

## **Serum regulates adipogenesis of mesenchymal stem cells via MEK/ERK-dependent PPAR $\gamma$ expression and phosphorylation**

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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. PPAR $\gamma$  had been considered as an important molecular marker of cells undergoing adipogenic differentiation. Here, we demonstrated that expression and phosphorylation of PPAR $\gamma$  could be found in bone marrow-derived MSCs cultured in expansion medium without any adipogenic additives (dexamethasone, IBMX, insulin or indomethacin). Then, PPAR $\gamma$  was dephosphorylated in MSCs during the process of adipogenic differentiation. We then found that inhibition of MEK activation by specific inhibitor (PD98059) counteracted the PPAR $\gamma$  expression and phosphorylation. However, expression and phosphorylation of PPAR $\gamma$  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 PPAR $\gamma$ . Moreover, exposure of MSCs to higher concentration of serum induced stronger PPAR $\gamma$  expression, and subsequently enhanced their adipogenesis. These data suggested that activation of the MEK/ERK signalling pathway by high serum concentration promoted PPAR $\gamma$  expression and phosphorylation, and subsequently enhanced adipogenic differentiation of MSCs.

**Keywords:** peroxisome proliferator-activated receptor  $\gamma$  • 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, car-

tilage, 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.

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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: PPAR $\alpha$ , PPAR $\beta$  (also called PPAR $\delta$ ) and PPAR $\gamma$  [11, 12], each has different ligands, target genes and biological functions [13]. PPAR $\gamma$ , 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 PPAR $\gamma$  in cellular differentiation and insulin sensitization has been demonstrated in PPAR $\gamma$  knockout animals [17–19]. Suppression of PPAR $\gamma$  function through RNA interference leads to cellular differentiation towards osteoblasts rather than adipocytes in multi-potent mesenchymal stem cells [20], suggesting that PPAR $\gamma$  may act as a gatekeeper of multi-potency in mesenchymal cells.

Similar to other nuclear receptors, PPAR $\gamma$  are phosphoprotein and its transcriptional activity is severely affected by cross-talk with kinases and phosphatases. The regulation of PPAR $\gamma$  activity through phosphorylation is complex and controversial. The major phosphorylation site of PPAR $\gamma$ , which is used by both extracellular signal-regulated kinase (ERK)- and JNK-MAPK [21], was mapped at serine 82 of mouse PPAR $\gamma$ 1, which corresponds to serine 112 of mouse PPAR $\gamma$ 2 [22]. Phosphorylation of PPAR $\gamma$  by activating mitogen-activated protein kinase (MAPK) signalling pathway reduces its transcriptional activity and subsequently inhibit adipogenic differentiation, whereas PPAR $\gamma$ 2 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 knockout increases adipogenesis in embryonic stem cells [24]. Mutation of the main MAPK site of phosphorylation in PPAR $\gamma$ 2 (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 PPAR $\gamma$  and enhances the TZD-induced PPAR $\gamma$  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 PPAR $\gamma$  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, PPAR $\gamma$  found in bone marrow MSCs was phosphorylated. Thus, these cells provide us an excellent cellular model to investigate the regulation of PPAR $\gamma$  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  $\alpha$ -modified Eagle's medium ( $\alpha$ -MEM) containing 2, 10 or 20% foetal bovine serum (FBS), 100 U/ml penicillin and 100  $\mu$ g/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 Gibco, Invitrogen (Grand Island, NY) unless otherwise stated.

For inhibition of MAPK signalling pathway, specific inhibitors were dissolved in DMSO, and then 50  $\mu$ M of PD98059 (Calbiochem, San Diego, CA) or 10  $\mu$ M of PD169316 (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  $\alpha$ -MEM supplemented with 10% FBS, 1  $\mu$ M dexamethasone (Sigma, Beijing, China), 10  $\mu$ M insulin (Sigma), 200  $\mu$ M 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-ml 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 PPAR $\gamma$  in MSCs was then quantified by real-time PCR by using the SYBR Green I 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

**Table 1** Forward (F) and Reverse (R) primer pairs used for RT-PCR to detect gene expression pattern in MSCs

Gene name	Primer sequence	Product size	Gene bank no.
C/EBP $\alpha$	F: 5' TTACAACAGGCCAGGTTTCC 3'	232	NM_007678
	R: 5' CTCTGGGATGGATCGATTGT 3'		
PPAR $\gamma$	F: 5' GACCACTCGCATTCTTT 3'	266	NM_011146
	R: 5' CCACAGACTCGGCACTCA 3'		
PPAR $\gamma$ 1	F: 5' TGTGAGACCAACAGCCTGAC 3'	109	NM_001127330
	R: 5' AGTGGTTCACCGCTTCTTTCC 3'		
PPAR $\gamma$ 2	F: 5' TGCACTGCCTATGAGCACTT 3'	131	NM_011146
	R: 5' TGATGTCAAAGGAATGCGAG 3'		
LPL	F: 5' AGGGTGAGGAATCTAATG 3'	270	NM_008509
	R: 5' CAGGTGTTTCAACCGTA 3'		
aP2	F: 5' CATCAGCGTAAATGGGGATT 3'	182	NM_024406
	R: 5' TCGACTTTCCATCCCACTTC 3'		
MEK1	F: 5' TGCCAGGCTGAACTACAGTA 3'	168	NM_008927
	R: 5' CACAAGGCTCCCTCTCAGAC 3'		
ERK1	F: 5' TCCAAGGGCTACACCAAATC 3'	208	NM_011952
	R: 5' TCCAAGGGCTACACCAAATC 3'		
ERK2	F: 5' AGAAGTCAGAGGCAGGTGGA 3'	192	NM_011949
	R: 5' GGTGCCATCATCAACATCTG 3'		
p38	F: 5' ATGGTGCAGGAAAACAGGAC 3'	161	NM_011951
	R: 5' CGTCTCTCCCTTTGTTTCAGC 3'		
JNK1	F: 5' GCCACAAAATCCTCTTTCCA 3'	175	NM_016700
	R: 5' CACATCGGGGAACATTCT 3'		
GAPDH	F: 5' ACCACAGTCCATGCCATCAC 3'	492	NM_001001303
	R: 5' TCCACCACCCTGTTGCTGTA 3'		

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 dimer formation and false priming. The primers for real-time PCR were as follows: PPAR $\gamma$ : 5'-TTTTCAAGGGT-GCCAGTTTC-3' (forward) and 5'-AATCCTTGGCCCTCTGAGAT-3' (reverse);  $\beta$ -actin: 5'-TGTTACCAACTGGGACGACA-3' (forward) and 5'-GGGGTGTGAAGGTCTCAAA-3' (reverse). Relative quantification of PPAR $\gamma$  was carried out according to  $\Delta\Delta$ Ct 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 PPAR $\gamma$  (AbCam, Cambridge, UK) or rabbit monoclonal antibody against PPAR $\gamma$  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 PPAR $\gamma$  and pho-PPAR $\gamma$  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 $\times$  magnifications) were randomly chosen to make sure at least 100 of total cells were analyzed.

## 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 $\gamma$ 1 and PPAR $\gamma$ 2. It turned out to be only PPAR $\gamma$ 2 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.

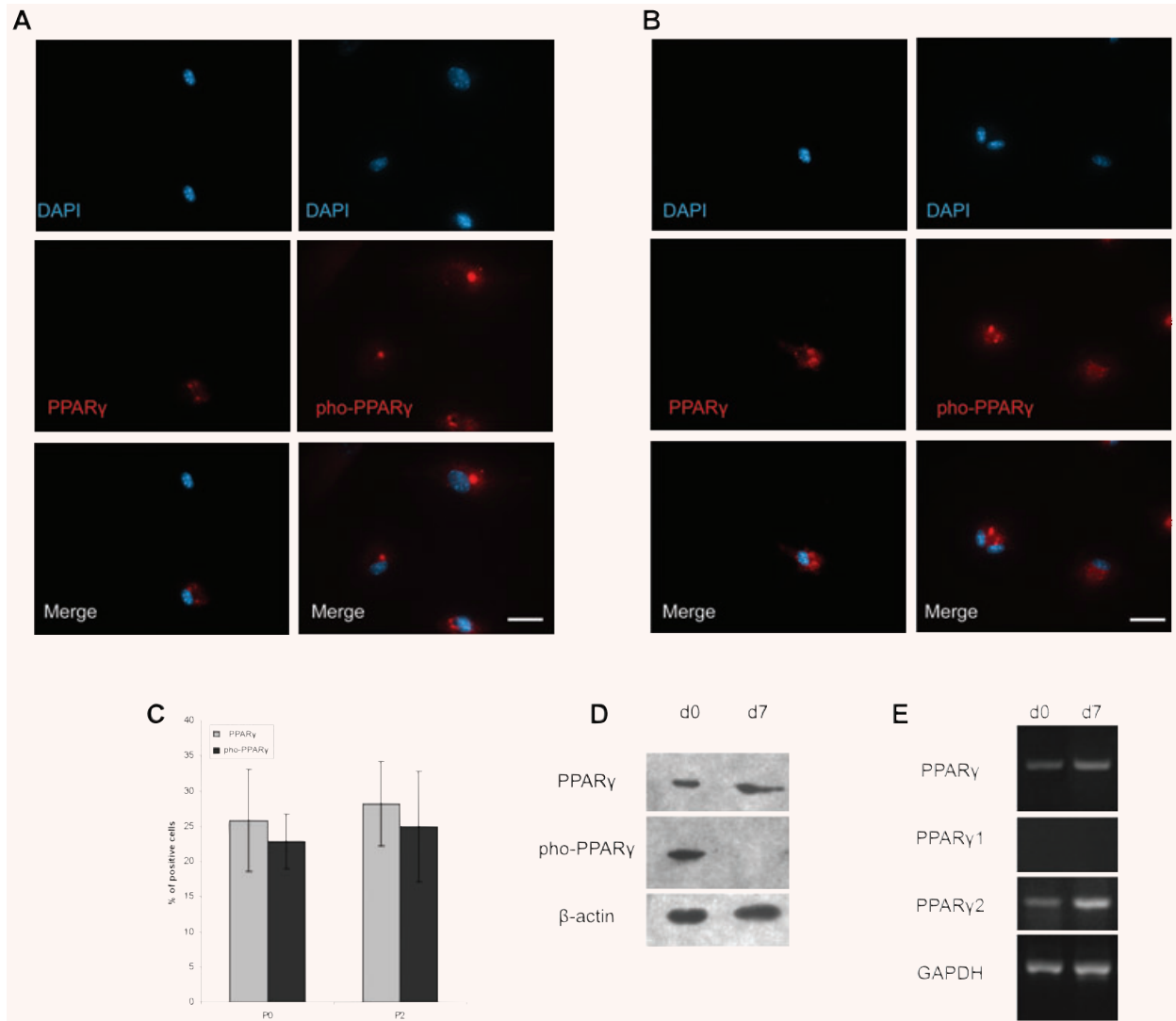
We also performed immunofluorescence to show the expression and localization of total PPAR $\gamma$  (non-phosphorylated and phosphorylated) during the adipogenic differentiation of MSCs (Fig. 2B). The expression of total PPAR $\gamma$  was detected from day 1 to day 7 during adipogenic differentiation. 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 pho-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

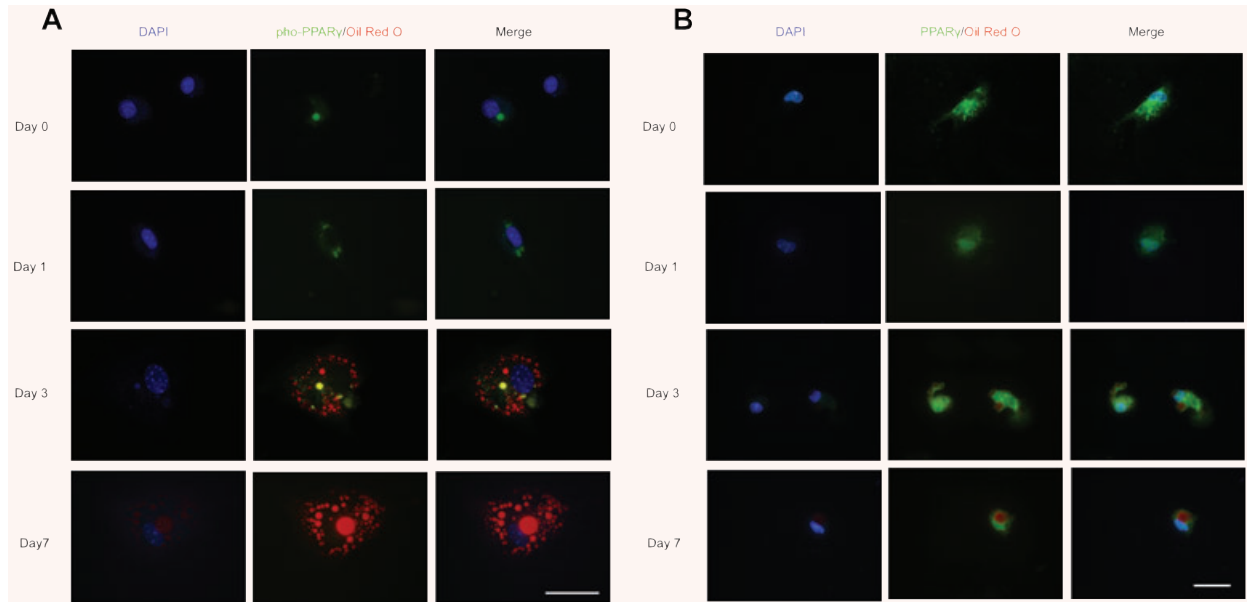
Then we isolated MSCs from murine bone marrow with low serum medium (2% FBS) to see if serum concentration affected the phosphorylation status of PPAR $\gamma$ . We used antibodies against either PPAR $\gamma$  or pho-PPAR $\gamma$  to show its expression and phosphorylation.



**Fig. 1** Expression and phosphorylation of PPAR $\gamma$  in undifferentiated MSCs. **(A)** PPAR $\gamma$  was expressed and phosphorylated in undifferentiated MSC. MSCs were sub-cultured to passage 2 in expansion medium containing 10% FBS, before immunofluorescent analysis; secondary antibodies conjugated to rhodamine were applied for fluorescent detection. Scale bar = 50  $\mu$ m. **(B)** PPAR $\gamma$  was also expressed and phosphorylated in primary MSCs. Primary MSCs cultured in expansion medium containing 10% FBS were directly applied to immunofluorescent analysis. Secondary antibodies conjugated to rhodamine were used. Scale bar = 50  $\mu$ m. **(C)** Percentages of positive cells are similar between different passages of MSCs. Immunofluorescent images were analyzed to compare PPAR $\gamma$  expression and phosphorylation between primary culture and passage 2 of MSCs. Positive cell ratio was calculated as percentage of total cell number. Data were represented as mean  $\pm$  S.D. ( $n = 3$ ). **(D)** Western blotting confirmed the expression and phosphorylation of PPAR $\gamma$ . MSCs, cultured in expansion media containing 10% FBS, were applied to adipogenic differentiation. At day 0 (d0) and day 7 (d7), total proteins of MSCs were extracted for Western blot to detect the expression and phosphorylation of PPAR. **(E)** RT-PCR then showed the expression of PPAR $\gamma$ , PPAR $\gamma$  isoform 1 (PPAR $\gamma$ 1) and isoform 2 (PPAR $\gamma$ 2). MSCs cultured in expansion medium containing 10% FBS were applied to adipogenic differentiation. At day 0 and day 7, total RNA of MSCs were isolated for RT-PCR to detect the mRNA of PPAR $\gamma$ , PPAR $\gamma$ 1 and PPAR $\gamma$ 2.

In the first row of Fig. 4, MSCs cultured in low serum medium show no expression and phosphorylation of PPAR $\gamma$ . MSCs were then applied to adipogenic medium and examined by immunofluorescence at different time points. When PPAR $\gamma$  was expressed at

day 1, it localized in the nuclei of MSCs. Then with the continual accumulation of fat drops in cytoplasm, PPAR $\gamma$  retained expression in the nuclei; however, pho-PPAR $\gamma$  never showed up until the end of adipogenic differentiation.



**Fig. 2** PPAR $\gamma$  was dephosphorylated during the differentiation of MSCs. MSCs, previously grown in expansion medium with 10% FBS, were cultured in adipogenic medium for 0, 1, 3 and 7 days before immunofluorescent staining. Antibodies against PPAR $\gamma$  with phosphoserine at residue 82 (**A**) or against PPAR $\gamma$  (**B**) were used. Then, primary antibodies were visualized with secondary antibodies conjugated to FITC; Oil Red O staining was performed to show fat drops. Scale bar = 50  $\mu$ m.

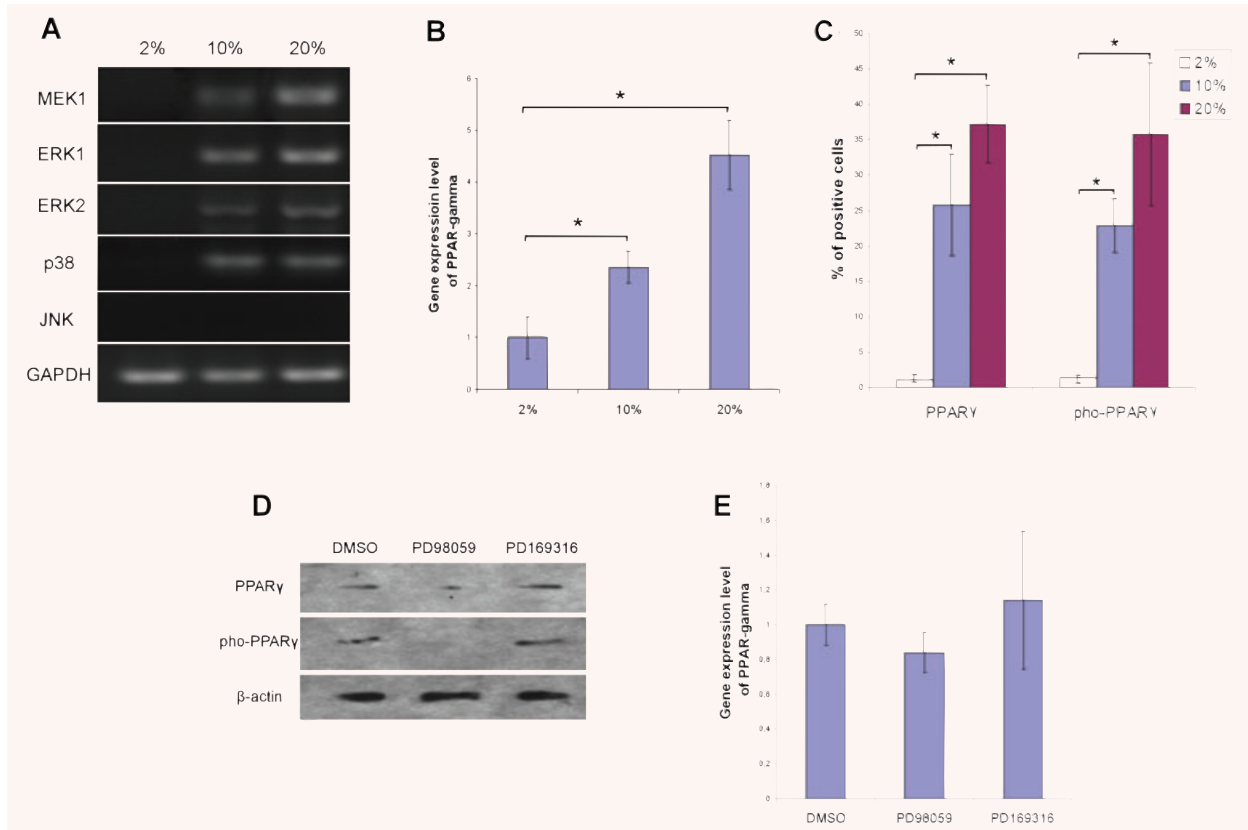
### Higher concentration of serum enhances adipogenic differentiation of MSCs

To investigate the consequences of PPAR $\gamma$  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

PPAR $\gamma$  was well recognized as a key regulator of adipogenic differentiation [18]. However, we recently reported that PPAR $\gamma$  could be found in undifferentiated MSCs derived from adipose tissue [30]. In the present study, we found PPAR $\gamma$  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 PPAR $\gamma$  expression and phosphorylation, and subsequently enhanced adipogenic differentiation of MSCs.

We first found PPAR $\gamma$  expressed in bone marrow-derived MSCs cultured in expansion medium without any adipogenic additives. This is different from previous studies indicating that PPAR $\gamma$  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 PPAR $\gamma$  protein in their cytoplasm [39]. More specifically, Moerman *et al.* reported that expression of PPAR $\gamma$ 2 was increased with the aging of murine bone marrow-derived MSCs [40]. Their findings indicated that microenvironment changes can induce the expression of PPAR $\gamma$ 2 in MSCs. But why the expression of PPAR $\gamma$  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 PPAR $\gamma$ : (i) PPAR $\gamma$  was found in the cytoplasm of MSCs in our study. As nuclear receptor transcription factor, PPAR $\gamma$  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, PPAR $\gamma$  had been previously reported to reside mainly in the nucleus rather than in the cytoplasm [42]. (ii) Phosphorylation of PPAR $\gamma$  in MSCs was confirmed by immunofluorescence and Western blot. Phosphorylation decreases PPAR $\gamma$  affinity to its ligand and by modulating the conformation of the unliganded receptor,



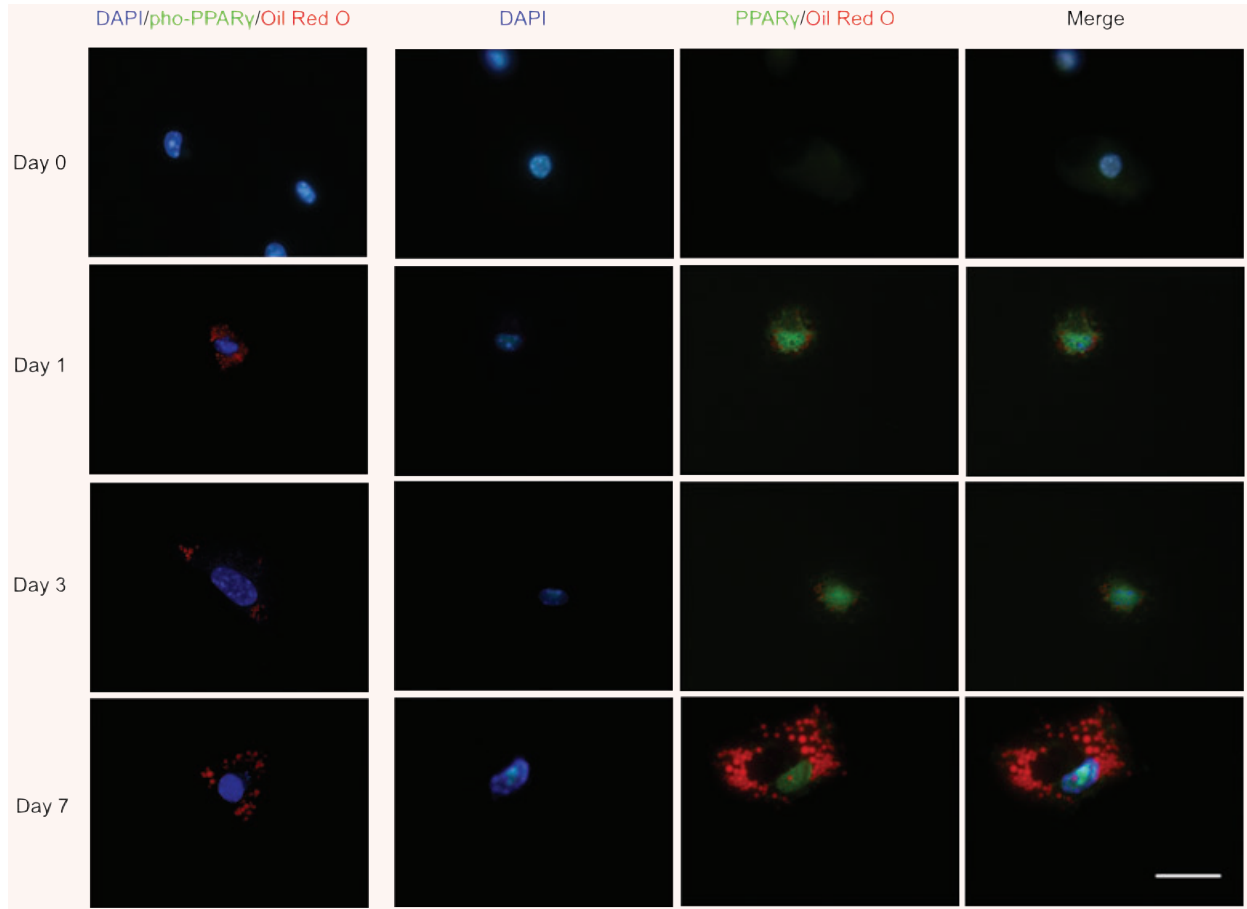
**Fig. 3** Inhibition of MEK activation reduced the expression and phosphorylation of PPAR $\gamma$ . **(A)** RT-PCR demonstrated the activation of MAPK signalling pathway. Total RNA of MSCs cultured in expansion medium was isolated for RT-PCR using the specific primers listed in Table 1. The values 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 PPAR $\gamma$  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  $\beta$ -actin and represented as mean  $\pm$  S.D. ( $n = 3$ ). \* ( $P < 0.05$ ) denotes statistical significance between the indicated pairs. **(C)** Percentages of PPAR $\gamma$  expressing and phosphorylating cells in MSCs. Immunofluorescent images were analyzed to compare PPAR $\gamma$  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  $\pm$  S.D. ( $n = 3$ ). **(D)** Inhibitor PD98059 reduced the expression and phosphorylation of PPAR $\gamma$ . Upon isolation of MSCs, 50  $\mu$ M of PD98059 or 10  $\mu$ M 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 PPAR $\gamma$  expression when MEK and MAPK p38 was inhibited by specific inhibitor. Upon isolation of MSCs, 50  $\mu$ M of PD98059 or 10  $\mu$ M 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  $\beta$ -actin and represented as mean  $\pm$  S.D. ( $n = 3$ ).

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 PPAR $\gamma$  localization and transcriptional activity remains unclear. Data from the present study revealed that there was a dephosphorylation process of PPAR $\gamma$  during the adipogenic differentiation of MSCs. It was

reported that genomic activity of PPAR $\gamma$  was reduced by a rapid MEK1-dependent export of PPAR $\gamma$  from the nucleus to the cytoplasm upon stimulation of mitogens (TPA, EGF) and PPAR $\gamma$ -ligands [44]. Although MEK1-mediated PPAR $\gamma$  translocation was independent of phosphorylation according to their results, PPAR $\gamma$  did exist in the nucleus of MSCs after the dephosphorylation process of PPAR $\gamma$  in our study.

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



**Fig. 4** Expression and phosphorylation of PPAR $\gamma$  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 PPAR $\gamma$  or against PPAR $\gamma$  with phosphoserine at residue 82 were used to detect the expression and phosphorylation of PPAR $\gamma$ . 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  $\mu$ m.

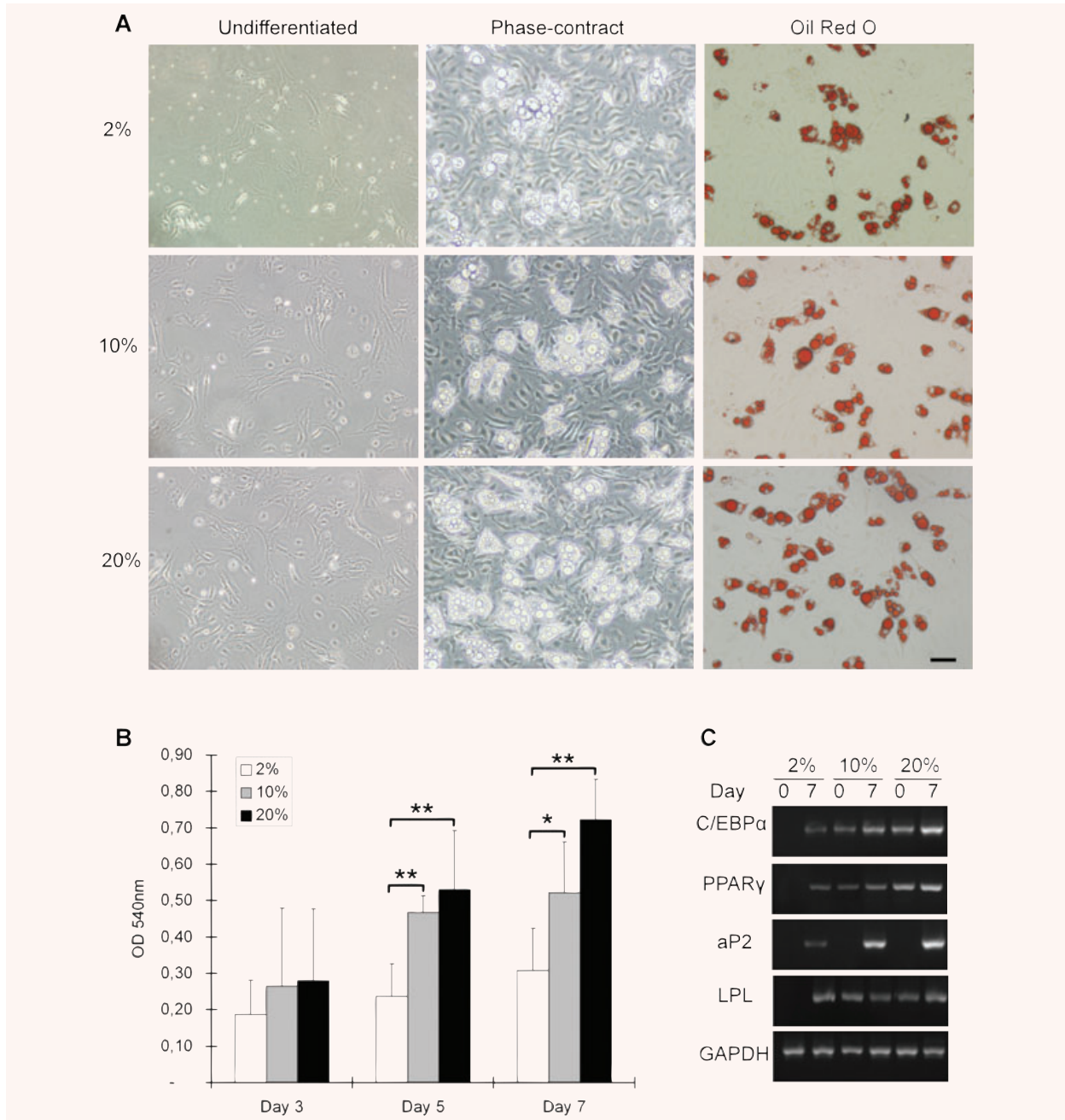
ligand-dependent transcriptional activity in adipocyte cell lines [21, 23]. Treatment of insulin and constitutive activation of ERK stimulated PPAR $\gamma$  phosphorylation *in vivo* [26]. *In vitro*, serine 84 of human PPAR $\gamma$  was phosphorylated by ERK2 and JNK, and this phosphorylation was 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 PPAR $\gamma$  as well as its phosphorylation. Surprisingly, PD169316, inhibitor for another MAPKs (p38MAPK), seemed to have no effect on the expression and phosphorylation of PPAR $\gamma$  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 PPAR $\gamma$  was not phosphorylated or even

expressed in these MSCs. Nevertheless, the expression and localization of PPAR $\gamma$  in these MSCs were quite normal upon adipogenic differentiation when compared with other cellular models [37]. With the accumulation of lipid droplets, PPAR $\gamma$  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 PPAR $\gamma$  [23]. So, it is clear that serum concentration in our cellular model caused the phosphorylation of PPAR $\gamma$ .

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





**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 media. 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  $\mu$ m. **(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  $\pm$  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 PPAR $\gamma$  expression [29]. Janderova *et al.* reported that inhibition of ERK1/2 phosphorylation by PD98059 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 preadipocytes 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 adipogenic 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 PPAR $\gamma$  in undifferentiated MSCs. Then our data

indicated that phosphorylation of PPAR $\gamma$  is dependent on the activation of MEK/ERK signalling pathway. Finally, we demonstrated that it was serum that caused the expression and phosphorylation of PPAR $\gamma$ , and subsequently promoted adipogenic differentiation of MSCs. These data sum up to suggest a dual effect of serum on the adipogenic differentiation of MSCs that determines the expression of PPAR $\gamma$  and at the same time induced its phosphorylation thus preventing it from translocation into nucleus to function as transcription factors.

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