Premix Ex Taq™) according to the manufacturer’s directions. The gene specific primers were as

follows: PPAR-γ2 (NM_011146), forward 5'-TCA AGC CCT TTA CCA CAG-3'; reverse 5'-ACA GAC TCG GCA CTC AAT-3';C/EBP-α (NM_007678), forward 5'- TGC GCA AGA GCC GAG ATA AAG -3'; reverse 5'- TCA CGG CTC AGC TGT TCC AC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NM_001001303), forward 5'-AAA TGG TGA AGG TCG GTG TG-3'; reverse 5'-TGA AGG GGT CGT TGA TGG-3'.

Statistical analysis

Data were presented as the means ± S.D. Statistical analysis was performed using one-way ANOVA. Comparisons between two groups were made using Student's *t*-test. Multiple comparisons were made by Ducan test. All statistical tests were performed using SPSS software. Statistically significant difference was set at $P < 0.05$.

RESULTS

Effect of serum starvation on cell cycle of 3T3-L1 preadipocytes

The relative percentage of confluent cells in the S and G2/M phases of the cell cycle was approximately 45% for 3T3-L1 preadipocytes. After reaching post-confluency the relative percentage of cells that entered into G0/G1 increased to 86.38%. These results indicated that post-confluency arrested 3T3-L1 preadipocytes in the G0/G1 phases of the cell cycle. FACS measurements revealed that the relative percentage of cells at the G0/G1 phase increased within 72 h maintained in serum deprivation medium for 24, 48, and 72 h. Compared to 86.38 ± 0.322% of the post-confluent cells in G0/G1 phase, serum deprivation treatment for 48 h by 0.5% and 72 h by 0.2% FBS had an equal effect (85.53 ± 0.656%, $P > 0.05$; 86.88±0.098%, $P > 0.05$, respectively) (Table 1 and Figure 1). In comparison, deprivation of 0.5% FBS caused the maximum percentage of cells in G0/G1 phase faster than that of 0.2% FBS. Therefore, deprivation of 0.5% FBS for 48 h was chosen to arrest the growth of 3T3-L1 preadipocytes.

Effect of serum starvation on growth of 3T3-L1 preadipocytes

Based on cell cycle results, we measured growth process of post-confluent 3T3-L1 cells and serum starvation treatment of 3T3-L1 cells via MTT. As shown in Table 2 and Figure 2, normal 3T3-L1 cell growth curve included four phases, the lag phase, logistic phase, decelerating phase and stationary phase. Compared with normal 3T3-L1 cells, post-confluency had no significant effect on cell growth during the most measured period, although the number of post-confluent 3T3-L1 cells was significantly fewer than that of normal cells at day 2 and day 4. Serum reduction led to a slow growth including a long lag phase, about one day after post-confluent cells, one and half day after normal cells. Cell growth had no obvious differences between the two serum-deprived treatments.

Differentiation process

Based on the previous results, we designed the new differentiation strategy of 3T3-L1 preadipocytes. Figure 3 showed the protocols for the differentiation of 3T3-L1 preadipocytes. After cells were cultured to subconfluent, cells were washed three times and maintained in DMEM supplemented with 0.5% FBS for 48 h. To induce differentiation, 2 days after 3T3-L1 preadipocytes under serum starvation condition (day 0), medium was changed for DMEM containing 10% FBS serum with 0.5 mM Mix, 0.25 μM Dex, and 1 μg/ml Ins (MDI). Then preadipocytes were replaced every 2 days with DMEM supplemented with 10% FBS and 1 μg/ml Ins until the end of experiment (day 19).

After cells were cultured to confluent, cells were washed three times and maintained in DMEM supplemented with 0.5% FBS for 48 h. To induce differentiation, 2-days serum-deprived 3T3-L1 preadipocytes (day 0) were stimulated for 72 h with 0.5 mM MIX, 0.25 μM DEX, and 1 μg/ml Ins (MDI) in DMEM containing 10% FBS serum. Then preadipocytes were maintained in and refed every 2 days with DMEM supplemented with 10% FBS and 1 μg/ml Ins until the end of experiment on day 19.

Representative phase images of 3T3-L1 cells under the inverted microscope were shown in Figure 4. During the growth phase, 3T3-L1 preadipocytes were morphologically similar to fibroblasts. After serum starvation for 48 h, although a few cells detached and floated to the medium, the number of cells increased, which indicated serum reduction didn't completely inhibit the proliferation. Marked changes in cells morphology were observed. Serum starvation changed cell morphology to a more round and flat cell body with less polar. The edge and inner structures of cells were less visible. After 48 h serum-deprived treatment, induction of differentiation by MDI and insulin leads to drastic cell shape changes. Incubation with MDI for 72 h (day 3) led to the quick proliferation and the cell size was larger than confluent and serum-deprived cells. With induction preceding, the 3T3-L1 preadipocytes progressively converted to a spherical shape and accumulated lipid droplet. On day 7, a few lipid droplets were observed in cytoplasm. Until day 19, more than 90% of cells accumulated the lipid droplets (Figure 5). These results indicated that 3T3-L1 preadipocytes completely differentiated the mature adipocytes.

PPAR-γ and C/EBP-α mRNA expression

As shown in Figure 5A, PPAR-γ expression wasn't detectable in serum-deprived 3T3-L1 preadipocytes (day 0), was detectable at day 3, then increased day by day. At day 11, the maximal levels of PPAR-γ expression were

Table 1. Effect of serum starvation on cell cycle of 3T3-L1 preadipocytes.

Treatment	G0/G1 (%)	S (%)	G2/M (%)
0.2% FBS-24 h	81.93 ± 0.36^{b}	2.56 ± 0.12^{b}	13.69 ± 0.22^{b}
0.2% FBS-48 h	80.07 ± 1.85^{a}	2.20 ± 0.12^{a}	15.34 ± 2.14^{b}
0.2% FBS-72 h	86.88 ± 0.10^{c}	2.11 ± 0.30^{a}	9.67 ± 0.40^{a}
0.5% FBS-24 h	80.9 ± 0.39^{ab}	2.05 ± 0.01^{a}	14.91 ± 0.91^{b}
0.5% FBS-48 h	85.53 ± 0.66^{c}	2.30 ± 0.07^{ab}	10.72 ± 0.96^{a}
0.5% FBS-72 h	81.57 ± 0.40^{b}	2.55 ± 0.06^{b}	13.23 ± 0.18^{b}
Post-confluent	86.38 ± 0.32^{c}	2.96 ± 0.08^{c}	9.33 ± 0.37^{a}
Confluent	54.21 ± 1.44^{d}	23.53 ± 0.08^{d}	21.16 ± 1.45^{c}

Within columns, values with different supercripts are significantly different (P < 0.05).

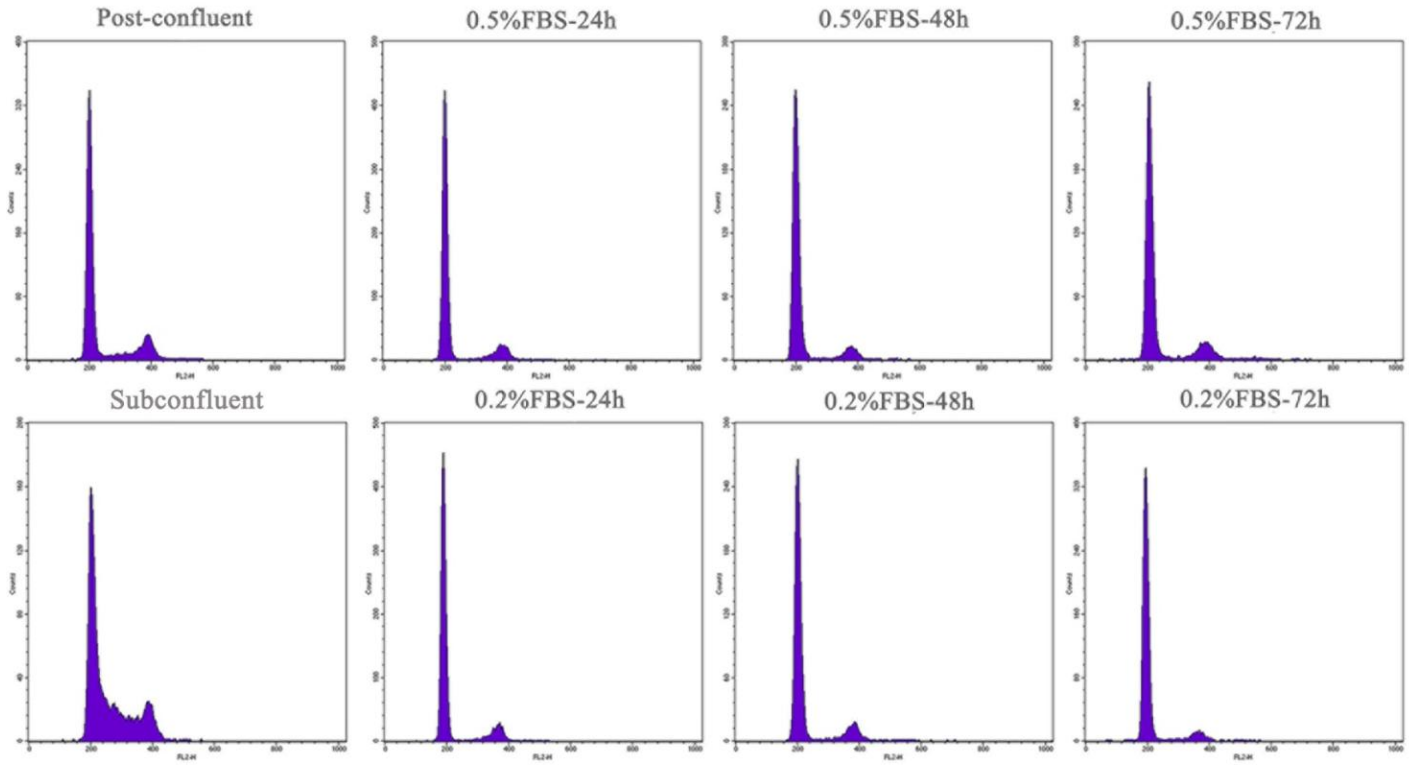

Figure 1. Typical histograms of DNA content obtained using flow cytometry of 3T3-L1 preadipocytes, under a variety of conditions. 3T3-L1 preadipocytes were maintained in DMEM containing low concentration of FBS for the indicated times. Cellular DNA content was determined by staining cells with propidium iodide and measuring fluorescence in a Becton Dickinson FACScan. Cellular DNA content of confluent or post-confluent cells was also measured.

observed and maintained until day 17. Interestingly, on day 19, PPAR-γ expression significantly decreased. Quantification of the changes in expression by q-RT-PCR indicated that PPAR-γ mRNA levels went up approximate 3 fold from day 0 to day 11 (Figure 6A). Compared with the undetectable levels of PPAR-γ in preadipocytes, C/EBP-α expression was detectable in serum-deprived 3T3-L1 preadipocytes (day 0) and reached to full expression on day 5 after the initiation of differentiation program. Then, the maximal level of expression was maintained until day 19. Quantification of the changes in expression by real-time PCR indicated that C/EBP-α mRNA levels were shown in Figure 6B.

DISCUSSION

One new observation described here is the growth-arrested status by serum deprivation prevented six-day-old differentiated 3T3-L1 preadipocytes by the adipogenic cocktail from detaching from the culture plates. Although the 3T3-L1 preadipocytes have been widely used as a

Table 2. Effect of serum starvation on the growth of 3T3-L1 preadipocytes.

Treatments	day1	day2	day3	day4	day5	day6	day7
Post-confluent	0.118 ± 0.005	0.269 ± 0.009	0.519 ± 0.051	0.753 ± 0.022	1.144 ± 0.110	1.484 ± 0.030	1.818 ± 0.067
0.5% FBS-48h	0.092 ± 0.004*	0.144 ± 0.005*	0.258 ± 0.015*	0.448 ± 0.027*	0.773 ± 0.048	1.162 ± 0.044*	1.461 ± 0.059*
0.2% FBS-72h	0.104 ± 0.004*	0.128 ± 0.007*	0.213 ± 0.003*	0.459 ± 0.031*	0.778 ± 0.040*	1.100 ± 0.021*	1.447 ± 0.034*
Growing	0.112 ± 0.007	0.338 ± 0.026*	0.626 ± 0.021	0.925 ± 0.000*	1.289 ± 0.088	1.696 ± 0.033	1.706 ± 0.052

*$P < 0.05$ compared to post-confluent.

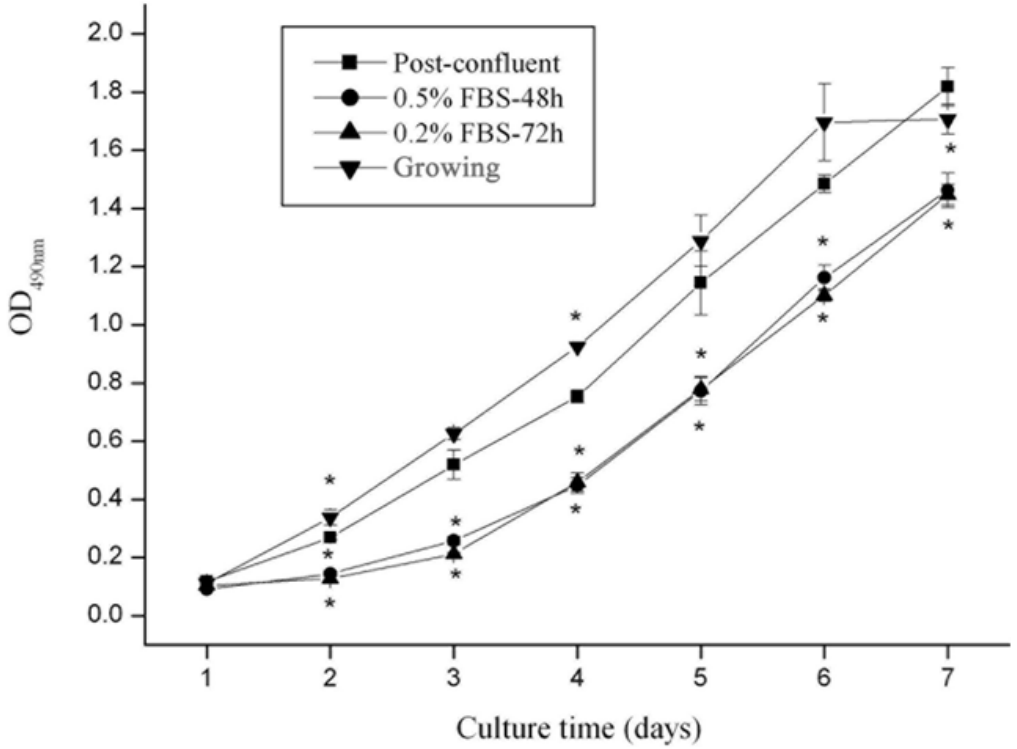

Figure 2. Effect of serum starvation on the proliferation of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were maintained in DMEM containing low concentration of FBS for indicated time. The growth curve of serum-starved 3T3-L1 preadipocytes were assessed by MTT. Normal and post-confluent 3T3-L1 preadipocytes were used to compare with serum-deprived cells. Cell proliferation assays were conducted every day for seven days. Each time point was run in triplicate. The results are reported as means±S.D. *$P < 0.05$ compared to post-confluent cells.

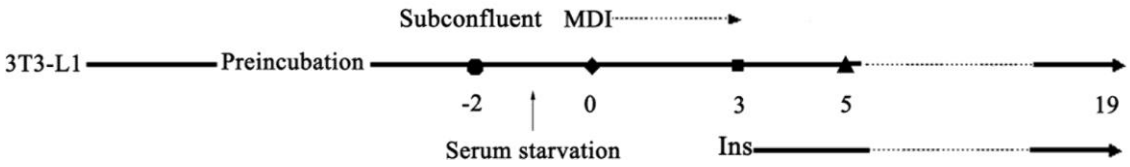

Figure 3. Experimental protocol of induced differentiation of 3T3-L1 preadipocytes.

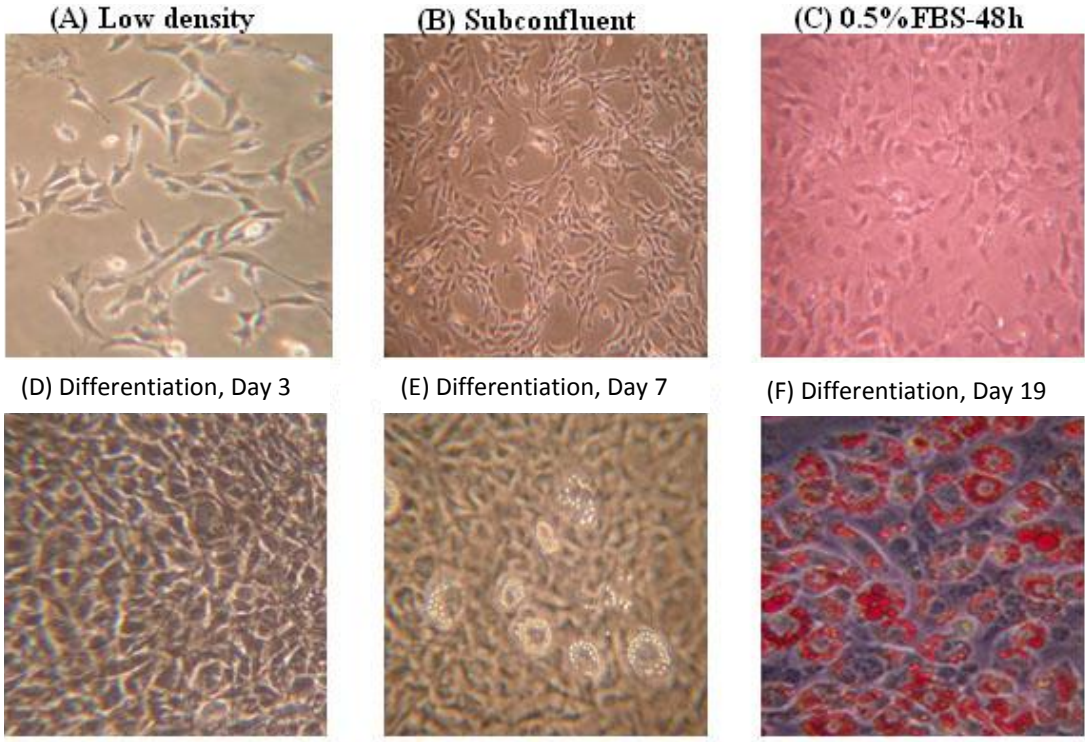

Figure 4. Microscopic appearance of 3T3-L1 preadipocytes during the differentiation process. (A.C.D. ×400 magnification, B.E.F. ×200 magnification).

system in fat metabolism related studies and the detachment during the differentiation of 3T3-L1 preadipocytes to adipocytes often happens, less attention has not been paid to it. In this study, according to our knowledge, we first reported that growth-arrested 3T3-L1 preadipocytes achieved by serum starvation did not detach from the culture plates and afterwards they successfully differentiated into mature adipocytes.

Differentiation of 3T3-L1 preadipocytes requires withdrawal from cell cycle. Therefore, we firstly investigated effects of different serum starvation conditions on 3T3-L1 cell cycle. Our data indicated that both serum starvation and full confluency significantly increased the percentage of cells at the G0/G1 phase and at least 85% cells were arrested at this phase. This study demonstrated that serum deprivation (0.2 or 0.5% FBS) has drastic effects on the cell cycle status of 3T3-L1 preadipocytes. The data are consistent with the previous studies although the synchronization duration is variable (Zandbergen et al., 2005; Goldfine et al., 2006). Statistical analysis has shown similar grow-arrested efficiencies in G0/G1 stage after treated with 0.5% FBS for 48 h and 0.2% FBS for 72 h, compared to the full confluent statement. These results indicated that serum starvation efficiently arrested the growth in 3T3-L1 preadipocytes and these cells are committed to differentiate.

Research results have shown that serum deprivation affects cell viability and proliferation (Jiang and Xu, 1998; Ishii et al., 2004). This led us further determine the growth of 3T3-L1 preadipocytes under serum deprivation conditions by MTT. Our results indicated that post-confluency had no significant effect on 3T3-L1 cell growth during the most measured period, compared with the growing cells. In the two serum-deprived treatment groups, 0.5% FBS for 48 h and 0.2% FBS for 72 h, cells growth slowed, although cell cycle analysis revealed the comparable cell ratio in G0/G1 phase under both serum-starvation condition to that under post-confluent

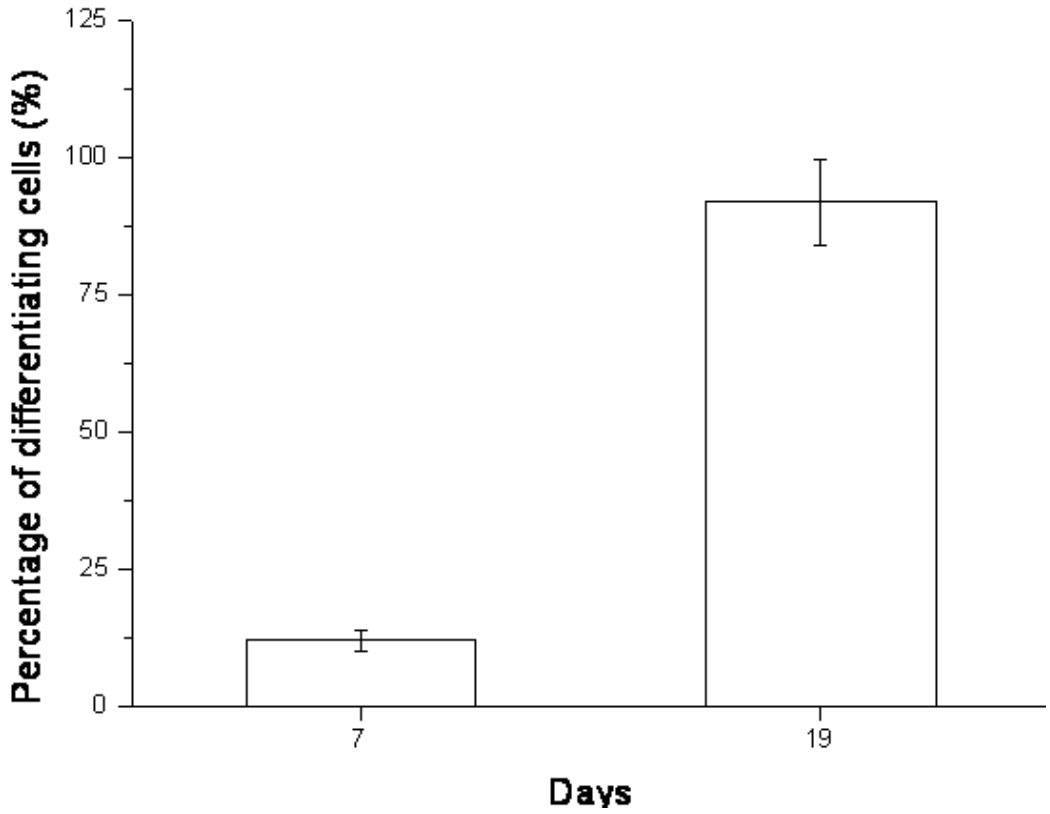

Figure 5. Percentage of differentiating cells. The cells accumulating lipid drops were counted and the percentage of differentiating cells were caculated. Data were from three independent experiments. The results are reported as means ± S.D.

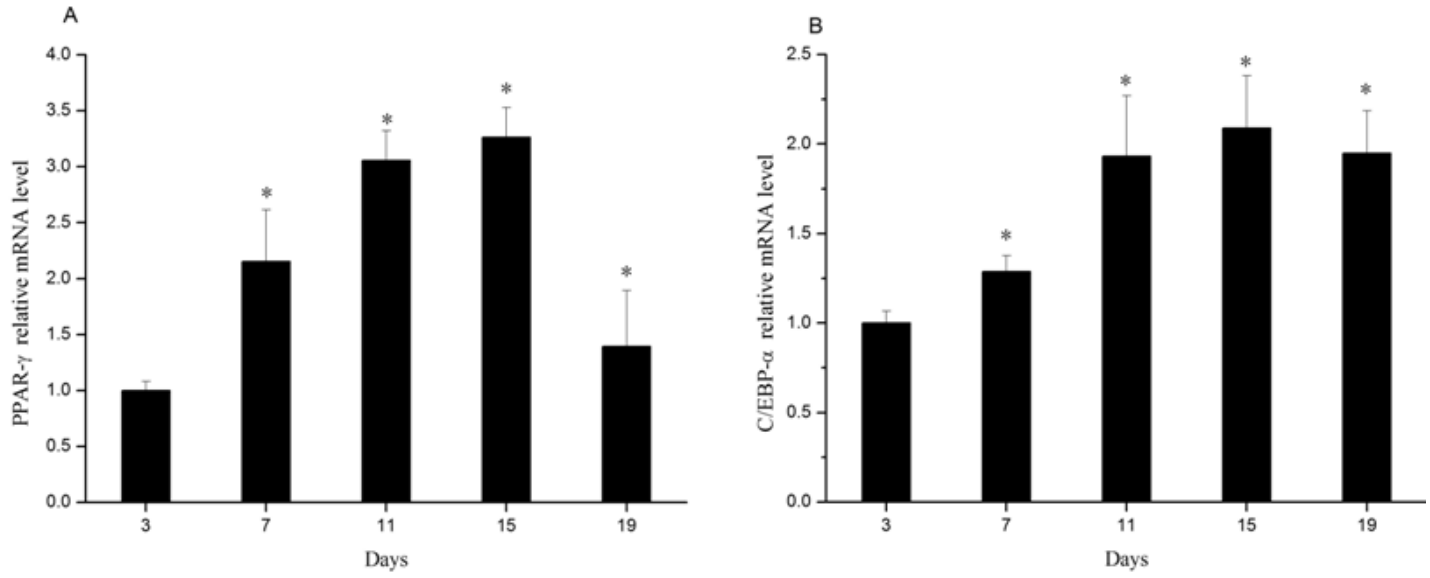

Figure 6. PPAR-γ and C/EBP-α mRNA expression during 3T3-L1 preadipocytes adipogenesis. 3T3-L1 preadipocytes were cultured to confluent. After incubated with DMEM supplemented with 0.5% FBS for 48 h (day 0), 3T3-L1 preadipocytes were stimulated with MDI for 72 h (day 3) and then refed with DMEM containing 1μg/ml insulin every two days until the end of experiment (day 19). (A) On day 3, 7, 11, 15, and 19, PPAR-γmRNA levels were measured by Real-time RT-PCR. (B) C/EBP-α mRNA levels were measured by q-RT-PCR. Values are normalized to GAPDH mRNA expression level and are expressed relative to differentiated 3T3-L1 cells on day 3. The results are reported as means ± S.D. *P < 0.05 compared to differentiated 3T3-L1 cells on day 3.

condition. The mechanism involved in quiescence between serum starvation and contact inhibition might differ (Blomena and Boonstraa, 2007). However, little is known about the proliferation after addition of mitotic factor in both cases. Serum starvation has suggested to cause time-dependent changes in some mitogen pathways (Pirkmajer and Chibalin, 2011), suggesting serum deprivation might impact the following mitosis.

Based on the above results, we determined that 0.5% FBS for 48 h was the optimal method to arrest 3T3-L1 preadipocytes in the G0/G1 phase. Trudel et al. (2004) have reported that serum starvation failed to induce the morphologic differentiation of multiple myeloma despite arresting the cells in G1. This led us to modify the current adipogenic cocktail-based protocol to differentiate 3T3-L1 preadipocytes growth-arrested by serum starvation to mature adipocytes. On the preliminary experiment, we firstly induced the differentiation as follows: subonfluent 3T3-L1 cells were maintained in DMEM containing 0.5% FBS for 48 h (day 0). Growth arrested cells were stimulated with DMEM containing with 0.5 mM MIX, 0.25 µM Dex, and 1 µg/ml Ins (MDI) in DMEM containing 10% FBS serum for 72 h (day 3). Then preadipocytes were maintained in DMEM supplemented with 10% FBS and 1 µg/ml Ins for 48 h (day 5) and replaced with DMEM supplemented with 10% FBS every 2 days. However, 3T3-L1 preadipocytes didn't show adipocytic phenotype and accumulate the lipid droplet in cytoplasm until day 19 (data not shown). Ins acts through insulin-like growth factor-1 (IGF-1) receptor on the membrane to induce differentiation and IGF-1 receptor initiates the adipocyte differentiation in post-confluent 3T3-L1 preadipocytes (Xu and Liao, 2004). Growth arrest sensitizes the IGF-1signal pathway in post-confluent 3T3-L1 preadipocytes (Zhu et al., 2009). However, growth-arrested 3T3-L1 preadipoctyes achieved by serum starvation may not respond short time stimulation by Ins. Therefore, we modified differentiation protocol as shown in Figure 3. After stimulated by MDI, 3T3-L1 preadipocytes were refed every two days with DMEM supplemented with 10% FBS and 1 µg/ml Ins. On day 19, over 90% of 3T3-L1 cells performed the characteristic of mature adipocytes, ring lipid droplet in cytoplasm. These results indicated that growth arrested 3T3-L1 preadipocytes induced by serum starvation were completely differentiated into the mature adipocytes. Additionally, there was an interesting finding that while establishing 3T3-L1 cell culture conditions in either BD Falcon® or Grenier® six-well plates to investigate the differentiation into mature adipocytes, we observed disappointingly low differentiation efficiency. However, good differentiation was seen when these cells were plated in Corning® ∆35mm dishes. These results are consistent with the previous reports on variability in 3T3-L1 adipocyte differentiation depending on cell culture dish (Anisha et al., 2007). The variation in differentiation in these may possibly be dependent on the airflow characteristics to the cells and media, assuming that the culture surfaces are identical (Anisha et al., 2007).

At the molecular level, adipogenesis is driven by a complex transcriptional cascade involving the sequential activation of C/EBPs and PPAR-γ (Farmer, 2006). C/EBP-β and C/EBP-δ are rapidly and transiently expressed after the hormonal induction of differentiation. These factors act synergistically to induce the expression of C/EBP-α and PPAR-γ, the master adipogenic transcription factors. C/EBP-α and PPAR-γ together promote terminal differentiation by activating the transcription of genes involved in creating and maintaining adipocyte phenotype.

In addition, PPAR-γ is one of adipogenic marker. We measured the mRNA expression of C/EBP-α and PPAR-γ during the differentiation process. Expression of PPAR-γ rose significantly during differentiation of growth-arrested 3T3-L1 adipocytes caused by serum starvation. PPAR-γ mRNA level increased from undetectable level in serum-starved 3T3-L1 preadipocytes to detectable level 3 days after MDI stimulation and reached maximum on day 11 to 17. On day 19, PPAR-γ expression decreased quickly. Expre-ssion of C/EBP-α was observed in serum-starved 3T3-L1 preadipocytes and was maintained the higher level until the end of differentiation. The previous studies which adopt full confluency plus the typical adipogenic cocktail to differentiate the 3T3-L1 preadipocytes into the adipocytes have shown that PPAR-γ is easily detectable during the second day of 3T3-L1 adipocyte differentiation, and maximal levels of expression are attained in mature adipocytes (Chawla and Lazar, 1994; Brun et al., 1996). In addition, C/EBP-α increases from undetectable levels in preadipocytes to detectable levels 2 days after MDI stimulation and to full expression, 5 days after initiation of the differentiation program (Christy et al., 1989; Lin and Lane, 1994). Expression of C/EBP-α and PPAR-γ with the proceeding differentiation confirmed the complete differentiation of 3T3-L1 preadipocytes into the mature adipocytes at the mRNA level. Growth arrest appears to be required for adipocyte and is accompanied by complex changes in the pattern of gene expression which plays central role in controlling lipid accumulation and terminal differentiation. The previous studies have shown that the specific genes of growth arrest are differentially expressed in serum deprived and contact inhibited quiescent NIH3T3 cells and C3H10T1/2 cells (Gustincich and Schnedier, 1993; Nelson and Gelman, 1997; Gos et al., 2005). The pattern of gene expression during growth arrest can differ with the specific differentiation protocols employed. In the present study, we used serum starvation instead of contact inhibition to lead to growth arrest. Thus, the differences between new differentiation strategy and the typical one may partly contribute to different methods used for establishing growth arrest.

Together, we demonstrated 3T3-L1 preadipocytes were growth-arrested under serum starvation. Because the typical cocktail protocol didn't successfully differentiate

the growth-arrested 3T3-L1 achieved by serum deprivation into adipocytes, we modified the differen-tiation protocol. There were significant differences between our differentiation protocol employed and the typical cocktail protocol, including differentiation process and expression of critical transcriptional factors. This may partly contribute to the differences between serum starvation and contact inhibition for establishing growth arrest. However the mechanism of serum starvation effect on 3T3-L1 preadipocytes differentiation needs further investigation.

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