In vitro visualization 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 evaluate the lineage commitment of BMSCs. Here we provide a mixed osteo-adipogenic (OA) differentiation medium that enabled BMSCs differentiation into osteoblasts and adipocytes in vitro, and establish a method for simultaneous assessment of bone-fat lineage choice of BMSCs based on the Alizarin red S and Oil red O staining of specific mineralized nodules and lipid droplets, respectively. This assay provides a specifically simple but effective and low-cost method to visualize the osteogenesis and adipogenesis of BMSCs, and evaluate the efficiency of lineage allocation of BMSCs.

Research highlights

► A bivalent differentiation medium is prepared for both osteogenic and adipogenic lineage commitment of BMSCs in vitro.

► A specifically simple but effective and low-cost method is established to simultaneously analyse the efficiency of osteogenesis and adipogenesis of BMSCs.

Abbreviations

ARS: Alizarin red S staining; BMSCs: Bone marrow stromal/stem cells; ORO: Oil red O staining; OA: osteo-adipogenic;

Keywords

BMSCs; Osteo-adipogenic differentiation; Lineage choice; Alizarin red S staining; Oil red O staining

1. Introduction

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 that the lineage choice of BMSCs may preferentially shift towards into adipo-lineage in vivo microenvironment. Therefore, rejuvenation of aged BMSCs that balance the osteogenic and adipogenic lineage commitment of BMSCs is essential for bone homeostasis. 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 differentiate into 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 BMSCs subsets 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 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.

2. Materials and methods

2.1. 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 purchased 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) was from Beijing Solarbio Science & Technology Co., Ltd (Solarbio Inc.). Bone morphogenetic protein 2 (BMP2) was a kind gift from Dr. Erwei Hao (Guangxi University of Chinese Medicine). For detection of osteogenesis and adipogenesis, Alizarin red S (ARS; G8550) and Oil red O (ORO; O8010) powder were purchased from Solarbio Inc..

2.2. 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 20ng/ml EGF. Cells were maintained in a humidified incubator at 37□ with 5% CO_2. When 70% to 80% confluent, adherent cells were trypsinized with 0.05% trypsin-1mM EDTA at 37°C for 2 minutes, harvested, and expanded for future use.

2.3. Preparation of differentiation medium

The in vitro differentiation medium of BMSCs was prepared as previously described[9, 11]. Specifically, for osteogenic medium, 90% high-glucose DMEM (Gibco,11965, 092) and 10% FBS were mixed, supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate and 0.2 mM ascorbic acid. For adipogenic medium, 90% high-glucose DMEM and 10% FBS were mixed, supplemented with 1 μM

dexamethasone, 500μM IBMX, and 200μM indomethacin and 20 ng/ml insulin. These prepared mediums could be stored at 4□ 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.

2.4. In vitro differentiation 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. 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. 10 days later, lipid droplets would appear in the culture dish. Then cell cultures were fixed and stained with ORO to visualize lipid droplets.

For osteo-adipogenic 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. After 14 days, the cell cultures could be fixed and stained with ARS/ORO to detect the mineralized nodules and lipid droplets.

2.5. Detection of osteogenesis and adipogenesis

To prepare ARS working solution, 2g 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% Alizarin Red S solution for 5 min. After washing with PBS, the plate was removed to an inverted microscope (DMI8, Leica, Germany) for image capture. 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.

2.6. Quantitative real-time PCR

Gene analysis of runt-related transcription factor 2 (*Runx2*) and peroxisome proliferator-activated receptor γ (*Pparγ*) by real-time PCR as we did before[12] were used to validate the bivalent differentiation. 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 primers used for gene amplification were listed as following: 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 relative expression of target genes was normalized to *Gapdh* and calculated by the $2^{-\Delta CT}$ method.

2.7. 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 **.

3. Results

BMSCs can be differentiated into osteoblasts in the presence of osteogenic induction medium (Figure 1A), or adipocytes in the presence of adipogenic induction medium (Figure 1C), as revealed by ARS and ORO staining respectively and also confirmed by the gene expression (Figure 1B, D). Interestingly, lipid droplets derived from adipogenesis were also appeared in ARS staining (Figure 1B, arrowheads and insertions), while little mineralized nodules were found in ORO staining. These results suggested that BMSCs could only be differentiated into osteoblasts under osteogenic condition. But in fact, as a heterogenous population, what happens to the adipo-bias subsets is largely unknown. This question also exists in the case of adipogenesis. To study the osteo-adipo lineage changes of BMSCs subsets, young BMSCs were treated with a mixed differentiation medium. Surprisingly, BMSCs could be simultaneously differentiated into osteoblasts (pink arrowheads) and adipocytes (green arrowheads), as revealed by ARS-chelate mineralized nodules and ORO-combinative lipid droplets (Figure 2A). These results were also confirmed by the expression of *Runx2* and *Pparγ* (Figure 2B). These results were quite different from the osteogenic or adipogenic medium, which only showed osteoblasts or adipocytes formation (Figure 1). Therefore, these results suggested that the OA medium could be a bivalent differentiation medium that permited both osteogenic and adipogenic differentiation of BMSCs.

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 study, we also used the OA medium to investigate its effects on aged BMSCs. As shown in Fig 2A in the same condition, although aged BMSCs could also be differentiated into osteoblasts and adipocytes, less mineralized nodules (pink arrowheads) and more lipid droplets (green arrowheads) were formed compared with young BMSCs (Figure 2 E and F). In according with the staining, gene level of *Pparγ* was significantly higher than that of *Runx2* in aged BMSCs (Figure 2C). These results validated that aged BMSCs show preferentially differentiation into 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 (Figure 2A, E, F). It also seemed that BMP2 could restore the differentiation abilities of aged BMSCs (Figure 2E, F), and there was no significance between the gene levels of *Runx2* and *Pparγ* (Figure 2D). These results augmented that it was easy to estimate the fate changes of BMSCs with the help of bidirectional differentiation medium.

4. Discussion and Conclusion

Mature cells derived from BMSCs, especially osteoblasts and adipocytes, play key roles in the homeostasis of bone marrow. In turn, as the only tissue where bone and fat coexist in the same microenvironment, bone marrow offers a unique signal to direct the lineage commitment of BMSCs. Therefore, it is reasonably important to uncover the osteogenic and adipogenic choice of BMSCs in the same condition, especially in the case of research into new drugs for senile osteoporosis. To our knowledge, there is little evidence for simultaneously detecting the subsets formation of BMSCs in vitro. In this work, we provided a special differentiation medium and method to monitor the osteoblasts and adipocytes formation of BMSCs. Additionally, we performed the *situ* translational expressions of RUNX2 and PPARγ of BMSCs upon with OA induction, and the same results were found (data not shown). This method provided a simple, effective and low-cost but specific approach to directly visualise the lineage choice of osteogenesis and adipogenesis of BMSCs. Moreover, our results not only confirmed that cultured young BMSCs were closer to osteoblasts than adipocytes[5], but also verified that aged BMSCs preferentially differentiation into adipocytes instead of osteoblasts[1, 2, 13]. However, whether the doses of supplemental drugs that were used to induce BMSCs differentiation are optimization combination still needed further studies.

In summary, we have developed a bivalent differentiation medium that permited both osteogenic and adipogenic differentiation of BMSCs. This discovery would also provide convenience for researching the lineage changes of BMSCs, especially in the case of regulating the skewed differentiation of BMSCs upon drugs against senile osteoporosis.

Declaration of competing interest

The authors declared that there was no conflict of interest in this paper.

Author contributions

Dawei Qiu and **Wanyi Wei:** conceptualization, methodology and writing. **Jia Chen:** investigation, data curation. **Jingwen Huang:** investigation, data curation. **Yong Yang:** formal analysis and supervision. **Ziwei Luo:** conceptualization, methodology, supervision, formal analysis and editing.

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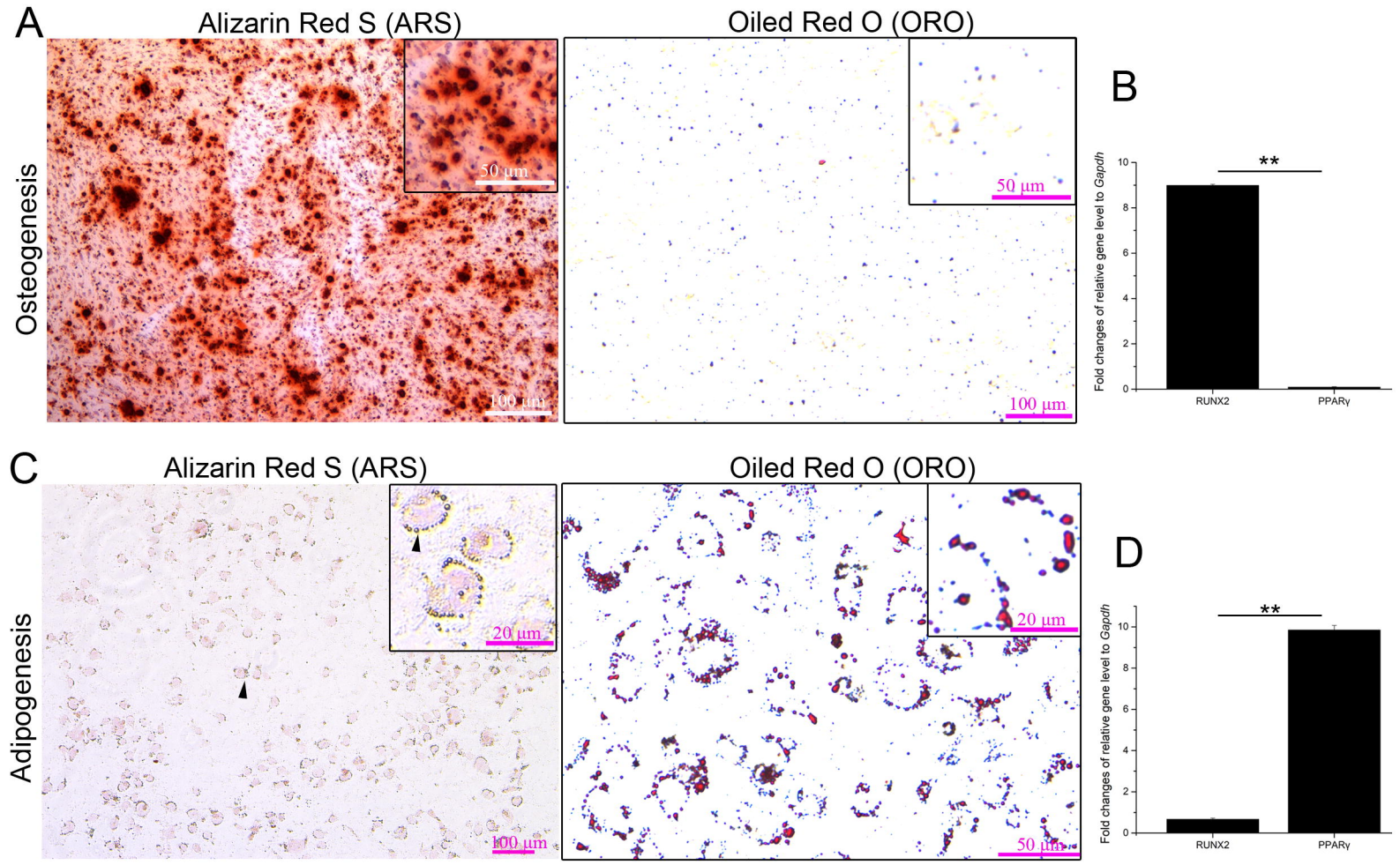
Figure legends

Figure 1. Osteogenic and adipogenic detection of young BMSCs in vitro. (A-B) Representative images and gene levels of *Runx2* of BMSCs upon to osteogenic differentiation. **(C-D)** Representative images and gene levels of *Pparγ* of BMSCs upon to adipogenic differentiation. Inserts are locally magnified area.

Figure 2. Osteo-adipogenic detection of young and old BMSCs treated with BMP2 or not. (A) Representative alizarin red S and Oil red O staining images of young BMSCs (upper), old BMSCs (middle) and BMP2 treated old BMSCs (lower) upon to OA differentiation. Inserts are locally magnified area. **(B-D)** Gene analysis of *Runx2* and *Pparγ* in young BMSCs (B), old BMSCs (C) and old BMSCs treated with BMP2 (D)

upon to OA differentiation medium. **(E-F)** Quantitatively analysis of numbers of mineralized nodules and lipid droplets in A. Results are presented as means ± SD, n ≥ 3. *$p < 0.05$, **$p < 0.01$, n.s. means no significance.


A
Alizarin Red S (ARS)
Oiled Red O (ORO)
Osteogenesis
50 µm
100 µm
50 µm
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B
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Fold changes of relative gene level to Gapdh
RUNX2
PPARγ
C
Alizarin Red S (ARS)
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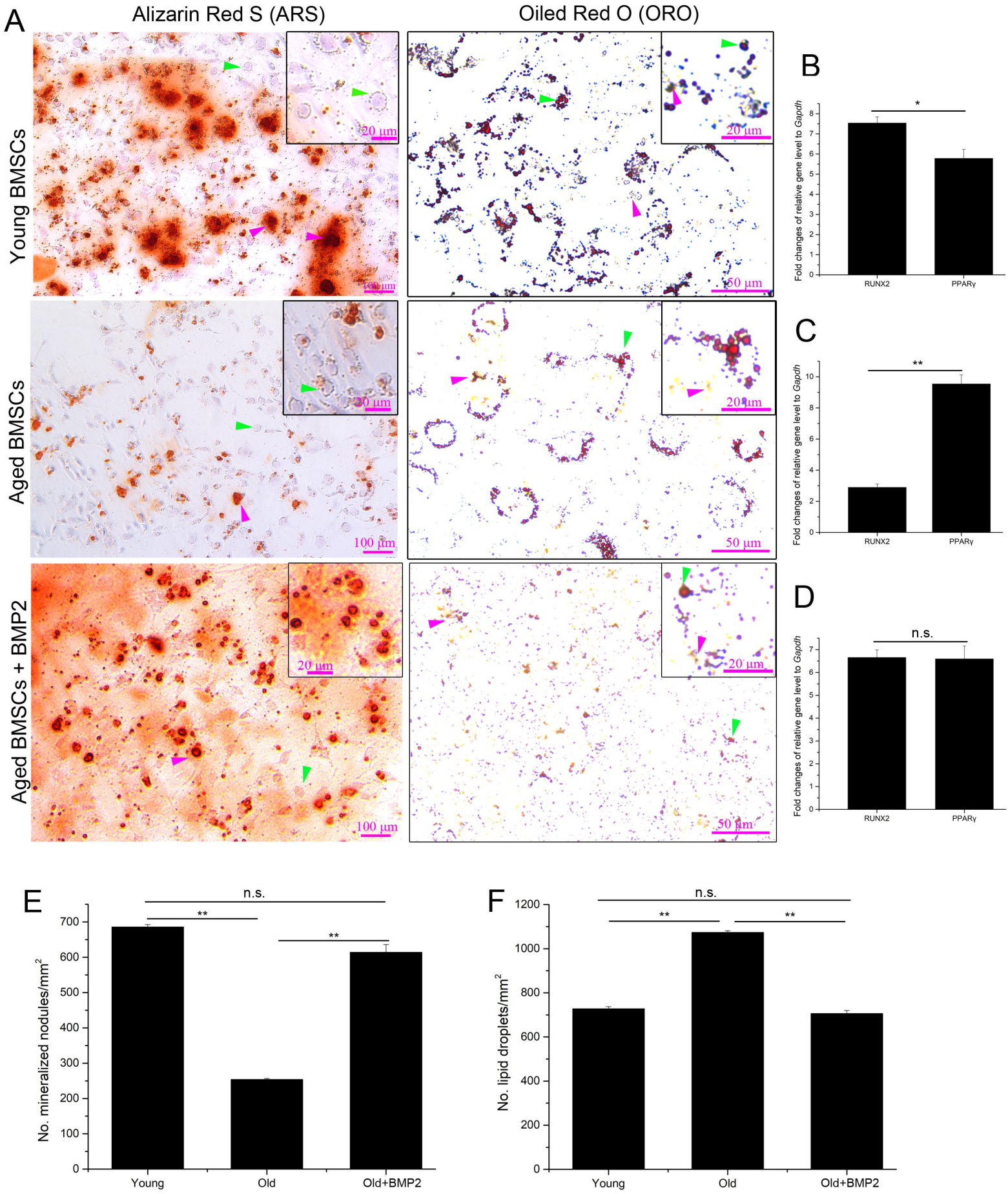
Fold changes of relative gene level to Gapdh
RUNX2
PPARγ


A
Alizarin Red S (ARS)
Oiled Red O (ORO)
Young BMSCs
Aged BMSCs
Aged BMSCs + BMP2
20 μm
100 μm
50 μm
B
C
D
Fold changes of relative gene level to Gapdh
RUNX2
PPARγ
*
**
n.s.
E
No. mineralized nodules/mm2
Young
Old
Old+BMP2
F
No. lipid droplets/mm2