Specific Labeling of Mouse 3T3-L1 Preadipocyte Cell Line with Green Fluorescent Protein

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Abstract   A vector of p *aP2-promoter- EGFP* was constructed and introduced into mouse 3T3-L1 preadipocyte cells, a cell line derived from mouse Swiss3T3 cells that were isolated from mouse embryo, to make the cells labelled with enhanced green fluorescent protein (EGFP) whose expression was controlled by the promoter of adipose-specific gene *aP*2. The cells were then induced to differentiate and the expression of *aP*2 was detected by EGFP-microscopy and RT-PCR assays. The EGFP gene was transferred into the mouse 3T3-L1 preadipocyte cells, and *EGFP* expression and lipid accumulation were observed during differentiation. The expression of *aP*2 was stable and similar to the expression of *EGFP*. A preadipocyte cell line expressing EGFP was obtained under the control of the promoter of adipocyte-specific expression gene *aP*2, and the preadipocyte cell line was specifically labelled. The cell line provides a powerful approach for the research of adipocyte differentiation and for the screening of anti-obesity and anti-diabetes drugs.

Key words   EGFP, adipocyte fatty acid binding protein (aP2), labeling, cell line

3T3-L1

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3T3-L1   .   p *aP2-promoter- EGFP*

,   3T3-L1   ,   RT-PCR   *aP*2   . *EGFP*

3T3-L1   ,   *EGFP*   . RT-PCR   ,

EGFP   *aP*2   .   *aP*2

EGFP   3T3-L1   ,

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Adipose tissue is now regarded as not only an energy storage organ but also an important endocrine one because it secrets many hormones and hormone-like peptides that play important roles in adipocyte differentiation, obesity formation and type 2 diabetes development[1 3].

Adipocyte fatty-acid-binding protein (aP2) is a member of the intracellular fatty-acid-binding protein (FABP) family[4], and also an important protein in regulating lipid metabolism and systemic insulin resistance[5,6]. It was

well established that the expression of *aP2* gene in 3T3-L1 cells was completely dependent on the cell's differentiation, that is, the gene is not expressed at all in undifferentiated preadipocytes, and begins to be expressed when the cells are induced to differentiate into adipocytes[7,8], implicating that the gene *aP2* is a marker indicating the differentiation status of preadipocytes into adipocytes.

Mouse 3T3-L1 preadipocyte is the initially established cell line[9] that has been widely used in the researches of lipid metabolism, adipocyte differentiation, and the researches for specific genes from adipose tissue and their functions. It would be more convenient for the above mentioned researches if the cells were labelled with a reporter that could indicate the differentiation process. EGFP is a mutant of GFP that absorbs blue light and emits green fluorescence so that it provides an efficient way to identify the labelled cells and suit to be used as such a reporter[10].

This research was carried out to label the mouse 3T3-L1 preadipocyte cell line with *EGFP* that was expressed under control of the promoter of *aP2*, a gene specifically expressed in adipose tissue, in order to provide a useful tool for the researches of adipocyte differentiation and for the screening of anti-obesity and anti-diabetes drugs.

1　Materials and Methods

1.1　Cell culture

3T3-L1 preadipocytes were cultured and induced to differentiate as we reported previously[11].

1.2　Construction of p aP2-promoter- EGFP plasmid

The *aP2* promoter was cloned by PCR from mouse genome using oligonucleotide primers (5′-TTTATTAATTCCCATTCGTAAATAGCCATG-3′) and (5′-TTTGTACCAAGACACAGCTCCTCCTCGA-3′) including *Ase* Ⅰ and *Kpn* Ⅰ linkers for the two ends. PCR products were digested with *Ase* Ⅰ and *Kpn* Ⅰ and cloned into the *Ase* Ⅰ/*Kpn* Ⅰ site of p *EGFP-N₃*. The resultant construct p *aP2- EGFP* was linearized with *Apa*L Ⅰ before transfection.

1.3　Gene transfer

The linearized p *aP2*-promoter- *EGFP* plasmid (20 μg) was introduced into 3T3-L1 preadipocytes by electroporation in 0.8 ml PBS at 300 V, 960 μF. G418 (400 mg/L) was added into the medium and the stable transfected cells were selected after 7～10 days.

1.4　RNA isolation and RT-PCR assays

Total RNA was isolated from cells every day using Cartrimox (TaKaRa INC) as described by the supplier. First-strand cDNA was synthesized with random primers by RT-PCR using total RNA as template. PCR was performed for each pair of specific primers of 5′-TTGTGAAGTGCTCATAGGCAGTG-3′ and 5′-TGGCTCATGCCCTTTCATAAAC-3′ for mouse *aP2* mRNA, and

5′-TTCCTTCTTGGGTATGGAAT-3′ and 5′-GAGCAATGATCTTGATCTTC-3′ for the house-keeping gene β-actin mRNA. The PCR products were quantitatively analyzed by agarose gel electrophoresis in the presence of EtBr.

2　Results

2.1　Vector construction and gene introduction

The insertion of *aP2* promoter into *Ase* Ⅰ/*Kpn* Ⅰ sites of p *EGFP-N3* forms the p *aP2*-promoter- *EGFP* vector as shown in Fig. 1. The vector p *aP2*-promoter- *EGFP* was introduced into 3T3-L1 preadipocytes by electroporation followed by G418 resistance selection.
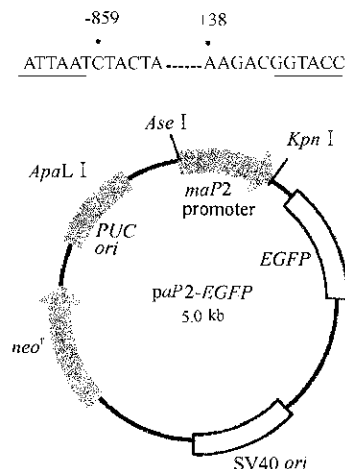

Fig. 1　Map of plasmid p *aP2*-promoter- *EGFP*

2.2　Differentiation induction

Before differentiation, the labelled 3T3-L1 cells displayed the mophology of fibroblast and showed no *EGFP* expression as shown in Fig. 2A and Fig. 2B. The cells began to become round and showed high *EGFP* expression when being induced to differentiate as shown in Fig. 2C and Fig. 2D. These results suggested that the EGFP gene and the promoter of *aP2* might be integrated into the cell's genome and that the gene *aP2* began to express when the preadipocytes were induced to differentiate. The mature adipocytes after induction for 6 days became morphologically round and accumulated large amount of lipid when stained with Oil Red O as shown in Fig. 3. The above results implied that the preadipocyte cells labelled with the marker of *aP2* gene expression was well established.

2.3　Confirmation of endogenous aP2 expression

RT-PCR assays were performed to confirm the endogenous *aP2* expression in these cells. The assays showed that the *aP2* mRNA could not be detected in the cells before differentiation induction for 1 day, and is detected in the cells of more than 1 day after differentiation induction (Fig. 4). These results were similar to that of *EGFP* expressions in these two types of
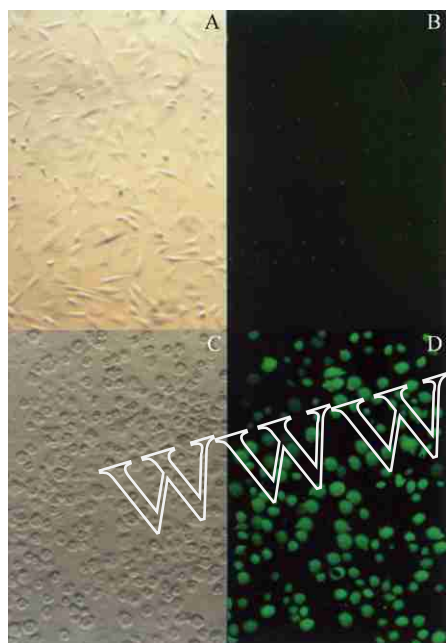
Fig. 2   *EGFP* expressions in the cells before and after differentiation induction
A：Cells before differentiation induction under visible light
B：Cells before differentiation induction under fluorescence
C：Cells after 6 days of differentiation induction under visible light
D：Cells after 6 days of differentiation induction under fluorescence
（Original magnification 400 ×）

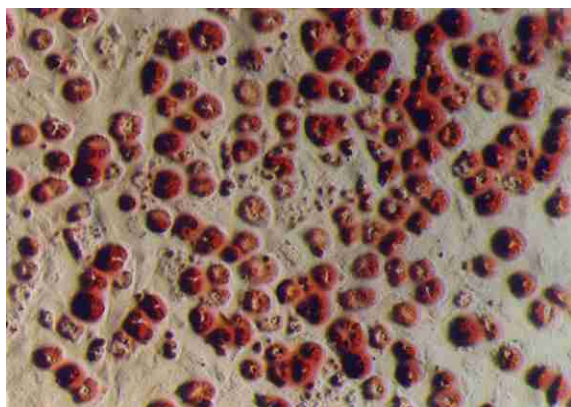

Fig. 3   Cells after 6 days of differentiation induction stained with Oil Red O
（Original magnification 400 ×）

cells as shown in Fig. 2 , indicating that the expression of *EGFP* authentically represented the expression of endogenous *aP2* gene and that the preadipocyte cell line labelled with *EGFP* worked well.

3   Discussion

Obesity is a serious problem in industrialized countries and contributing to several diseases including type 2 diabetes , hypertension and atherosclerosis[12] , and results from an excessive accumulation of white adipose tissue composed of preadipocytes and adipocytes , which play a central role in energy storage[12] . Recent studies showed that fat tissue secrets many hormones and peptides

Fig. 4   RT-PCR assays showing the expressions of *aP2* and -actin during the differentiation of 3T3-L1 cells
Total RNAs were extracted from the 3T3-L1 cells of differentiation induction for day 0（lane 1）, day 1（lane 2）, day 2（lane 3）, day 3 （lane 4）, day 4（lane 5）, day 5（lane 6）and day 6（lane 7） respectively. The mRNAs were used as templates for amplifications by RT-PCR using primers specifically designed for *aP2* and -actin. Lane 8 was DNA marker DL2000

that play crucial roles in maintaining energy balance in vertebrates and in the development of obesity and type 2 diabetes[1 3] . As the investigations into preadipocytes and adipocytes are more and more of importance , an *in vitro* system suitable for studying adipogenesis and differentiation process is desirable.

The mouse 3T3-L1 preadipocyte cell line has been the most studied cell line for these purposes because it can be differentiated into adipocytes by hormonal treatment. Nevertheless , the preadipocyte cell lines established to date are lack of a specific marker that can indicate the cell differentiation status.

The adipocyte fatty-acid-binding protein , aP2 , plays an important role in regulating systemic insulin resistance , lipid metabolism , and obesity formation[5 ,6] . The promoter of *aP2* contains two adipose-specific elements （FSE1 and FSB2）[7 ,8] , so the expression of *aP2* gene can be used as the marker indicating the cell differentiation status.

In this research , we established an *EGFP*-labeled mouse 3T3-L1 preadipocyte cell line in which the expression of *EGFP* was under the control of *aP2* promoter. The expression patterns of *EGFP* and RT-PCR assays demonstrated that the expression of *EGFP* authentically represented the endogenous expression of *aP2*. The establishment of this specifically labelled cell line provides a powerful tool for investigating adipocyte differentiation , understanding the aetiology of lipid metabolic disorders , and screening anti-obesity and anti-diabetes drugs.

Further studies are being focused on the screening of small molecules with the cell model we established in this research from Chinese herbs , a vast resource of chemicals with anti-obesity and anti-diabetes activities. The advantage of EGFP as a reporter is a great convenience to observe its expression with naked eyes so that one can

easily follow the whole differentiation process. Nevertheless , a reporter , whose expression intensity can be concisely quantitatively determined , is strongly recommended , because a cell model with such a reporter can be used in high-throughput screening for active molecules. Given this , we are now establishing the cell line with luciferase as a reporter in order to quantitatively determine *aP*2 expression during the cell differentiation.

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