

Expression Patterns of *PPAR γ 2* in Differentiation of Mouse Embryonic Stem (ES) Cells into Adipocytes

小鼠胚胎干细胞向脂肪细胞分化过程中 *PPAR γ 2* 基因的表达模式

Li Cheng-Jian, ZHANG Xiao-Lan, YANG Yang and ZHANG Chong-Ben*

李成健, 张晓兰, 杨 扬, 张崇本*

北京大学生命科学学院, 北京 100871

College of Life Sciences, Peking University, Beijing 100871, China

摘 要 将 *PPAR γ 2* 基因启动子和报告基因荧光素酶相连接克隆于特定载体构建成表达质粒, 电穿孔转染小鼠 ES 细胞, 筛选阳性克隆。诱导 ES 细胞向脂肪细胞分化, 通过定量检测荧光素酶活性跟踪 *PPAR γ 2* 基因的表达情况, 以此研究脂肪细胞分化过程中该基因的表达模式。结果显示 *PPAR γ 2* 基因在未分化的 ES 细胞和 EB 形成的前两天中不表达, 从 EB 形成的第 3 天开始表达, 直至脂肪细胞分化完成。该基因在已完成分化的脂肪细胞中的表达远强于在分化中的前脂肪细胞中的表达。首次报道了从小鼠 ES 细胞到脂肪细胞分化过程中 *PPAR γ 2* 基因的表达模式, 支持了 *PPAR γ 2* 基因为脂肪组织特异性表达基因的已有报道, 并为脂肪细胞分化机理研究提供了线索。

关键词 胚胎干细胞, 分化, *PPAR γ 2* 基因, 表达模式

中图分类号 Q786 文献标识码 A 文章编号 1000-3061(2005)02-0187-05

Abstract To investigate the expression patterns of peroxisome proliferator activated receptor2 (*PPAR γ 2*) gene in the differentiation of mouse embryonic stem (ES) cells into adipocytes, mouse ES cells were transfected with the vector of p*PPAR γ 2*-promoter-luciferase, and *PPAR γ 2* expressions were analyzed by detecting luciferase activities and by detecting the protein expressions using western blotting. The results showed that the gene *PPAR γ 2* did not express in undifferentiated mouse ES cells and in embryoid bodies (EBs) within the first 2 days of differentiation induction after EB formation, and began to express from the third day of differentiation induction after EB formation to the finish of the differentiation. The gene's expression in differentiated adipocytes was much stronger than that in differentiating preadipocytes. In Conclusion our results reported for the first time the five-step expression patterns of the gene *PPAR γ 2* during the whole differentiation procedures from mouse ES cells into adipocytes via preadipocytes, and supported the previous studies that *PPAR γ 2* is a fat-specific gene that expresses only in developed and developing adipose tissues.

Key words embryonic stem (ES) cell, differentiation, peroxisome proliferator activated receptor2 (*PPAR γ 2*), expression patterns

Received: October 18, 2004; Accepted: December 28, 2004.

The project was supported by the grant of the National High Technology Research and Development Program of China (863 Program) (No. 2003AA2Z3432).

* Corresponding author. Tel: 86-10-62751858; E-mail: zhangcb@pku.edu.cn

国家 863 高技术研究发展计划资助(No.2003AA2Z3432)。

Mouse embryonic stem cells are pluripotent cells derived from the inner cell mass of 3.5 days blastocysts^[1,2], and they retain the abilities to differentiate into all types of cells in an embryonic and adult mouse *in vivo*, and can be induced to differentiate into many cell types *in vitro*. The differentiation and regulation of ES cells into different cell types are providing important approaches for identification of the roles of genes expressed during the adipose development and for the potential stem cell therapy.

Mammalian fat tissue is now regarded as not only an energy storage organ but an important endocrine one because it secretes many hormones and hormone-like peptides that play important roles in adipocyte differentiation, obesity formation and type 2 diabetes development^[3-5]. It was proved that the treatment of mesodermal stem cells with 5'-azacytidine led to the activation of regulatory genes that commit the cells to adipocytes^[6], and that the treatment of mouse ES cells with retinoic acid induced the differentiation of the cells into adipocytes^[7]. These results suggested that ES cells are the origins of differentiated adipocytes, and so that the investigations for the differentiation of ES cells into adipocytes and the regulatory genes expression patterns in the differentiation are becoming more and more importance for fully understanding the adipogenesis and adipose development in mammals.

Peroxisome proliferator activated receptor $\gamma 2$ (*PPAR* $\gamma 2$) is a ligand-activated transcription factor that belongs to the nuclear hormone receptor family^[8,9]. It has been demonstrated that the *PPAR* $\gamma 2$ gene plays a crucial role in mammalian triacylglycerol synthesis and fat development *in vivo*. Previous studies by us^[10] and by others^[11,12] revealed that *PPAR* $\gamma 2$ expressed in both differentiating preadipocytes and in differentiated adipocytes, and the expression in differentiated adipocytes is much stronger than that in differentiating preadipocytes. Nevertheless, the above studies were all carried out with mouse 3T3-L1 preadipocytes as a cell model, and to date there is no description on the expression patterns of *PPAR* $\gamma 2$ during differentiation of mouse ES cell into adipocytes.

This study is aimed to investigate the expression patterns of the gene *PPAR* $\gamma 2$ during mouse ES cell differentiations into adipocytes by detecting the luciferase reporter activities and by analyzing the protein expression through western blotting. These expression patterns will be helpful in understanding the differentiation mechanisms from ES cells into adipose committed cells and for elucidating *PPAR* $\gamma 2$'s functions in triacylglycerol synthesis and fat development.

1 MATERIALS AND METHODS

1.1 Maintenance and differentiation induction of ES cell line

The mouse ES cell line, MESPU 22, was established in our lab in 1999^[13]. ES cells were cultured in ES-medium [DMEM medium supplemented with 20% of fetal bovine serum (Gibco, USA), L-glutamine (final concentration of 2 mmol/L), 2-mercaptoethanol (final concentration of 0.1 mmol/L), penicillin (50 IU/mL) and streptomycin (50 IU/mL)]. ES cells were maintained on PMEF feeder layer^[14] in ES-medium supplemented with 10^6 u/L of recombinant mouse leukaemia inhibitor factor (LIF, Chemicon, USA). At the formation of embryoid bodies (EB), the ES-medium was replaced by differentiation one that is referred to as EB-medium. The EB-medium was formulated by removing PMEF feeder-layer and LIF away from the ES-medium and by adding adipogenic hormones into the medium as described by Dani C.^[7]. For the first 3 days of differentiation induction the EB-medium was also supplemented with retinoic acid (RA, Sigma, USA) of certain concentration as described^[7].

1.2 Plasmid construction, transfection and selection of positive clones

For constructing p*PPAR* $\gamma 2$ -promoter-luciferase, the *PPAR* $\gamma 2$ promoter was cloned by PCR from mouse genome using oligonucleotides primers (5'-TTTATTAATGAATTTGGATAGCAGTAACATTTTGGACC-3') and (5'-TTTGCTACCAGAGATTTGCTGTAATTCACACTGGTG-3') including the *Ase* I and *Kpn* I linkers at the two ends. The PCR products were digested with *Ase* I and *Kpn* I and cloned into the *Ase* I / *Kpn* I sites of promoterless luciferase-reporter vector pGL3 Basic (Promega). The constructed vector p*PPAR* $\gamma 2$ -promoter-luciferase and the empty vector pGL3Basic were linearized with *Apa* I before transfection. The mouse ES cells (MESPU 22) were electroporated with 20 μ g of linearized p*PPAR* $\gamma 2$ -promoter-luciferase vector DNA and 20 μ g of linearized pGL3Basic vector DNA in 0.8 mL PBS at 300V, 960 μ F. G418 was added into the medium and positive clones were selected after 7 days.

1.3 Analysis of *PPAR* $\gamma 2$ promoter activities

PPAR $\gamma 2$ promoter activities were tested by measuring the luciferase activities. Luciferase activity was determined using the luciferase reporter assay system according to the manufacturer's instructions (Promega) in a 9507 Luminometer (Berthold). The relative promoter activity is expressed as the ratio of luciferase activities of p*PPAR* $\gamma 2$ -promoter-luciferase vector and the luciferase activities of the co-transfected pRL-CMV vector. Triple independent experiments were made and analyzed with ONE-WAY ANOVA and the results were expressed as means \pm standard deviation.

1.4 Western blotting

Cell lysate was prepared by adding 1.0 mL of boiling lysis buffer (1% SDS, 1.0 mmol/L sodium ortho-vanadate, 10 mmol/L Tris, pH 7.4) to the wells of the plates. Protein

concentrations were determined using BCA protein assay (Pierce Chemical Co.). Total cell lysate was electrophoresed on 8% SDS polyacrylamide gels. The proteins were transferred to polyvinylidene difluoride membrane, blocked for 60 min in 5% nonfat dry milk, and then incubated for 60 min with polyclonal antibodies against PPAR (SANTA CRUZ, Co.). The membrane was then washed and developed using super signal chemiluminescence reagent (Pierce Chemical Co.). Densitometry was performed using Bio-Rad Molecular Analyst Software (Hercules, CA). Triple independent experiments were made for the blots and the results were analyzed with ONY-WAY ANOVA and expressed as means \pm standard deviation.

2 RESULTS

2.1 Differentiation induction of mouse ES cells into adipocytes

Before differentiation induction, the ES cells were phe-

notypically elliptical (Fig.1A). ES cells aggregated to form embryoid bodies (EB) at about the sixth day of suspension culture. At EB formation, the ES-medium was replaced by EB-medium, and the cells were induced to differentiate. Within the first 2 days of differentiation induction, the EBs appeared nearly round (Fig.1B). Many cells outgrown from the EBs were observed at the third day of differentiation induction, and they took the shape of fibroblasts (Fig.1C). The cells outgrown from the EBs fulfilled differentiation process from fibroblasts into adipocytes in the next 11 days, and the cell shapes changed from spindle (Fig.1C, Fig.1D) to round (Fig.1E) gradually during the process. At the finish of the differentiation, large amount of lipid was observed in the cells (Fig.1F). These shape changes and lipid accumulation resembled the typical patterns in cell phenotypes from ES cells into adipocytes⁶, indicating that the mouse ES cells were successfully induced to differentiate into adipocytes.

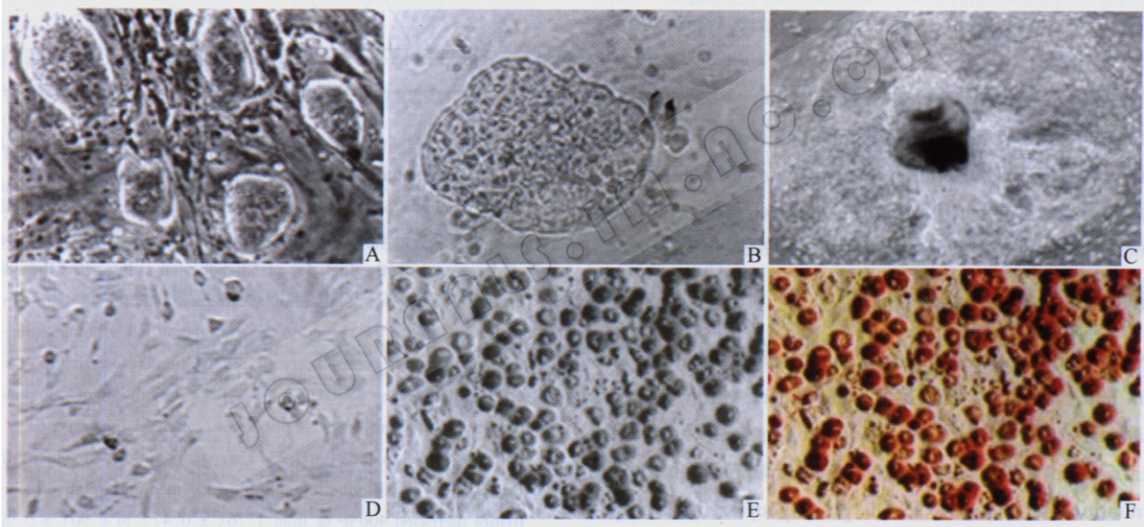


Fig.1 The phenotypic changes and lipid accumulation of the cells during differentiation induction

A: undifferentiated ES cells ($\times 400$); B: an EB ($\times 400$), the first day after EB formation; C: an EB and the outgrown cells ($\times 200$), the 5th day after EB formation; D: preadipocytes in differentiation ($\times 200$), the 8th day after EB formation; E: differentiated adipocytes ($\times 200$), 14th day after EB formation; F: differentiated adipocytes ($\times 200$), stained with oil red O, 14th day after EB formation.

2.2 The *PPAR* γ 2 gene expression patterns indicated by luciferase activities

In the first 3 days of differentiation induction after EB formation, the relative luciferase activities in EB and EB-derived cells remained at the basal level. From the 4th to the 6th day of differentiation induction after EB formation, relative luciferase activities increased 51% from 1.05 (± 0.19) to 1.59 (± 0.28); from the 7th to the 12th day of differentiation induction after EB formation, relative luciferase activities increased 406% from 1.76 (± 0.47) to 8.90 (± 0.92). From the 13th day to the finish of the differentiation, relative luciferase activities increased 2.8% from 8.90 (± 0.92) to 9.15 (± 1.12). These results were indicated by Fig.2.

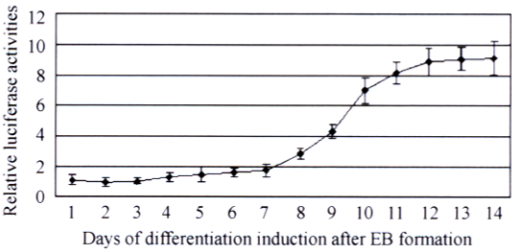


Fig.2 *PPAR* γ 2 expression patterns indicated by luciferase activities

The relative promoter activity is expressed as the ratio of luciferase activities of *pPPAR* γ 2-promoter-luciferase vector and the luciferase activities of the co-transfected pRL-CMV vector. Triple independent experiments were performed and the results were expressed as means \pm SED.

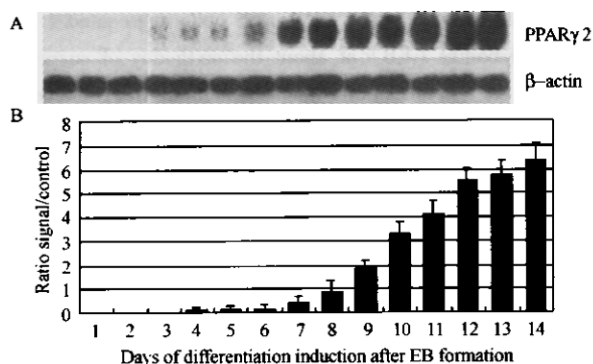


Fig.3 *PPAR*γ2 expression patterns indicated by western blotting

A: Total cell lysate was electrophoresed on 8% SDS polyacrylamide gels. The proteins were transferred to polyvinylidene difluoride membrane, blocked for 60 min in 5% nonfat dry milk, and then incubated for 60 min with polyclonal antibodies against PPAR. The membrane was then washed and developed using super signal chemiluminescence reagent; B: Densitometry was performed using Bio-Rad Molecular Analyst Software. Triple independent experiments were made for the blottings and the results were expressed as means \pm standard deviation.

2.3 The *PPAR*γ2 gene expression patterns indicated by Western blotting

The *PPAR*γ2 protein expression patterns revealed by Western blotting were identical to that indicated by luciferase activities (Fig.3). That similar results were obtained by the two means suggested that the reporter expression authentically represented the gene's endogenous expressions.

3 DISCUSSIONS

No investigation was reported up to date about the expression patterns of the *PPAR*γ2 gene during differentiations of mouse ES cells into adipocytes, although some researches were carried out about the gene's expression patterns during differentiations of mouse 3T3-L1 preadipocytes into adipocytes^[10-12]. In the present study, we cloned the promoter of mouse *PPAR*γ2 gene and constructed the vector of p*PPAR*γ2-promoter-luciferase, and transfected mouse ES cells with this vector. By inducing the ES cell to differentiate into preadipocytes and adipocytes, we investigated the expression patterns of this gene by detecting luciferase activities and by detecting the protein expressions. This is the first time to report this gene's expression patterns during the whole differentiation procedures from mouse ES cells into adipocytes via preadipocytes.

The results in this study showed that the whole differentiation procedure from mouse ES cells into adipocytes comprises five steps. Step one includes the first 6 days before EB formation (period a and b, Fig.4), step two includes the first 3 days after EB formation (period c, d and e, Fig.4), step three includes the 3 days of from the 4th to the 6th day after EB formation (period f, g, and h, Fig.4), step four

includes the 6 days of from the 7th to the 12th days after EB formation (period i, j, k, l, m and n, Fig.4), and step five includes the last period of from the 13th day to the fulfillment of the differentiation (period o and p, Fig.4). *PPAR*γ2 gene expression features indicated by luciferase activities along with the five steps are that the gene does not express at all in step one and step two, begins to express and the expression increases slowly in step three, exhibits strong expressions in step four, and remains at a steady level in step five. The above features are schematically illustrated in Fig.4, and is referred to as the five-step expression patterns of the gene *PPAR*γ2 during mouse ES cells' differentiations into adipocytes. These patterns imply that, when the induction protocols are constant to that used in this study, the expression patterns could be used as a normal in function and regulation investigations for the gene *PPAR*γ2.

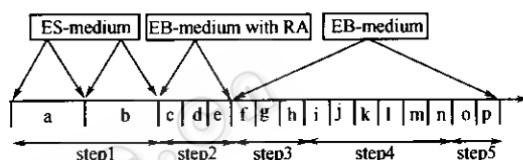


Fig.4 The five-step expression patterns of the gene *PPAR*γ2 in mouse ES cells' differentiations into adipocytes

Step1: includes period a, 3 days culture of undifferentiated ES cells in ES-medium, and period b, 3 days culture of EBs in ES-medium; step2, includes period c, the first day of EB adhesion culture in EB-medium, no outgrown cells observed, relative luciferase activities at basal level, and and periods d-e, the second to the third day of EB adhesion culture in EB-medium, outgrown cells observed, relative luciferase activities at basal level; step3, includes periods f-h, the 4th to the 6th day of EB adhesion culture in EB-medium, relative luciferase activities increased 51% of from 1.05 (\pm 0.19) to 1.59 (\pm 0.28); step4, includes periods i-n, the 7th to the 12th day of EB adhesion culture in EB-medium, relative luciferase activities increased 406% of from 1.76 (\pm 0.47) to 8.90 (\pm 0.92); and step5, includes periods o-p, the 13th day to the finish of the differentiation, relative luciferase activities increased 2.8% of from 8.90 (\pm 0.92) to 9.15 (\pm 1.12).

Previous studies described that the gene *PPAR*γ2 expressed in both mouse differentiating preadipocytes and in differentiated adipocytes, and the expressions in differentiated adipocytes were much stronger than that in differentiating preadipocytes^[10-12]. The last three steps in the five-step expression patterns shown in this study are similar to the expression features in mouse differentiating preadipocytes and in differentiated adipocytes described in the above previous work, implying that our results supported the previous studies that *PPAR*γ2 is a fat-specific gene that expresses only in developed and developing adipose tissues.

We once labeled a mouse ES cell line with reporter gene EGFP to provide a cell model for differentiation studies and for genes' functions identifications^[15]. One limitation of ES

cell lines labeled with EGFP is that the marker genes' expressions can not be detected quantitatively. Our work in this study provided an efficient ES cell model labeled with reporter gene luciferase. The cell line labeled with luciferase allows a quantitative analysis in investigating the differentiation process from ES cells into adipocytes and for identifying new genes and cytokines during the differentiation, and for screening small molecules that may be potential in regulating fat accumulation and obesity formation.

REFERENCES

- [1] Evans MJ, Kaufman MH. Establishment in culture of pluripotent cells from mouse embryos. *Nature*, 1981, **292** (5819): 154 - 156
- [2] Martin G. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Nat Acad Sci USA*, 1981, **78**:7634 - 7638
- [3] Shuldiner AR, Yang R, Gong DW. Obesity and insulin resistance--the emerging role of the adipocyte as an endocrine organ. *N Engl J Med*, 2001, **345**(18):1345 - 1346
- [4] Guerre-Millo M. Adipose tissue hormones. *J Endocrinol Invest*, 2002, **25**(10):855 - 861
- [5] Havel PJ. Update on adipocyte hormones: regulation of energy balance and carbohydrate/lipid metabolism. *Diabetes*, 2004, **53** (Suppl 1):s143 - 151
- [6] Taylor SM, Jones PA. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5'-azacytidine. *Cell*, 1979, **17**:771 - 779
- [7] Dani C, Smith AG, Dessolin S *et al.* Differentiation of embryonic stem cells into adipocytes *in vitro*. *Journal of Cell Science*, 1997, **110**:1279 - 1285
- [8] Tontonoz P, Hu E, Spiegelman BM. Regulation of adipocyte gene expression and differentiation by Peroxisome proliferator activated receptor γ PPAR γ . *Cell*, 1994, **79**:1147 - 1156
- [9] Tontonoz P, Hu E, Spiegelman BM. mPPAR γ PPAR γ 2: tissue-specific regulator of an adipocyte enhancer. *Genes Dev*, 1994, **8**:1224 - 1234
- [10] Li CJ(李成健), Cheng JY(成俊英), Zhang XL(张晓兰) *et al.* The labeling of 3T3-L1 preadipocyte cells with enhanced green fluorescent protein. *Chinese Journal of Biotechnology* (生物工程学报), 2004, **20**(5):583 - 586
- [11] Guo X, Liao K. Analysis of gene expression profile during 3T3-L1 preadipocyte differentiation. *Gene*, 2000, **251**(1):45 - 53
- [12] Boeuf S, Klingenspor M, Van Hal NL *et al.* Differential gene expression in white and brown preadipocytes. *Physiol Genomics*, 2001, **7**(1):15 - 25
- [13] Han R(韩容), Chen WS(陈伟胜), Tong Y(童英), Shang KC(尚克刚). Establishment of two ES cell lines with good germline contribution. *Acta Genet Sinica* (遗传学报), 1999, **26**: 208 - 212
- [14] Wassarman PM, DePamphilis ML. Hints on using the guide to techniques in mouse development. *Methods Enzymol*, 1993, **225**:33 - 38
- [15] Teng Lu, Zhang Chongben, Shang Kegang *et al.* The labeling of C57BL/6j derived ES cells with EGFP. *Chinese Medical Journal*, 2003, **116**(1):151 - 153