Serum regulates adipogenesis of mesenchymal stem cells via MEK/ERK-dependent PPARγ expression and phosphorylation

Ling Wu a, b, †, Xiaoxiao Cai a, c, †, Hai Dong d, Wei Jing a, Yuanding Huang a, Xingmei Yang a, Yao Wu a, Yunfeng Lin a, *

a State Key Laboratory of Oral Diseases, West China College of Stomatology, Sichuan University, Chengdu, P. R. China
b Department of Tissue Regeneration, Institute for Biomedical Technology, University of Twente, Enschede, The Netherlands
c Dental Implant Center, West China College of Stomatology, Sichuan University, Chengdu, P. R. China
d Department of Stomatology, Medical College, Tibet University, Lhasa, P. R. China

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Abstract

Mesenchymal stem cells (MSCs) provide us an excellent cellular model to uncover the molecular mechanisms underlying adipogenic differentiation of adult stem cells. PPARγ had been considered as an important molecular marker of cells undergoing adipogenic differentiation. Here, we demonstrated that expression and phosphorylation of PPARγ could be found in bone marrow–derived MSCs cultured in expansion medium without any adipogenic additives (dexamethasone, IBMX, insulin or indomethacin). Then, PPARγ was dephosphorylated in MSCs during the process of adipogenic differentiation. We then found that inhibition of MEK activation by specific inhibitor (PD98059) counteracted the PPARγ expression and phosphorylation. However, expression and phosphorylation of PPARγ did not present in MSCs cultured in medium with lower serum concentration. When these MSCs differentiated into adipocytes, no phosphorylation could be detected to accompany the expression of PPARγ. Moreover, exposure of MSCs to higher concentration of serum induced stronger PPARγ expression, and subsequently enhanced their adipogenesis. These data suggested that activation of the MEK/ERK signalling pathway by high serum concentration promoted PPARγ expression and phosphorylation, and subsequently enhanced adipogenic differentiation of MSCs.

Keywords: peroxisome proliferator– activated receptor γ – mesenchymal stem cells – adipogenesis – mitogen-activated protein kinase – extracellular signal-regulated kinase

Introduction

Mesenchymal stem cells (MSCs) are a group of multi-potent adult stem cells that have been isolated from organs and tissues investigated so far, including bone marrow, dermis, muscular tissue, hair follicles, the periodontal ligament and placenta as well as adipose tissue [1]. MSCs may undergo self-renewal for several generations while maintaining their capacity to differentiate into multi-lineage tissues such as bone, cartilage, myocardial and adipose tissue [2]. MSCs derived from bone marrow are non-haematopoietic stem cells found in the stroma of the bone marrow [3]. They are easily isolated, cultured and expanded in the laboratory setting. All of these characteristics make MSCs an attractive cell source for clinical applications, including cell-based therapies and tissue engineering [4–6]. In addition, MSCs also provide us excellent model to uncover the regulation of adipocyte differentiation from precursor cells and the insulin sensitivity of mature adipocytes, which are vital for fat tissue regeneration and treatment of obesity and insulin resistance. Nowadays, most functional studies on adipogenic differentiation and function have been performed in the murine adipogenic 3T3-L1 cell line [7]. However, research based on adipogenic differentiation of adult stem cell model would be more useful in translating these data to clinical applications.
Peroxisome proliferator-activated receptors (PPARs) are a group of ligand-dependent nuclear receptors responsible for gene expression regulation [8, 9], and generally function as transcriptional regulators of adipogenic differentiation and lipid metabolism [10]. So far, three members of the PPAR sub-family has been identified: PPARα, PPARβ (also called PPARδ) and PPARγ [11, 12], each has different ligands, target genes and biological functions [13]. PPARγ, expressed predominantly in adipose tissue and the immune system [14], has been demonstrated to be a master regulator of adipogenesis and metabolic homeostasis [15, 16]. The critical role of PPARγ in cellular differentiation and insulin sensitization has been demonstrated in PPARγ knockout animals [17–19]. Suppression of PPARγ function through RNA interference leads to cellular differentiation towards osteoblasts rather than adipocytes in multi-potent mesenchymal stem cells [20], suggesting that PPARγ may act as a gatekeeper of multi-potency in mesenchymal cells.

Similar to other nuclear receptors, PPARγ are phosphoprotein and its transcriptional activity is severely affected by cross-talk with kinases and phosphatases. The regulation of PPARγ activity through phosphorylation is complex and controversial. The major phosphorylation site of PPARγ, which is used by both extracellular signal-regulated kinase (ERK)- and JNK-MAPK [21], was mapped at serine 82 of mouse PPARγ1, which corresponds to serine 112 of mouse PPARγ2 [22]. Phosphorylation of PPARγ by activating mitogen-activated protein kinase (MAPK) signalling pathway reduces its transcriptional activity and subsequently inhibit adipogenic differentiation, whereas PPARγ2 with a non-phosphorylatable mutation at serine-112 help cells to resist to inhibition of differentiation by mitogens [23]. Inhibition of p38MAPK with chemical inhibitors or p38MAPK gene knock-out increases adipogenesis in embryonic stem cells [24]. Mutation of the main MAPK site of phosphorylation in PPARγ2 (S112D) exhibits a decreased ligand-binding affinity [22]. It has been proposed that phosphorylation-mediated inhibition of transcriptional activity of nuclear receptors is an important ‘off-switch’ of ligand-induced activity [25]. However, not all phosphorylation events are inhibitory to adipogenic differentiation. Insulin treatment increases the ligand-independent transcriptional activity of PPARγ and enhances the TZD-induced PPARc transactivating function [26]. It was also reported that activation of MAPK/ERK pathway is required for adipogenic differentiation in an embryonic stem cells and in rat subcutaneous pre-adipocytes [27, 28]. It is possible that both claims are correct because the precise timing of MAPK activation during the initial stages of the differentiation process may determine its effect on the final destination of cells [29].

We recently reported that a group of MSCs derived from adipose tissue was found expressing PPARγ prior to adipogenic differentiation and lipid accumulation [30]. Here in this study, we found these cells also present in bone marrow derived MSCs. More interestingly, PPARγ found in bone marrow MSCs was phosphorylated. Thus, these cells provide us an excellent cellular model to investigate the regulation of PPARγ phosphorylation during the process of adipogenic differentiation of adult stem cells.

Materials and methods

Cell culture

In line with the International Guiding Principles for Animal Research (1985), bone marrow–derived MSCs were isolated as we previously reported [31]. Briefly, MSCs were harvested from the bone marrow of the femurs and tibias of 8-week-old BALB/c mice by inserting a 5-gauge needle into the shaft of the bone and flushing with α-modified Eagle’s medium (α-MEM) containing 2, 10 or 20% foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (expansion medium). Cells from one mouse were plated into one T25 flask. After 48 hrs, floating cells were discarded, and adherent cells were washed with phosphate-buffered saline (PBS). Cells were then incubated in expansion medium for 7–10 days to reach confluence and subcultured to passage 2 for further experiments. All experiments used passage 2 of MSCs unless otherwise stated. All cell culture reagents were purchased from Gibico, Invitrogen (Grand Island, NY) unless otherwise stated.

For inhibition of MAPK signalling pathway, specific inhibitors were dissolved in DMSO, and then 50 μM of PD98059 (Calbiochem, San Diego, CA) or 10 μM of PD168316 (Calbiochem) was added to the expansion medium. The same amount of DMSO was added in expansion medium containing 10% FBS as control.

Adipogenic differentiation, Oil Red O staining and quantification

For adipogenic differentiation, cells were cultured for 14 days in adipogenic medium containing α-MEM supplemented with 10% FBS, 1 μM dexamethasone (Sigma, Beijing, China), 10 μM insulin (Sigma), 200 μM indomethacin (Sigma) and 0.5 mM isobutyl-methylxanthine (IBMX; Sigma). The medium was replaced every 2 days. Differentiated MSCs were then applied to Oil Red O staining and quantification according to previously reported methods [32]. MSCs were fixed in a 10% formaldehyde solution (Sigma) for 1 hr, washed with 60% isopropanol (Sigma) and stained with Oil Red O solution (in 60% isopropanol) for 5 min. followed by repeated washing with PBS. After microscopy observation, Oil Red O was destained by 100% isopropanol for 15 min. The optical density (O.D.) of the solution was measured at 540 nm with HTS 7000 Plus Bio Assay reader (Perkin-Elmer, Boston, MA, USA).

RNA isolation, RT-PCR and real-time PCR

RNA samples of MSCs cultured in expansion medium or adipogenic medium were isolated with a Total Tissue/cell RNA Extraction Kits (Watson, Shanghai, China) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed into cDNA in a 20-μl reverse transcription system (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The cDNA samples were amplified with a Pfu PCR kit (Tiangen, Beijing, China), and the specific primers were displayed in Table 1. All PCR products were resolved on a 2% agarose gel. Expression of PPARγ in MSCs was then quantified by real-time PCR by using the SYBR Green l PCR master mix (Takara, Dalian, China). Reactions were carried out on an ABI 7300 (Applied Biosystems, Shanghai, China) under the following conditions: cDNA was denatured

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for 15 min. at 94°C, followed by 40 cycles, consisting 30 sec. at 94°C, 30 sec. 58°C and 1 min. at 72°C. For each reaction, a melting curve was generated to test the primer dimmer formation and false priming. The primers for real-time PCR were as follows: PPARα: 5`-TTTACAACGAGGCCAGTTC3‘ (forward) and 5`-CTCTGGATGGGTGATGTG3‘ (reverse); β-actin: 5`-TGTTACCAACTGGGACGACA3‘ (forward) and 5`-GGGGTGTTGAAGGTCTCAAA3‘ (reverse). Relative quantification of PPARα was carried out according to ΔΔCt method [33].

**Immunofluorescence and image analysis**

MSCs either cultured in expansion medium or adipogenic medium were plated on glass cover slips in six-well plates 24 hrs before staining. Cells were washed briefly with PBS, fixed in 4% paraformaldehyde for 30 min. at room temperature and then permeabilized and blocked in 0.5% Triton-X 100 and 0.5% bovine serum albumin (BSA) for 15 min. at room temperature. Slips were subsequently incubated overnight at 4°C with rabbit polyclonal antibodies against PPARγ (AbCam, Cambridge, UK) or rabbit monoclonal antibody against PPARγ with phosphoserine at residue 82 (Upstate, Lake Placid, NY). Sequentially, slides were incubated with secondary antibodies conjugated to Rhodamine or FITC (Pierce, Rockford, IL), and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR). For MSCs differentiated into adipocytes, Oil Red O staining was then performed to visualize fat drop. After rinsing in PBS, cells were observed and imaged with DMi 6000 B fluorescent microscope (Leica, Bensheim, Germany).

To determine the percentage of PPARγ and p-PPARγ expressing cells, the numbers of positive cells and total cells were counted manually. For each condition, at least three slides were stained and examined. For each slide, one to two fields (100× magnifications) were randomly chosen to make sure at least 100 of total cells were analyzed.

| Gene name | Primer sequence | Product size | Gene bank no. |
|-----------|----------------|--------------|---------------|
| C/EBPα | F: 5`-TTTACAACGAGGCCAGTTC3‘ | 232 | NM_007678 |
| | R: 5`-CTCTGGATGGGTGATGTG3‘ | 266 | NM_011146 |
| PPARγ | F: 5`-TGTTACCAACTGGGACGACA3‘ | 131 | NM_001127330 |
| | R: 5`-GGGGTGTTGAAGGTCTCAAA3‘ | 119 | NM_008509 |
| PPARγ1 | F: 5`-TTTACAACGAGGCCAGTTC3‘ | 232 | NM_007678 |
| | R: 5`-CTCTGGATGGGTGATGTG3‘ | 266 | NM_011146 |
| PPARγ2 | F: 5`-TGTTACCAACTGGGACGACA3‘ | 131 | NM_001127330 |
| | R: 5`-GGGGTGTTGAAGGTCTCAAA3‘ | 119 | NM_008509 |
| LPL | F: 5`-TGTTACCAACTGGGACGACA3‘ | 270 | NM_000827 |
| | R: 5`-GGGGTGTTGAAGGTCTCAAA3‘ | 182 | NM_024406 |
| aP2 | F: 5`-TTTACAACGAGGCCAGTTC3‘ | 232 | NM_007678 |
| | R: 5`-CTCTGGATGGGTGATGTG3‘ | 266 | NM_011146 |
| MEK1 | F: 5`-TTTACAACGAGGCCAGTTC3‘ | 232 | NM_007678 |
| | R: 5`-CTCTGGATGGGTGATGTG3‘ | 266 | NM_011146 |
| ERK1 | F: 5`-TTTACAACGAGGCCAGTTC3‘ | 232 | NM_007678 |
| | R: 5`-CTCTGGATGGGTGATGTG3‘ | 266 | NM_011146 |
| ERK2 | F: 5`-TTTACAACGAGGCCAGTTC3‘ | 232 | NM_007678 |
| | R: 5`-CTCTGGATGGGTGATGTG3‘ | 266 | NM_011146 |
| p38 | F: 5`-TTTACAACGAGGCCAGTTC3‘ | 232 | NM_007678 |
| | R: 5`-CTCTGGATGGGTGATGTG3‘ | 266 | NM_011146 |
| JNK1 | F: 5`-TTTACAACGAGGCCAGTTC3‘ | 232 | NM_007678 |
| | R: 5`-CTCTGGATGGGTGATGTG3‘ | 266 | NM_011146 |
| GAPDH | F: 5`-TTTACAACGAGGCCAGTTC3‘ | 232 | NM_007678 |
| | R: 5`-CTCTGGATGGGTGATGTG3‘ | 266 | NM_007678 |
Protein extraction and Western blot

Before cell dissolving, cell layers were washed three times with PBS buffer, then total proteins were extracted by protein extract reagents (Pierce), followed by centrifugation (13,000 \( \times \) g for 15 min.) to remove cellular debris. Protein concentration was assessed by BCA kit (Pierce) according to the manufacturer's instructions. Twenty-five microgram of total proteins was analyzed by Western blotting using polyclonal anti-PPAR\(\gamma\) antibody (AbCam) or anti-PPAR\(\gamma\) (phosphor 112) antibody (AbCam). Immunocomplexes were visualized using enhanced chemiluminescence reagent (Pierce) according to the manufacturer's instructions.

Statistical analysis

We performed three or more independent sets of the experiments, and each experiment was run at least 3 times. Data were shown as average with indicated standard deviation. Means \( \pm \) S.D. and P values were calculated using Student's t-test. \( P < 0.05 \) was considered as statistically significant.

Results

PPAR\(\gamma\) expressed and phosphorylated in undifferentiated MSCs

PPAR\(\gamma\) has been considered as a molecular marker of cells undergoing adipogenic differentiation [34]. However, we recently reported that PPAR\(\gamma\) was found expressing in a group of adipose-derived mesenchymal stem cells cultured in expansion medium [30]. Here, in this study, we examined the expression of PPAR\(\gamma\) in undifferentiated MSCs (derived from murine bone marrow) with immunofluorescence. Unsurprisingly, we found a group of cells that were positive for PPAR\(\gamma\) before adipogenic differentiation (Fig. 1A left panel). Further analysis showed that PPAR\(\gamma\) was phosphorylated in undifferentiated MSCs (Fig. 1A right panel). To test the potential effects of passaging on PPAR\(\gamma\) expression and phosphorylation, we repeated the experiments with primary MSCs and got a similar result (Fig. 1B). Furthermore, percentages of positive cells between different passages are not significantly different (Fig. 1C). These results were also confirmed by Western blot (Fig. 1D). Because PPAR\(\gamma\) is expressed as two protein isoforms, both of which can be detected by immunoreaction, we performed RT-PCR with primers specific to PPAR\(\gamma\), PPAR\(\gamma1\) and PPAR\(\gamma2\). It turned out to be only PPAR\(\gamma2\) expressed in the undifferentiated and differentiated MSCs.

PPAR\(\gamma\) dephosphorylated during adipogenic differentiation of MSCs

It was indicated by Western blot that phosphorylated PPAR\(\gamma\) was not found in MSCs differentiated into adipocytes. So we performed intensive analysis of immunofluorescence to show the dynamics of PPAR\(\gamma\) phosphorylation during the process of adipogenic differentiation. As shown in Fig. 2A, green fluorescence showing pho-PPAR\(\gamma\) became condensed at 3 days as compared with 1 day, and finally vanished at 7 days after adipogenic differentiation. The process of pho-PPAR\(\gamma\) condensation and vanishing was accompanied by the deposition of lipid drops in the cytoplasm.

We also performed immunofluorescence to show the expression and localization of total PPAR\(\gamma\) (non-phosphorylated and phosphorylated) during the adipogenic differentiation of MSCs (Fig. 2B). The expression of total PPAR\(\gamma\) was detected from day 1 to day 7 during adipogenic differentiation. With the expansion of lipid droplets in the cytoplasm, PPAR\(\gamma\) finally condensed in the nuclei of MSCs.

PPAR\(\gamma\) is phosphorylated by activation of MEK/ERK signalling pathway

In vitro assays demonstrate that ERK2 are able to phosphorylate PPAR\(\gamma\) [35], therefore, we first looked into the MAPKs to investigate the signalling pathway involved in the expression and phosphorylation of PPAR\(\gamma\). Meanwhile, it was believed that ERK can be preferentially activated by mitogens such as the serum [36], we also considered the influence of serum concentration in the medium when we examined the expression of MAPKs in MSCs. As revealed by RT-PCR, most MAPKs, except JNK, expressed in MSCs cultured in expansion medium containing 10% or 20% FBS (Fig. 3A). Data of real-time PCR indicated that higher concentration of serum led to higher expression level of PPAR\(\gamma\) in MSCs (Fig. 3B). It was also indicated by quantitative analysis of immunofluorescence that percentage of PPAR\(\gamma\)-expressing cells increased tremendously in MSCs cultured in expansion medium with 10% or 20% FBS compared with that with 2% FBS. Percentage of p38-PPAR\(\gamma\)-positive cells showed big differences as well. Then we blocked the signalling pathway by specific inhibitor. Western blot showed that PD98059, which inhibits the activation of MEK1, decreased the expression of PPAR\(\gamma\) as well as its phosphorylation (Fig. 3D). However, inhibition of p38 MAPK with PD169316 seemed to have no effects on the expression and phosphorylation of PPAR\(\gamma\). Meanwhile, real-time PCR also showed inhibition of MEK1 activation in MSCs’ reduced PPAR\(\gamma\) expression level, whereas inhibition of p38MAPK slightly increased the expression of PPAR\(\gamma\) (Fig. 3E).

PPAR\(\gamma\) is expressed without phosphorylation in MSCs cultured with low serum concentration

Then we isolated MSCs from murine bone marrow with low serum medium (2% FBS) to see if serum concentration affected the phosphorylation status of PPAR\(\gamma\). We used antibodies against either PPAR\(\gamma\) or pho-PPAR\(\gamma\) to show its expression and phosphorylation.
In the first row of Fig. 4, MSCs cultured in low serum medium show no expression and phosphorylation of PPARγ. MSCs were then applied to adipogenic medium and examined by immunofluorescence at different time points. When PPARγ was expressed at day 1, it localized in the nuclei of MSCs. Then with the continual accumulation of fat drops in cytoplasm, PPARγ retained expression in the nuclei; however, pho-PPARγ never showed up until the end of adipogenic differentiation.
Higher concentration of serum enhances adipogenic differentiation of MSCs

To investigate the consequences of PPARγ expression and phosphorylation caused by different concentration of serum, we performed Oil Red O staining and quantification to show the different capacity in adipogenic differentiation of MSCs cultured in medium containing 2, 10 and 20% of FBS. As shown in Fig. 5A, MSCs accumulated more fat droplets in their cytoplasm, with the increase of serum concentration in the medium. This trend was further confirmed by the quantitative analysis of Oil Red O captured by the fat drops (Fig. 5B). RT-PCR (Fig. 5C) revealed the expression pattern of a set of adipocyte marker genes in MSCs.

Discussion

PPARγ was well recognized as a key regulator of adipogenic differentiation [18]. However, we recently reported that PPARγ could be found in undifferentiated MSCs derived from adipose tissue [30]. In the present study, we found PPARγ expressed and phosphorylated a group of bone marrow–derived MSCs. Further analysis demonstrated that activation of the MEK/ERK signalling pathway by higher concentration of serum promoted PPARγ expression and phosphorylation, and subsequently enhanced adipogenic differentiation of MSCs.

We first found PPARγ expressed in bone marrow–derived MSCs cultured in expansion medium without any adipogenic additives. This is different from previous studies indicating that PPARγ was expressed in differentiated MSCs [37, 38] and activated transcription of its target genes in lipogenic pathways, including lipoprotein lipase (LPL), adipocyte fatty acid-binding protein (A-FABP or aP2), acyl-CoA synthase and fatty acid transport protein (FATP) [18]. However, our result was in agreement with other studies showing that undifferentiated 3T3-L1 fibroblasts contained significant levels of PPARγ protein in their cytoplasm [39]. More specifically, Moerman et al. reported that expression of PPARγ2 was increased with the aging of murine bone marrow–derived MSCs [40]. Their findings indicated that microenvironment changes can induce the expression of PPARγ2 in MSCs. But why the expression of PPARγ in our cellular model did not trigger adipogenic differentiation and lipid accumulation in MSCs? Two differences in our study may provide reasonable explanations for the malfunction of PPARγ: (i) PPARγ was found in the cytoplasm of MSCs in our study. As nuclear receptor transcription factor, PPARγ has to bind to a specific element (PPRE) in the promoter region of its target genes, before it activates transcription in response to binding of the ligands [41]. So, PPARγ had been previously reported to reside mainly in the nucleus rather than in the cytoplasm [42]. (ii) Phosphorylation of PPARγ in MSCs was confirmed by immunofluorescence and Western blot. Phosphorylation decreases PPARγ affinity to its ligand by modulating the conformation of the unliganded receptor.
thus reducing its transcriptional activity and inhibiting adipogenic differentiation [22].

It is believed that the phosphorylation status of proteins can influence their nuclear translocation. For example, phosphorylation of carbohydrate response element binding protein (ChREBP), a transcription factor involved in the regulation of gene transcription by glucose, impairs its translocation into the nucleus [43]. Whether such a mechanism regulates PPARγ localization and transcriptional activity remains unclear. Data from the present study revealed that there was a dephosphorylation process of PPARγ during the adipogenic differentiation of MSCs. It was reported that genomic activity of PPARγ was reduced by a rapid MEK1—dependent export of PPARγ from the nucleus to the cytoplasm upon stimulation of mitogens (TPA, EGF) and PPARγ—ligands [44]. Although MEK1-mediated PPARγ translocation was independent of phosphorylation according to their results, PPARγ did exist in the nucleus of MSCs after the dephosphorylation process of PPARγ in our study.

There are piles of documents demonstrating PPARγ are phosphorylated by MAPK. Epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) increased phosphorylation of PPARγ through MAPK signalling, thus decreasing its

Fig. 3 Inhibition of MEK activation reduced the expression and phosphorylation of PPARγ. (A) RT-PCR demonstrated the activation of MAPK signalling pathway. Total RNA of MSCs cultured in expansion medium was isolated for RT-PCR using the specific primers listed in Table 1. The values 2%, 10% and 20% indicate the percentages of FBS in the expansion media MSCs were kept in before RNA isolation. (B) Relative gene expression of PPARγ in MSCs cultured in different concentration of FBS. The values 2%, 10% and 20% indicate the percentages of FBS in the expansion media MSCs were kept in before RNA isolation. Data were normalized to β-actin and represented as mean ± S.D. (n = 3). * (P < 0.05) denotes statistical significance between the indicated pairs. (C) Percentages of PPARγ expressing and phosphorylating cells in MSCs. Immunofluorescent images were analyzed to compare PPARγ expression and phosphorylation in MSCs cultured in the expansion media with 2%, 10% or 20% FBS. Positive cell ratio was calculated as percentage of total cell number. Data were represented as mean ± S.D. (n = 3). (D) Inhibitor PD98059 reduced the expression and phosphorylation of PPARγ. Upon isolation of MSCs, 50 μM of PD98059 or 10 μM of PD169316 were added to the expansion medium containing 10% FBS. At the end of primary culture (7 days), total proteins of MSCs were extracted for Western blot to detect the expression and phosphorylation of PPAR. (E) Quantitative analysis of PPARγ expression when MEK and MAPK p38 was inhibited by specific inhibitor. Upon isolation of MSCs, 50 μM of PD98059 or 10 μM of PD169316 were added to the expansion medium containing 10% FBS. At the end of primary culture (7 days), total RNA of MSCs were extracted for real-time PCR. Data were normalized to β-actin and represented as mean ± S.D. (n = 3).
ligand-dependent transcriptional activity in adipocyte cell lines [21, 23]. Treatment of insulin and constitutive activation of ERK stimulated PPARγ phosphorylation in vivo [26]. In vitro, serine 84 of human PPARγ was phosphorylated by ERK2 and JNK, and this phosphorylation was markedly reduced in the S84A mutant [35]. In our cellular model, expression of most MAPKs was revealed by RT-PCR, indicating the activation of the signalling pathway in MSCs. Moreover, inhibition of ERK activation by PD98059 decreased the expression of PPARγ as well as its phosphorylation. Surprisingly, PD169316, inhibitor for another MAPKs (p38MAPK), seemed to have no effect on the expression and phosphorylation of PPARγ in our cellular model.

Then, another interesting finding drew our attention: MAPK was not activated in MSCs cultured in medium with low concentration of serum (2% FBS). Further examination of immunofluorescence showed that PPARγ was not phosphorylated or even expressed in these MSCs. Nevertheless, the expression and localization of PPARγ in these MSCs were quite normal upon adipogenic differentiation when compared with other cellular models [37]. With the accumulation of lipid droplets, PPARγ was continually expressed in the nuclei, functioning as normal nuclear receptor transcription factors. Both in vitro and in vivo studies demonstrated that stimulation of Rat-IR cells and NIH 3T3 with serum caused an increase in the amount of phosphorylated PPARγ [23]. So, it is clear that serum concentration in our cellular model caused the phosphorylation of PPARγ.

Our data also suggested that serum concentration not only influence the phosphorylation of PPARγ but also determined the expression of PPARγ. Increase of serum concentration in the medium promoted the expression level of PPARγ. In the cellular model of pre-adipocytes 3T3-L1, activation of the MEK/ERK signalling pathway during the initial stages of adipogenesis enhanced

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Fig. 4 Expression and phosphorylation of PPARγ was undetectable in MSCs cultured in low concentration of serum. MSCs previously cultured in expansion medium with 2% FBS were differentiated into adipocytes for 0, 1, 3 and 7 days. Antibodies against PPARγ or against PPARγ with phosphoserine at residue 82 were used to detect the expression and phosphorylation of PPARγ. Then, primary antibodies were visualized with secondary antibodies conjugated to FITC; Oil Red O staining was then performed to show fat drops. Scale bar = 50 μm.
Fig. 5 MSCs cultured in different concentration of serum behaved differently in adipogenic differentiation and gene expression pattern. (A) Morphology of undifferentiated MSCs (left panel) and differentiated MSCs (middle and right panel) cultured in adipogenic medium. Differentiated MSCs were MSCs cultured in adipogenic medium for 7 days. The values 2%, 10% and 20% indicate the percentages of FBS in the expansion media MSCs were kept in before adipogenic differentiation. Scale bar = 100 μm. (B) Oil Red O quantification showed an increasing trend of O.D. value with the increase of serum in the medium. At day 3, day 5 and day 7 of adipogenic differentiation, Oil Red O staining was performed and then Oil Red O captured by the fat droplets was quantified. The values 2%, 10% and 20% indicate the percentages of FBS in the expansion media MSCs were kept in before adipogenic differentiation. Data are represented as mean values ± S.D. (n = 6). * (P < 0.05) or ** (P < 0.01) denotes statistical significance between the indicated pairs. (C) RT-PCR showed different expression pattern of adipogenic genes. At day 0 and day 7 of adipogenic differentiation of MSCs, total RNA were isolated for RT-PCR. The values 2%, 10% and 20% indicate the percentages of FBS in the expansion media MSCs were kept in before adipogenic differentiation.
the activity of factors that regulate PPARγ expression [29]. Janderova et al. reported that inhibition of ERK1/2 phosphorylation by PD098059 increase the adipogenesis of human MSCs [45].

As early as the 1980s, scientists had already noted that human serum effectively promote the terminal differentiation of pre-adipocytes 3T3-L1 cell [46, 47]. In vitro study on adipogenesis of rat stromal-vascular cells demonstrated that initial concentration of FBS increased the rate of adipocyte differentiation [48]. Oreffo et al. reported that continuous treatment of human serum was required for an adipogetic differentiation model of human MSCs. Their results demonstrate that human serum contains factors that exert dramatic effects on human bone marrow cell differentiation to adipocytes [49]. It was reported that chick serum contains factors for adipocyte induction not only in vitro but also in vivo, and that the adipogenic potential does not depend on the supplements used during the cell culture [50]. Our data also indicated that MSCs showed stronger capacity in adipogenic differentiation if they were previously cultured in medium containing higher concentration of serum.

Taken together, our results indicated the expression and phosphorylation of PPARγ in undifferentiated MSCs. Then our data indicated that phosphorylation of PPARγ is dependent on the activation of MEK/ERK signalling pathway. Finally, we demonstrated that it was serum that caused the expression and phosphorylation of PPARγ, and subsequently promoted adipogetic differentiation of MSCs. These data sum up to suggest a dual effect of serum on the adipogetic differentiation of MSCs that determines the expression of PPARγ and at the same time induced its phosphorylation thus preventing it from translocation into nucleus to function as transcription factors.

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