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In vitro determination of osteo-adipogenic lineage choice of bone marrow stromal/stem cells (BMSCs)

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ABSTRACT

Bone marrow stromal/stem cells (BMSCs) are primitive and heterogeneous cells that can be differentiated into osteoblasts, adipocytes and other subsets. Their bone-fat lineage commitment is responsible for the homeostasis of bone marrow microenvironment. However, there are little effective methods and evidence to simultaneously visualise the lineage commitment of BMSCs. Here we provide a bivalent differentiation medium that can enable BMSCs differentiation into osteoblasts and adipocytes *in vitro*, and establish a method to simultaneously distinguish osteoblasts or adipocytes from the heterogeneous BMSCs based on Alizarin red S and Oil red O staining, which have been used for detection of specific mineralized nodules and lipid droplets, respectively. This assay provides a specifically simple but effective and low-cost method to evaluate the efficiency of osteo-adipogenic (OA) allocation of BMSCs.

► Researchers can utilize the bivalent differentiation medium to evaluate the efficiency of osteogenic and adipogenic differentiation of BMSCs *in vitro*.

Specifications table

Subject area:	
More specific subject area:	Bone-fat determination of marrow stromal/stem cells
Name of your method:	Visualization of osteo-adipogenic lineage choice in cultured BMSCs
Name and reference of original method:	N.A.
Resource availability:	N.A.

Method details

Rationale

Bone marrow stromal/stem cells (BMSCs) are primitive and heterogeneous cells resident in the bone marrow, and can be differentiated into osteoblasts, adipocytes, chondrocytes and other cell types in certain conditions[1–3]. In fact, BMSCs are believed to be the common progenitors for both osteoblasts and adipocytes [4,5]. Under aging, BMSCs are most likely to differentiate into adipocytes instead of osteoblasts, leading to age-related fat accumulation in bone marrow and senile osteoporosis [2,6], indicating

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that the lineage choice of BMSCs may preferentially shift towards into adipo-lineage *in vivo* microenvironment. However, there are little effective methods and evidence to simultaneously evaluate the lineage commitment of BMSCs *in vitro*. So far, the vast majority of studies that induce the osteogenic or adipogenic differentiation of BMSCs are conducted separately. For example, when treated with osteogenic medium, only the osteogenic-bias BMSCs will generate osteoblasts [7–9]. But what happens to the adipogenic-bias subsets is largely unknown under the osteogenic condition. Consequently, it is necessary to develop an effective experiment to analyse the fate changes of heterogeneous subsets of BMSCs under the same conditions.

During differentiation of BMSCs, two hallmarks of osteogenesis and adipogenesis are the formation of mineralized nodules and lipid droplets, respectively. Since Alizarin red S (ARS) and Oil red O (ORO) staining have been specifically and widely used to detect the final osteogenesis and adipogenesis [8,10], little is known about the effects of ARS staining on adipogenesis and ORO staining on osteogenesis.

Here we provided a special differentiation medium, which contained equal osteogenic and adipogenic drugs, that could directly induce BMSCs differentiation into osteoblasts and adipocytes. This method would provide great convenience for assessment the lineage choice of BMSCs in future studies.

Method details

Reagents and drugs

All reagents used for cell culture, including low-glucose Dulbecco's Modified Eagle's Media (DMEM; Gibco,11054,020), fetal bovine serum (FBS; Gibco,10099,141C), non-essential amino acids (Gibco, 11140, 050), antibiotics (P/S; Gibco, 15070, 063) and trypsin (Gibco,15400, 054) were purchased from Thermo Fisher Scientific Incorporation. Recombinant basic fibroblasts growth factor (bFGF; 100-18B) and epidermal growth factor (EGF; AF-100-15) were from PeproTech Inc..

All drugs used for *in vitro* differentiation of hBMSCs, including dexamethasone (Sigma; D4902), β-glycerophosphate (Sigma, G9422), L-ascorbic acid (Sigma, A4544), indomethacin (Sigma, I7378), isobutylmethylxanthine (IBMX; Sigma, I7018) were purchased from Merck Group. Insulin (I8830) is from Beijing Solarbio Science & Technology Co., Ltd (Solarbio Inc.). Bone morphogenetic protein 2 (BMP2; PeproTech,120-02) was a kind gift from Dr. Erwei Hao (Guangxi University of Chinese Medicine). For detection of mineralized nodules and lipid droplets, Alizarin red S (ARS; G8550) and Oil red O (ORO; O8010) powder were purchased from Solarbio Inc..

Cell culture

Human BMSCs were maintained as described before [9,11]. Briefly, 5×10^5 cells/ml were plated in a 100 mm diameter cell culture dish containing low-glucose DMEM supplemented with 10 % FBS, 100 U/ml P/S, 0.1 mM non-essential amino acids, 20 ng/ml bFGF and 20 ng/ml EGF. Cells were maintained in a humidified incubator at 37 °C with 5 % CO_2. When 70 % to 80 % confluent, adherent cells were trypsinized with 0.05 % trypsin-1mM EDTA at 37 ˚C for 2 min, harvested, and expanded for future use.

Preparation of differentiation medium

The *in vitro* differentiation medium of BMSCs was prepared as previously described [9,11]. Specifically, for osteogenic medium, high-glucose DMEM (Gibco,11965, 092) was supplemented with 10 % FBS, 100 nM dexamethasone, 10 mM β-glycerophosphate and 0.2 mM L-ascorbic acid. For adipogenic medium, high-glucose DMEM was supplemented with 10 % FBS, 1 μM dexamethasone, 500 μM IBMX, and 200 μM indomethacin and 20 ng/ml insulin. These prepared mediums could be stored at 4 °C for 3 months. The osteo-adipogenic (OA) medium was prepared from mixture of equal osteogenic and adipogenic medium at a volume ratio of 1:1, plus 10 ng/ml bFGF when immediately needed.

In vitro *differentiation induction of hBMSCs*

For osteogenic induction, 2×10^5 cells per well were seeded into a 12-well plate and then incubated with 500 μl osteogenic medium, followed by exchange with fresh medium every 3 days. 7 days later, total RNAs of cell cultures were prepared as described in **Method validation.** 10 days later, cell cultures were fixed and stained with ARS to visualize mineralized nodules.

For adipogenic induction, 1×10^6 cells per well were seeded into a 12-well plate and then incubated with 500 μl adipogenic medium, followed by exchange with fresh medium every 3 days. 7 days later, total RNAs of cell cultures were prepared as described in **Method validation.** 10 days later, lipid droplets would appear in the culture dish. Then cell cultures were fixed and stained with ORO to visualize the lipid droplets.

For osteo-adipogenic (OA) induction, 1×10^6 cells per well were seeded into a 12-well plate and then incubated with 500 μl OA medium and 20 ng/ml BMP2 or not, followed by exchange with fresh OA medium every 3 days. 10 days later, total RNAs of cell cultures were prepared as described in **Method validation.**14 days later, the cell cultures could be fixed and stained with ARS and ORO to detect the mineralized nodules and lipid droplets.

Detection of osteogenesis and adipogenesis

To prepare ARS working solution, 2 g ARS powder was dissolved in 100 ml distilled water and filtered by filter paper, then the solution was adjusted to pH 4.2. This solution could be stored at room temperature for at least 6 months. To detect the mineralized nodules, cells were fixed with absolute ethyl alcohol for 10 min at room temperature and then stained with 2 % ARS solution for 5 min. After washing with PBS, the plate was removed to an inverted microscope (DMI8, Leica, Germany) for image capture. The mineralized nodules were appeared as bright red or black sediment shown in Figs. 1A and 2A (green arrowheads).

For detection of adipogenesis, a stored solution of ORO staining was prepared. Briefly, 0.5 g ORO powder was dissolved in 100 ml isopropanol. This solution could be stored away from light at room temperature for at least 6 months. The stored solution will be diluted by distilled water at a volume ratio of 3:2 when to prepare working solution. To detect the lipid droplets, cells were fixed with 4 % paraformaldehyde for 10 min at room temperature and then stained with ORO working solution for 5 min. After washing with PBS, the plate was removed to an inverted microscope for image capture. The lipid droplets were appeared as dark red or black red shown in Figs. 1A and 2A (pink arrowheads).

It should be noted that only mineralized nodules were formed in osteogenic induction of BMSCs, as lipid droplets formed only in adipogenic induction. However, both mineralized nodules and lipid droplets were formed in OA induction medium (Fig. 1). Although the lipid droplets stained by ARS were not the same as appeared in ORO staining, the morphological features of adipocytes were preserved intactly, suggesting that adipocytes could be distinguished from osteoblasts by ARS staining (Fig. 1). In parallel to ARS staining, mineralized nodules in ORO staining pictures were not obviously visualized. Meanwhile, this method also provided quantitative analysis of mineralized nodules and lipid droplets (Fig. 2D, E). In summary, both ARS and ORO staining should be combinedly used to detect the bivalent differentiation efficiency of BMSCs.

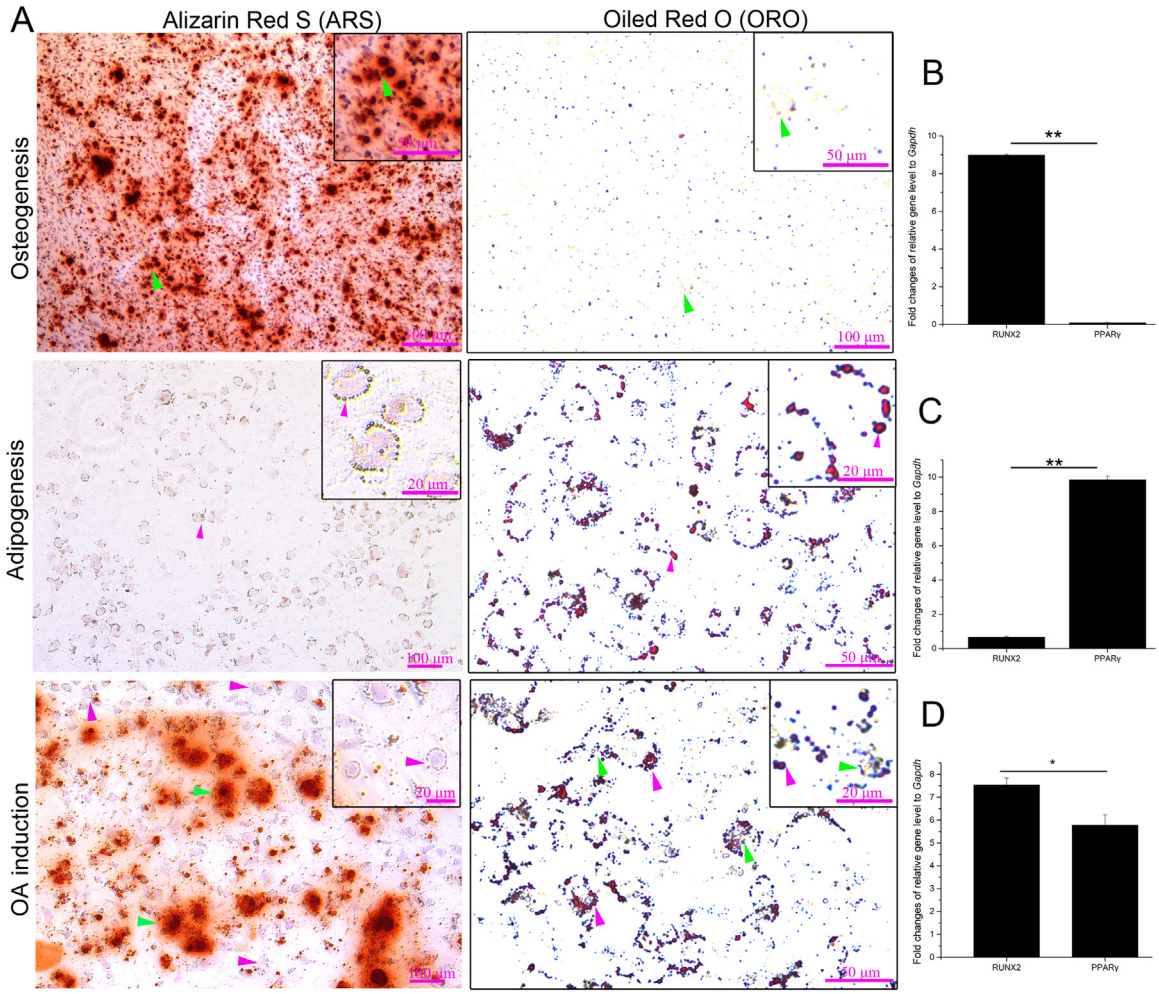

Fig. 1. Osteogenic and adipogenic detection of young BMSCs *in vitro*. (A) Representative alizarin red S and Oil red O staining images of young BMSCs upon to osteogenic, adipogenic and osteo-adipogenic induction. (B–D) Relative gene levels of *Runx2* and *Pparγ* of BMSCs in A. Results are presented as means ± SD, $n \geq 3$. $^{*}p < 0.05$, $^{**}p < 0.01$.

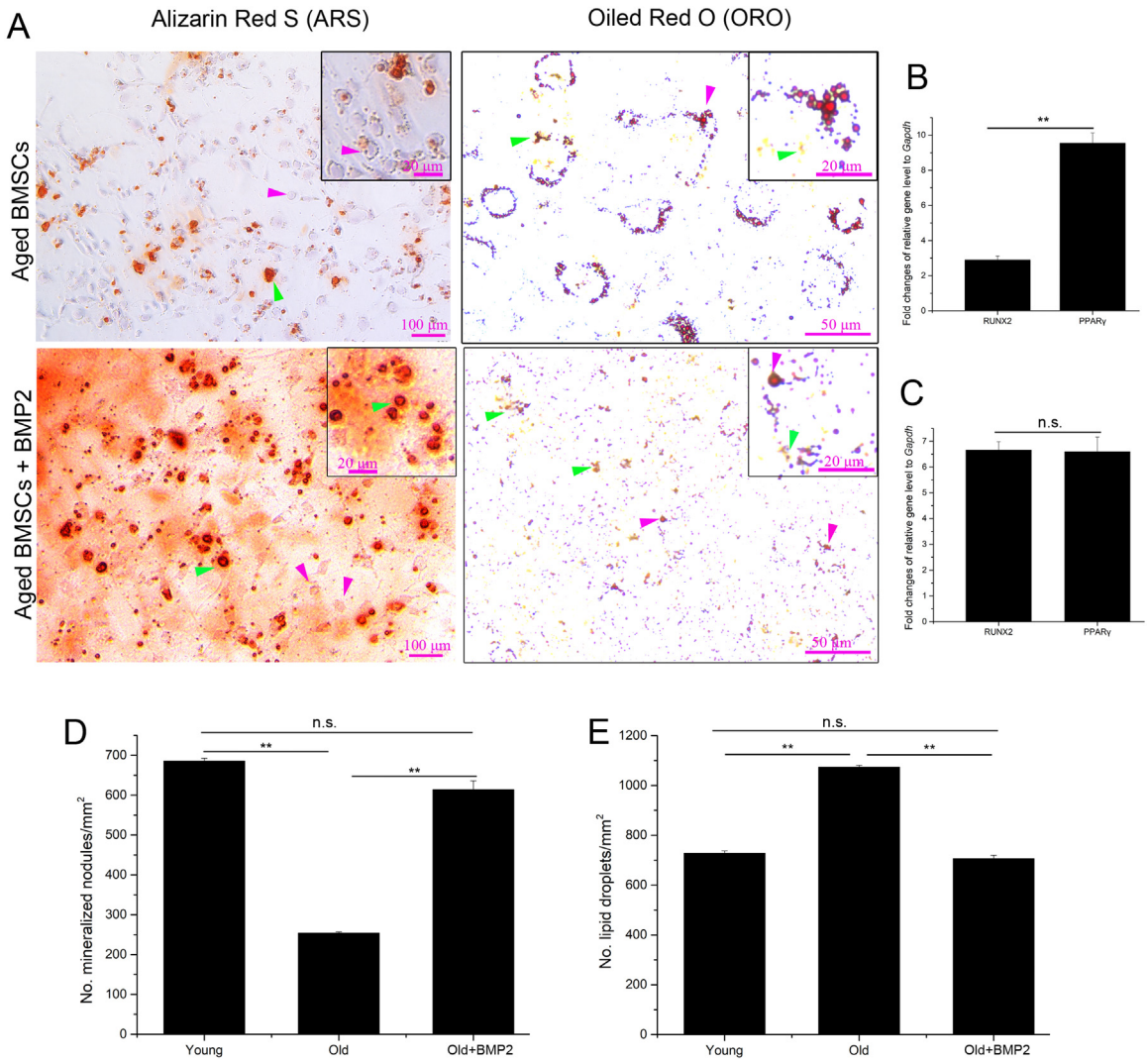

Fig. 2. Osteo-adipogenic detection of aged BMSCs treated with BMP2 or not. (A) Representative alizarin red S and Oil red O staining images of aged BMSCs treated with BMP2 or not. (B-C) Gene analysis of *Runx2* and *Pparγ* in A. (D-E) Combined analysis of numbers of mineralized nodules and lipid droplets in Figs. 1A and 2A. Results are presented as means ± SD, $n \geq 3$. $^{*}p < 0.05$, $^{**}p < 0.01$, n.s. means no significance.

Method validation

Transcription factors like runt-related transcription factor 2 (RUNX2) and peroxisome proliferator-activated receptor γ (PPARγ) play key roles in determining the lineage commitment of BMSCs at early stage. To confirm the bivalent effects of OA medium on the lineage choice of BMSCs, the gene levels of *Runx2* and *Pparγ* were quantitatively analyzed by real-time PCR. The following primers were used in this study. For *Runx2*: Forward-5′-TGACATCCCCATCCATCCAC-3′; Reverse-5′-AGAAGTCAGAGGTGGCAGTG-3′; for *Pparγ*: Forward-5′-TATCACTGGAGATCTCCGCCAACAGC-3′; Reverse-5′-GTCACGTTCTGACAGGACTGTGTGAC-3′; for glyceraldehyde phosphate dehydrogenase (*Gapdh*): Forward-5′-ACTCCACTCACGGCAAATTC-3′; Reverse-5′-TCTCCATGGTGGTGAAGACA-3′. The standard procedures of qPCR were described as we did before [12]. Briefly, Total cellular RNAs were isolated using RNA Extraction Kits (Solarbio, R1200) according to the manufacturer's instructions and quantified by Nanodrop One C (Thermo Fisher Scientific, Madison, USA). 1 μg RNA was analyzed by using a TaqMan One Step RT-qPCR Kit (Solarbio, T2210) on LightCycle 96 Instrument (Roche, Mannhein, Germany) according to the manufacturer's instructions. The relative expression of target genes was normalized to *Gapdh* and calculated by the $2^{-\Delta CT}$ method (Fig. 1B–D).

It is widely accepted that aged BMSCs have decreased abilities to be differentiated into osteoblasts and increased abilities to be adipocytes [1,13]. In this section, we also used the OA medium to investigate its effects on aged BMSCs. As shown in Fig 2A, although aged BMSCs could be differentiated into osteoblasts and adipocytes, less mineralized nodules (green arrowheads) and more lipid droplets (pink arrowheads) were formed compared with young BMSCs (Fig. 1A, OA induction). In according with the staining,

gene level of *Pparγ* was significantly higher than that of *Runx2* in aged BMSCs (Fig. 2B). These results validated that aged BMSCs preferentially generated adipocytes instead of osteoblasts.

To better view the fate changes of osteogenesis and adipogenesis of BMSCs, we additionally treated the aged BMSCs with BMP2, which has been widely employed in many preclinical and clinical studies exploring osteoinductive potential in several animal model defects and in human diseases [14,15]. Compared with the untreated aged BMSCs, BMP2 remarkedly increased mineralized nodules and decreased lipid droplets under the OA induction (Fig. 2A), and there was no significance between the gene levels of *Runx2* and *Pparγ* (Fig. 2C). Finally, numbers of mineralized nodules and more lipid droplets can truly reflect the lineage choice of BMSCs. Combined analysis from Figs. 1 and 2 demonstrated that aged BMSCs showed less osteogenic and more adipogenic abilities than young BMSCs, while BMP2 could inhibit adipogenesis and restore osteogenesis (Fig. 2D and E).

These results augmented that it was easy to estimate the fate changes of BMSCs with the help of bidirectional differentiation medium.

Statistical analysis

Results are represented as means ± standard deviations. Statistical analysis was performed using Student's t-test as well as one-way analysis of variance (ANOVA) followed by the Tukey HSD test for post hoc comparison (Origin 8.0, OriginLab). Difference was considered significant when $P < 0.05$ indicated as *, while more significant when $P < 0.01$ indicated as **.

Ethics statements

None.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Dawei Qiu: Conceptualization, Methodology, Investigation. **Wanyi Wei:** Data curation, Writing – original draft, Validation. **Jia Chen:** Validation, Data curation. **Jingwen Huang:** Investigation, Visualization. **Yong Yang:** Supervision. **Ziwei Luo:** Conceptualization, Methodology, Writing – review & editing.

Data availability

Data will be made available on request.

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