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Asian Journal of Animal and Veterinary Advances

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Asian Journal of Animal and Veterinary Advances 6 (5): 482-487, 2011
ISSN 1683-9919 / DOI: 10.3923/ajava.2011.482.487

3T3-L1 Cell Line Revealing Partially White Adipogenesis of Mice

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ABSTRACT

3T3-L1 cell line is a model for studying adipose differentiation. Transcription factor 21 (Tcf21) positively regulates bone morphogenetic protein 4 (BMP4) expression, while BMP4 can up regulate differentiation of white adipocytes. So Tcf21 is a gene in the pathway of regulation of white adipocyte differentiation. In the present study, results of oil and red O staining showed that 3T3-L1 preadipocytes successfully differentiated into adipocytes by inducers. The RNA of mice white adipose tissues were harvested from the peri-uterine fat pads of female and the epididymal fat pads of male KUNMING (KM) mice and RT-PCR results showed that Tcf21 expressed in primary white adipocytes and tissues of mice while its expression was not detectable in 3T3-L1 cell differentiation (from 3 to 192 h). So conclusion was draw that 3T3-L1 cell line can not reveal completely white adipogenesis *in vivo* and Tcf21 was absent in the pathway of regulation of 3T3-L1 adipose differentiation.

Key words: Fat, oil and Red O staining, adipose tissues, preadipocytes, adipose differentiation

INTRODUCTION

Diabetes (containing type 1 and 2) occurs worldwide and its incidence is rising, type 2 diabetes occurs more frequently in obese animals (Ladan *et al*., 2007; Guilherme *et al*., 2008; Ozcelik and Ucar, 2008). Adipose tissues play an essential role in energy homeostasis and have numerous physiological and pathological functions (Oishi *et al*., 2005). At the present time, adipogenesis is a research hot spot that is studied widely in human disease and livestock production. *In vitro*, adipogenesis has been studied using some cell lines, in which 3T3-L1 cell line is a widely used model. 3T3-L1 cell line is a substrain of 3T3 cells (Swiss albino) developed through clonal isolation. The cells can undergo a preadipocyte to adipocyte like conversion and accumulate triglyceride in cytoplasm (Gregoire *et al*., 1998). 3T3-L1 cells have two shapes which are fibroblast shape (preadipocyte) and round shape (adipocyte), respectively. Induced with insulin, dexamethasone and IBMX, 3T3-L1 preadipocytes convert into adipocytes after 8 day differentiation. Adipocytes differentiated from 3T3-L1 cells are evaluated using Oil and Red O staining, which could bind to triglyceride in adipose cytoplasm and is a fast and simple method to evaluate the extent of conversion from preadipocyte to adipocyte (Ramirez-Zacarias *et al*., 1992).

Transcription factor 21 (Tcf21) is a basic-helix-loop-helix transcription factor that is highly expressed in the mesenchyme of developing organs (Cui *et al*., 2003). Previous research showed that Tcf21 expresses in primary white adipocytes of mice, but not in primary brown adipocytes and C2C12 cells of mice (Timmons *et al*., 2006). Tcf21 positively regulates the expression of Bone Morphogenetic Protein 4 (BMP4) (Quaggin *et al*., 1999), furthermore BMP4 expression can promote

differentiation of white adipocytes (Tseng *et al.*, 2008). Therefore, Tcf21 is likely a gene in the pathway regulating 3T3-L1 cells differentiation. In the present research, we detected Tcf21 expression in the differentiating process of 3T3-L1 cells to study its expression patterns in the model cells.

MATERIALS AND METHODS

All of the research work was conducted in the laboratories at College of Animal Science and Technology, China Agricultural University in the year of 2009 and 2010.

Cell culture and differentiation: 3T3-L1 cells (American Type Culture Collection) were cultured in high glucose DMEM (GBICO, America), with 10% newborn calf serum (GBICO, America) at 37°C and 5% CO_2.

3T3-L1 cells differentiated with DMEM, 10% Fetal Bovine Serum (FBS), 1uM bovine insulin (Sigma, America), 0.5 mM 3-Isobutyl-1 methylxanthine (IBMX, Sigma, America) and 1 μM dexamethasone (Sigma, America) for 2 days. From day 3 to day 4, the cells were induced with DMEM, 10% FBS and 1 μM insulin. From day 5 to day 8, the cells were maintained with DMEM and 10% FBS (Bai *et al.*, 2007).

Oil red O staining: After washing with PBS for three times, cells were fixed with 3.7% formaldehyde (Sigma, America) for 5 min at room temperature. After a second wash, cells were stained with a filtered solution of 0.3% Oil and Red O (Sigma, America) for 30-40 min. Finally, cells were washed three times with water (Mariani *et al.*, 2007).

Mouse white adipose tissue preparation: Mouse White Adipose Tissue (WAT) was obtained from peri-uterine fat pads of three 6-week-old female KUNMING (KM) mice. Other white adipose tissue was obtained from epididymal white adipose tissues of three 6-week-old male KM mice.

RNA isolation and reverse transcription: Cells for extracting RNA were harvested using RNAprep pure Cell (TIANGEN BIOTECH, China) at an interval of 3 h during the period from 3 to 192 h after the cells differentiated. The RNA of mice white adipose tissues was isolated using Trizol reagents (Invitrogen, America) and DNA in the samples was digested with DNase I (Promega, America). Reverse transcription of total RNA was performed as 25°C 5 min, 42°C 60 min, 70°C15 min and 4°C conservation using Reverse Transcriptase (Promega, America).

Amplification and primer sequence: The following primers were used for detecting Tcf21 and b-actin expression:

- Tcf21 forward, 5'-CCACCTCAAACCCAACAC-3'
- Tcf21 reverse, 5'-GTTCCCAGACTCGCACCT-3'
- b-actin forward, 5'-AGGTCATCACTATTGGCAAC-3'
- b-actin reverse, 5'-ACTCATCGTACTCCTGCTTG-3'

The PCR Amplification was performed as 94°C 5 min and 30 cycles for 94°C 30 sec, 58°C 30 sec, 72°C 40 sec, 72°C 7 min, 4°C conservation.

RESULTS AND DISCUSSION

Evaluating 3T3-L1 differentiation with oil and red O staining: *In vitro* differentiation of 3T3-L1 cells include following stages: growth arrest (day 0), cells undergoing limited clonal expansion (days 1-2) and terminal differentiation (days 3-10). Adipocytes are evaluated using oil and red O staining and the degree of staining was proportional to the extent of cell differentiation. Adipocytes could be stained with oil and red O, because triglyceride accumulates in cytoplasm during 8-10 day. In the present study, the cytoplasm of 3T3-L1 adipocytes filled with triglyceride and nucleolus was pushed into a corner (Fig. 1c). Under insulin, IBMX and dexamethasone inducing, most 3T3-L1 cells had accumulated plentiful triglyceride (Fig. 1a, b). It was demonstrated that the differentiation of the 3T3-L1 cells was successful. In the present research, we discovered that confluence of cells was a critical factor influencing differentiation. The higher the ratio of cell confluence was and the more 3T3-L1 cells differentiated into adipocytes.

Detecting Tcf21 expression in 3T3-L1 cells and WAT with RT-PCR: For mouse, WAT female peri-uterine fat pad and male epidydimal fat pad, which have been researched widely, are major White Adipose Tissues (WAT) of mouse. Generally, the 6-week-old mice had mature WAT. Adipose tissue, like muscle and bone, is generally regarded as a mesodermal tissue. Tcf21 is a gene of bHLH family that has essential roles in the embryonic development of mesodermal tissues, which has been reported expressed in primary white adipocytes *in vivo*. 3T3-L1 preadipocytes differentiated into mature white adipocytes during 8 days. In the present study, Tcf21 expression was found in white adipose tissues of female mice and male mice (Fig. 3), but it was not detectable during adipose differentiation of 3T3-L1 cell (Fig. 2 a-h). The results showed that Tcf21 expressed in primary white adipocytes and white adipose tissue of female and male mice, but didn't express in 3T3-L1 adipocytes.

It is a complicated transition that preadipocytes differentiate into adipocytes. In the transition some key genes positively regulating differentiation start to express sufficiently, such as CCAAT/enhancer binding protein family C/EBPα, C/EBPβ, C/EBPδ and peroxisome proliferator-activated receptor-γ (PPARγ) (Rosen and MacDougald, 2006).

Adipogenesis is a complicated process *in vivo*, while adipocyte cell lines could differentiate with several inducers *in vitro*, which facilitates the researches on it. So the model cells have been extensively used to study adipocyte differentiation for the past several decades. The mouse adipose 3T3 cells is a popular model for the study of adipocyte differentiation *in vitro* (Rosen and Spiegelman, 2006), which includes two types of cells, 3T3-L1 cell line and 3T3-F442A cell line.

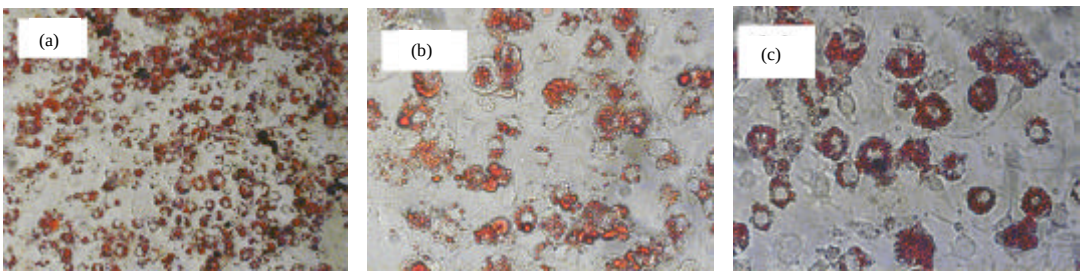

Fig. 1: 3T3-L1 cells were stained with Oil and Red O after the cells inducted for 8 days. The red color indicates the lipid droplets in adipocytes. The stained cells amplified with (a) 100, (b) 200 and (c) 400 times under microscope, respectively

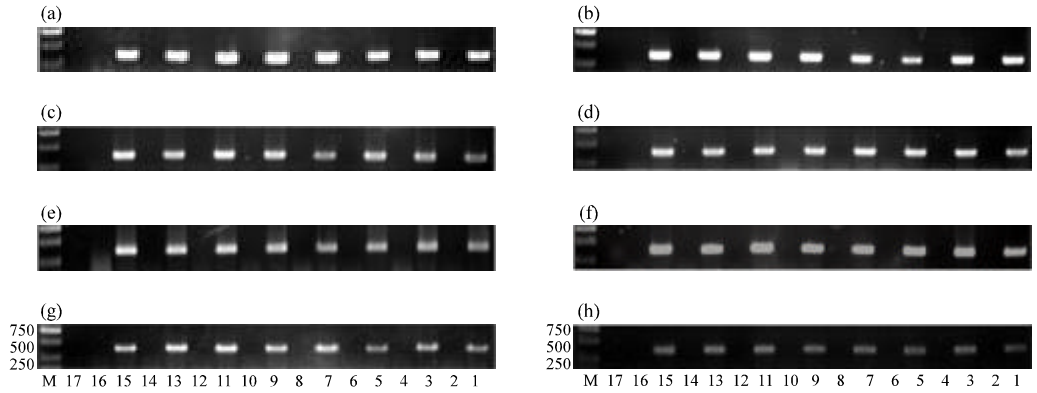

Fig. 2: Tcf21 expression in 3T3-L1 cells detected with RT-PCR. Figure a-h showed the results of detecting Tcf21 expression on day 1 to day 8 of the cell differentiation, respectively. In each Figure, Lane 2, 4, 6, 8, 10, 12, 14 and 16 demonstrated detection of Tcf21 expression (667 bp) at 3 h interval in the same day while Lane1, 3, 5, 7, 9, 11, 13 and 15 showed positive control (b-actin 370 bp) and Lane17 was negative control

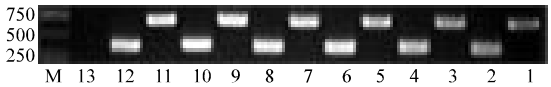

Fig. 3: Tcf21 expression in white adipose tissue of mice detected with RT-PCR. Lane 1, 3, 5 and Lane 7, 9, 11 showed the RT-PCR results of Tcf21 expression in peri-uterine tissues of three 6-week-old female KM mice and epidydimal white adipose tissues of three 6-week-old male KM mice, respectively. Lane 2, 4, 6, 8, 10 and 12 were positive control (b-actin 370 bp) and lane 13 was negative control

Although, 3T3 cell lines have been researched as adipose model *in vitro*, their differentiation has some discrepancy with adipogensis *in vivo*. These discrepancies mainly display in secreting adipokines and gene express profiles. Adipose tissue not only stores excess energy derived from food intake, but also secretes a large number of peptide hormones and cytokines, which affect energy metabolism in other tissue such as the liver and muscle (Guilherme *et al*., 2008). Leptin is an adipocyte-derived hormone that effects a number of cell types and modulates nutritional state (Soukas *et al*., 2000). *In vitro* adipocytes express leptin level unexpectedly lower than *in vivo* implanted adipocytes (Mandrup *et al*., 1997). There are some genes which are highly expressed *in vivo*, but the genes are absent in differentiated 3T3-L1 adipocytes revealed with oligonucleotide microarrays (Soukas *et al*., 2001). Comparing with oligonucleotide microarrays experiment, we selected densely differentiation time points in order to detect Tcf21 gene expression to avoid any missing.

Tcf21 is a number of the bHLH transcription factor family that is involved in various cell differentiation processes (Funato *et al*., 2003). Tcf21 positively regulates BMP4 expression, while BMP4 can promote pluripotent mesenchymal cells to form white adipocytes (Quaggin *et al*., 1999; Tseng *et al*., 2008). The db/db mouse is a genetic model of type 2 diabetes with obesity and insulin resistance (Makino *et al*., 2006). Tcf21 mRNA levels of diabetic db/db mice significantly upregulated at 5 weeks and downregulated at 7 weeks of age than nondiabetic db/m mice (Makino *et al*., 2006) which showed that Tcf21 participates in adipose differentiation *in vivo*. In the present study, the

results showed that Tcf21 expressed in primary white adipocytes and white tissues of mice, but its expression was absent in 3T3-L1 cells during differentiation (from 3 to 192 h).

There are two possible reasons explaining the discrepancy. Firstly, conditions to maintain cell lines are not comparable to the environment *in vivo*. Secondly, 3T3-L1 is an eternal cell line with aneuploid and unstable kartotypes, which is different from cells *in vivo*. Because of immortal state and unstable kartotypes, gene expression profile of the cells changes. Primary cells are diploid and may therefore reflect the context *in vivo*, which is better than aneuploid cell lines (Gregoire *et al*., 1998). Therefore, it is easy to understand that there are some differences of gene expression between adipocytes of white adipose tissues *in vivo* and 3T3-L1 cells.

In conclusion, Tcf21 does not express in 3T3-L1 adipocytes and participate in regulating 3T3-L1 cell differentiation. As an adipogenesis model *in vitro*, 3T3-L1 cell line can not reveals completely adipogenesis mechanism *in vivo*.

ACKNOWLEDGMENT

The research was supported by the National Basic Research Program of China (Code: 2006CB102100). We are grateful to Prof. Wu for his great support to the present study.

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