The Labeling of 3T3-L1 Preadipocyte Cells with Enhanced Green Fluorescent Protein

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Abstract A cell model is desired for adipocyte differentiation investigation and for high-throughput screening of anti-obesity and anti-diabetes molecules from chemical resources due to the world wide epidemic of obesity and diabetes. In order to establish such a cell model, a plasmid of pPPARγ2-promoter-EGFP was constructed by inserting a 660bp sequence of mouse PPARγ2 promoter into the *Ase* Ⅰ and *Kpn* Ⅰ sites of pEGFP-N_3 and transferred into 3T3-L1 preadipocyte cells. The cells were induced to differentiate and the expression of PPARγ2 was detected by the microscopic observation of EGFP and by RT-PCR assays. The results showed that the EGFP gene expression patterns were similar to that of pPPARγ2 's, which indicated that the EGFP gene was transferred into the mouse 3T3-L1 preadipocyte cells, and its expression was under the control of pPPARγ2 promoter. RT-PCR assays showed that the EGFP expression authentically represented the stable expression of PPARγ2. In conclusion, a preadipocyte cell line expressing EGFP under the control of the promoter of adipocyte-specific expression gene PPARγ2 was generated. The cell line provides a powerful approach for the research of adipocyte differentiation and for the high-throughput screening of anti-obesity and anti-diabetes chemicals.

Key words EGFP, PPARγ2, labeling, cell line

Fat tissue is now regarded as not only an energy storage organ but 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[1,2]. Fat tissue is composed of preadipocytes and adipocytes, and it is crucial to make known the differentiation process from pre-adipocytes into adipocytes to fully understand the endocrine diseases such as obesity and type 2 diabetes[3].

Mouse 3T3-L1 preadipocyte is the most early established cell line[4] that has been widely used in the researches of lipid metabolism, adipocyte differentiation, adipose tissue specifically expressed gene 's discovery and their function 's investigation. It would be more convenient for the above researches if the cells be labelled with a reporter that can 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 is suitable to be used as such a reporter[5].

Peroxisome proliferator activated receptor γ2 (PPARγ2) is a ligand-activated transcription factor that belongs to the nuclear hormone receptor family and its expression represents the differentiation patterns of preadipocytes into adipocytes because it specifically expresses in differentiating adipocytes[6,7]. This research was carried out to label the mouse 3T3-L1 preadipocyte cell line with EGFP, of which the expression is controlled by the promoter of PPARγ2, 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[8].

1.2 Construction of pPPARγ2-EGFP vector

For constructing the plasmid of pPPARγ2-promoter-EGFP, the PPARγ2 promoter was cloned by PCR from mouse genome using oligonucleotides primers (5′-TTTATTAATGAATTTGGATAGCAGTAACATTTTGGACC-3′) and (3′-GTGGTCACACTTAATGTCGTTTAGAGACCATGTTT-5′) including *Ase* Ⅰ and *Kpn* Ⅰ linkers at the two ends. PCR product was digested with *Ase* Ⅰ and *Kpn* Ⅰ and cloned into the *Ase* Ⅰ/*Kpn* Ⅰ site of pEGFP-N_3. The constructed plasmid was named pPPARγ2-promoter-EGFP. The plasmid was linearized with *Apa*LⅠ before transfection.

1.3 Gene transfer and positive clones ' selection

3T3-L1 preadipocyte cells were electroporated with 20μg of linearized pPPARγ2-promoter-EGFP plasmid DNA in 0.8mL PBS at 300V, 960μF. G418 was added into the medium and the stable transfected cells were selected after 7 ~ 10 days.

The anti-G418 cells were diluted and seeded in a 96-well plate to make every well have 5 ~ 10 cells. Each clone obtained as such was divided into two parts, and one was frozen for reservation and the other was induced for differentiation. EGFP expressions were microscopically observed. The percentage of green cells in each clone was determined by cell counting.

1.4 RNA isolation and RT-PCR assays

Total RNA was isolated from cells using Cartrimox (TaKaRa INC) according to the manufacturer 's guidance and used for analysis of PPARγ2 mRNA and EGFP mRNA by RT-PCR. First-strand cDNA was synthesized from total RNA by using random primers. PCR was performed for each set of specific primers of 5′-TTGTGAAGTGCTCATAGGCAGTG-3′ and 5′-TGGCTCATGCCCTTTCATAAAC-3′ for mouse PPARγ2 mRNA, 5′-GCTGCCCGTGCCCTG-3′ and 5′-AGTTCACCTTGATGCCGTTC-3′ for EGFP mRNA, and 5′-TTCCTTCTT-

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GGGTATGGAAT-3′ and 5′-GAGCAATGATCTTGATCTTC-3′ for the house keeping gene β-actin mRNA, an internal control. The PCR products were separated by electrophoresis on a 1.2% agarose gel with ethidium bromide staining.

2 Results

2.1 Plasmid construction, gene transfer and positive clones' selection

The insertion of PPARγ2 promoter into *Ase* Ⅰ/*Kpn* Ⅰ site of pEGFP-N3 forms the pPPARγ2-promoter-EGFP plasmid as shown in Fig. 1. The plasmid pPPARγ2-promoter-EGFP was transferred into 3T3-L1 preadipocytes by electroporation and selected by adding G418. The selected anti-G418 cells were diluted into the wells of a 96-well plate, and 55 clones obtained as such had green cells of different percentages from about 10% to about 80%. A clone with 80% green cells was inducted for differentiation as shown in Fig. 2. The photomicrographs of Fig. 2A and Fig. 2A′ represented the cells before differentiation induction. Fig. 2B and Fig. 2B′ represented the cells after 3 days differentiation induction. Fig. 2C and Fig. 2C′ represented the cells after 5 days differentiation induction. These results indicated that most (about 80%) preadipocytes differentiated into adipocytes by differentiation induction.

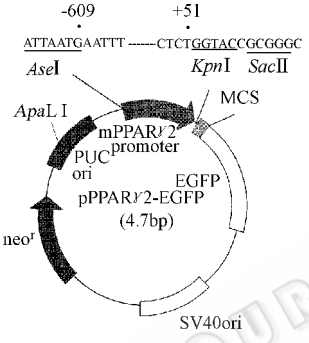

Fig. 1 The map of plasmid pPPARγ2-promoter-EGFP
The plasmid was constructed by inserting a 660bp sequence of mouse PPARγ2 promoter into the *Ase* Ⅰ and *Kpn* Ⅰ sites of pEGFP-N_3

2.2 The expressions of EGFP in the cells before and after induction for differentiation

When induced to differentiate, the transfected cells began to express EGFP, while the cells before differentiation induction did not express any EGFP as shown in Fig. 2. Fig. 2A showed no EGFP expression before differentiation induction, Fig. 2B showed the expression of EGFP in cells of 3 days after differentiation induction, and Fig. 2C showed the expression of EGFP in cells of 5 days after differentiation induction. Fig. 2A′, Fig. 2B′ and Fig. 2C′ represented the same background as Fig. 2A, Fig. 2B and Fig. 2C under visible light respectively. These results implied that the reporter gene was successfully transferred into this preadipocyte cell line, and the reporter's expression could be used as a marker that indicates PPARγ2's expression.

2.3 Confirmation of endogenous PPARγ2 expression

RT-PCR assays were performed to confirm the endogenous PPARγ2 expression in these cells. As shown in Fig. 3, the assays showed that, in the cells before differentiation (lane 4) and in differentiation (lane 3 and lane 2), the endogenous PPARγ2 gene appeared no expression and highly expression respectively. In accordance with PPARγ2 expressions, EGFP mRNA exhibited the same expression patterns. These results were homogenous to the expressions of

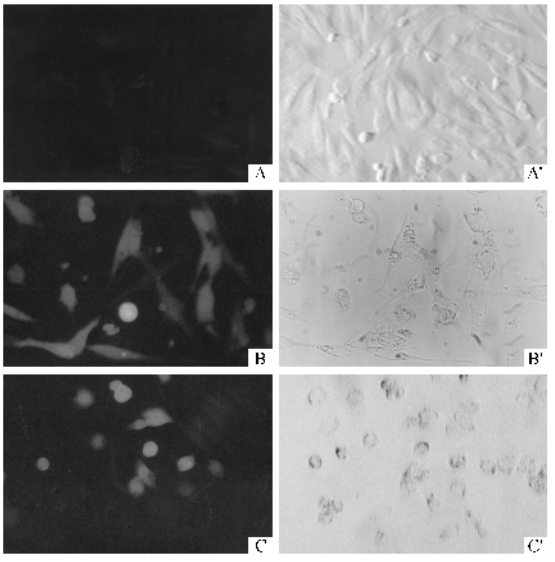

Fig. 2 The expressions of EGFP in the cells before and after induction for differentiation (Original magnification ×400)
A: Before differentiation induction;
A′: the same background as A under visible light.
B: 3 days after differentiation induction; B′: the same background as B under visible light.
C: 5 days after differentiation induction; C′: the same background as C under visible light. Original magnification ×400

EGFP as a reporter protein in these two types of cells as shown in Fig. 2. The above results indicated that the expressions of EGFP authentically represented the endogenous PPARγ2 expressions and implied that the preadipocyte cell line labelled with the marker of PPARγ2 gene expression was well established.

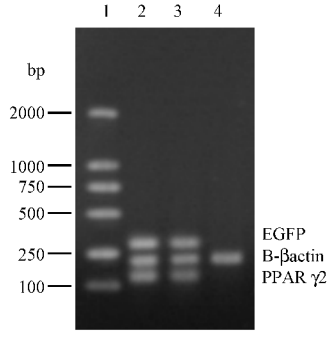

Fig. 3 RT-PCR assays showing the mRNA levels of PPARγ2, EGFP and β-actin during the differentiation of 3T3-L1 cells
Total RNA were extracted from the 3T3-L1 cells of day 0 (lane 4), day 3 (lane 3), and day 5 (lane 2) after differentiation induction. The mRNA were amplified by RT-PCR using primers specifically designed for PPARγ2, EGFP and β-actin. Lane 1 was DNA marker DL2000

3 Discussion

Obesity is a serious problem in industrialized countries and contributing for several diseases including type 2 diabetes, hypertension and atherosclerosis[9], and it results from an excessive accumulation of white adipose tissue, composed of preadipocytes and adipocytes, which play a central role in energy storage. Because the investigations into preadipocytes and adipocytes and the screening of anti-obesity and anti-diabetes substances are emerging more and more importance, an *in vitro* system suitable for studying adipogenesis and differentiation

process is desirable. For this purpose, we selected PPARγ2, the fat tissue-specific expressed gene, as a marker indicating the differentiation status to establish such a cell model. In order to make the marker PPARγ2 easier to be observed and detected, a reporter whose expression is under the control of the marker should be linked to it. The reporters mostly used for this purpose are EGFP, luciferase, and so on. In one of our previous studies a stem cell line was labelled with EGFP as a reporter[10].

In this research we established an EGFP-labeled mouse 3T3-L1 preadipocyte cell line in which the expression of EGFP is under the control of PPARγ2 promoter. The expression patterns of EGFP and RT-PCR assays demonstrated that the expression of EGFP authentically represents the endogenous expression of PPARγ2. The establishment of this specifically-labeled cell line provides a powerful tool for investigating adipocyte differentiation, understanding the aetiology of lipid metabolic disorders, and for screening anti-obesity and anti-diabetes drugs.

The advantage of EGFP as a reporter is that it is very convenient to observe its expression with human naked eyes so that one can follow the whole differentiation process easily. Nevertheless, a reporter, of which the expression intensity can be concisely quantitatively determined is strongly recommended, for a cell model with such a reporter can be used in high-throughput screening for active small molecules. Given this, we are now establishing the cell line with luciferase as a reporter in order to quantitatively determine PPARγ2's expressions during the cells' differentiation.

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小鼠 3T3-L1 前脂肪细胞系的增强绿色荧光蛋白标记

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摘　要　细胞模型是研究细胞分化原理以及进行高通量筛选的有效工具。为了建立特异性标记的脂肪细胞分化模型，构建了包括脂肪细胞分化特异性表达基因 PPARγ2 的启动子在内的载体（pPPARγ2-promoter-EGFP），用电穿孔方法转染小鼠 3T3-L1 前脂肪细胞，用显微荧光观察和 RT-PCR 确认 PPARγ2 基因的内源表达。结果显示，EGFP 基因成功转入 3T3-L1 前脂肪细胞，观察到细胞分化过程中 EGFP 表达和脂肪积累，RT-PCR 分析表明 EGFP 代表了稳定而真实的 PPARγ2 基因的内源性表达。建立了由脂肪组织特异表达基因 PPARγ2 的表达控制的 EGFP 标记的小鼠 3T3-L1 前脂肪细胞系，目前国内外尚未见用同样方法对前脂肪细胞进行特异性标记。该细胞系将为脂肪细胞分化机理研究以及为抗肥胖症和抗糖尿病药物筛选提供有力工具。

关键词　增强绿色荧光蛋白，过氧化物酶体增殖物激活受体 γ2，标记，细胞系

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