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Osteogenic differentiation of adipose-derived stem cells promoted by quercetin

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Abstract

Objectives: The present study aimed to investigate overall effect of quercetin on proliferation and osteogenic differentiation of mouse adipose stem cells (mASCs) *in vitro*.

Materials and methods: Mouse adipose stem cells were isolated from subcutaneous fat pads and induced into the osteogenic lineage. Effects of quercetin on cell proliferation were assessed using MTT assay. Then they were treated by quercetin for 3, 7 and 11 days in a range of concentrations. Finally, effects of quercetin on osteogenic differentiation of mASCs were analysed by real-time PCR.

Results: Data of MTT assay showed that quercetin did not enhance mASC proliferation in a dose-dependent or time-dependent manner. Results of qPCR indicated that quercetin promoted expressions of Osx, Runx2, BMP-2, Col-1, OPN and OCN at the mRNA level in the presence of osteo-induction medium.

Conclusions: Our data demonstrated that quercetin was not active in terms of enhancing mASCs proliferation; however, it increased osteogenesis of mASCs by up-regulation of genes including *Osx*, *Runx2*, *BMP-2*, *Col-1*, *OPN* and *OCN*.

Introduction

Mesenchymal stem cells (MSCs) derived from adult organs and tissues have the capacity of self-renewal and differentiation into multi-lineage tissues (1). MSCs can be isolated from multiple organs and tissues including umbilical cord blood, bone marrow, muscle, adipose tissue and pulp of deciduous teeth (2–4). Accordingly, MSCs derived from adipose tissues are usually called adipose-derived stem cells (ASCs). Previous studies have demonstrated that ASCs could be a source of stem cells with easy isolation from fat tissue and relatively high frequency of clonogenic cells (5). As MSCs isolated from other sources, ASCs can also differentiate into cells of osteogenic (6) chondrogenic (7), myogenic (8), adipogenic (9) and hepatic (10) lineages. Thus, ASCs are considered to be a promising cell source for tissue engineering purposes.

Flavonoids are a large group of natural compounds, including catechin, quercetin, rutin and more. They are found in plants and can be consumed by humans as food stuffs. A kind of bioflavonoid, quercetin is present widely in extracts of Ginkgo biloba and edible plants (fruit, vegetables, tea, wine, amongst others) (11); it is the most widely studied product of its kind with potential pharmacological properties and beneficial health effects (12,13). It has been reported to have advantageous properties, such as being an antioxidant (14), anti-inflammatory and anti-carcinogenic (15,16). Quercetin (17) is claimed to be one of the most prominent antioxidants and able to inhibit activity of enzymes such as those that scavenge free radicals (18). It has been reported that quercetin inhibits both acute and chronic inflammation in rats (18) and exerts growth inhibitory activity on human cancer cells (19–23). Several recent studies (24–26) have indicated that quercetin might play a protective role against bone loss. Prouillet *et al.* (24) found that quercetin significantly increased alkaline phosphatase activity and stimulated MG-63-cultured human osteoblasts. Thus, flavonol derivatives may represent a new pharmacological tool for treatment of osteoporosis. A further study (25) has shown that addition of 0.25% rutin (a glycoside containing quercetin) to the diet provided for ovariectomized rats slows down bone resorption, and specially inhibits oestrogen deficiency-induced bone loss of the femoral trabecular by enhancing osteoblast activity. Meanwhile, other studies have also (26) demonstrated that quercetin could potentially inhibit *in vitro* bone resorption of osteoclasts and

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reduce bone resorption in a time- and dose-dependent manner. Up to now, however, no studies had been performed to investigate effects of quercetin on mouse ASCs (mASCs).

In the present study, we investigated overall effect of quercetin on osteogenesis of mASCs. Between quercetin pre-treated groups and controls, we compared expression of osterix (Osx), runt-related transcription factor 2 (Runx2), bone morphogenetic protein-2 (BMP-2), collagen-1 (Col-1), osteopontin (OPN) and osteocalcin (OCN) after osteogenic induction, and analysed effects of quercetin on cell proliferation.

Materials and methods

Isolation and culture of mASCs

Work with 4-week-old female Kunming mice used in this study was approved by the Sichuan University Experimental Animal Center, in accordance with the International Guiding Principles for Animal Research (1985). Subcutaneous fat pads were dissected from the groin and back of the mice and washed extensively in germ-free PBS to remove remaining debris. Tissues were then chopped and digested in 0.075% type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) in α-MEM medium without FBS, for an hour at 37 °C with agitation. Sequentially, cells were filtered and collected at 300 ***g*** for 8 min by centrifugation. Resulting pellets were suspended and washed three times in medium. Then, cells were seeded in tissue culture flasks with α-MEM medium plus 10% FBS and cultured at 37 °C in a humidified atmosphere with 5% CO_2. After 3–5 days, adherent cells were incubated in a monolayer while non-adherent cells were removed. mASCs were passaged three times before all assays.

Immunofluorescence staining for mASCs

To demonstrate phenotype of mASCs, the third passage cells were placed on glass coverslips for immunofluorescence staining. They were briefly washed three times in PBS, fixed in cold paraformaldehyde for 20 min at room temperature, then blocked in 0.5% bovine serum albumin for 20 min. Coverslips were incubated at room temperature in alexa fluor 488 anti-mouse CD44 (Biolegend, San Diego, CA, USA) for 0.5 h, CD90 (Biolegend) for 2 h, CD146 (Biolegend) for 1.5 h and α-SMA (Abcam, Cambridge, MA, USA) for 2 h, respectively. Sequentially, cells were incubated in secondary antibodies conjugated to FITC (Goat Anti-Mouse IgG; Zhongshanjinqiao, Beijing, China) for 0.5 h at room temperature. Nuclei were counterstained with DAPI (Molecular Probes, Eugene, OR, USA) for 5 min. After being rinsed in PBS, cells were observed and photomicrographs were taken using a DMi 6000-B fluorescence microscope (Leica, Wetzlar, Germany). Image analysis was performed using Image-Pro Plus 6.0.0.260 (Media Cybernetics, Inc, Rockville, MD, USA), and integral optical density was measured to evaluate CD44, CD90, CD146 and α-SMA concentration.

MTT testing

Effects of quercetin on cell proliferation were assessed using the MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. mASCs were seeded in 96-well plates at 1000 cells per well in growth medium for 24 h, and treated with quercetin at different concentrations (10, 50 and 100 μM) for 1–5 days, respectively. Cells cultured in control medium (CC group) and osteogenic medium (OM group) served as controls. Each plate was treated with a solution of 20 μl (1 mg/ml) MTT (Zhongshanjinqiao) and incubated for 4 h at 37 °C. After medium was removed, formed formazan crystals were dissolved in 150 μl dimethyl sulphoxide (DMSO). Control cultures were treated without test samples and with DMSO. Subsequently, optical density was read at test wavelength of 570 nm on the plate reader referring to wavelength 630 nm. Each experiment was repeated twice and mean value of the three experiments was considered as the result. Cell number was determined according to the standard curve.

Quercetin treatment and osteogenic induction

After passaging three times, mASCs were seeded into six-well plates at initial density 2×10^5 cells/ml, and cultured in basic medium for 2 days, to reach confluence. Once 60% confluence was reached, all wells were divided into five experimental groups: one negative control group (Control), one positive control (OM) and 3 quercetin-treated groups (10, 50 and 100 mM). At least three parallel wells were used in each group. Quercetin (Sigma) was dissolved in sterile PBS to obtain stock solution of 1 mM, which was then diluted with basic medium to desired concentrations. Cells cultured in basic medium alone were set up as negative control. Cells cultured in OM were used as positive control. In quercetin groups, mASCs were cultured in osteogenic media containing quercetin at different concentrations (10, 50 and 100 μM, respectively). Total RNA was extracted from all groups at days 3, 7 and 11. OM was made by dissolving dexamethasone (10 nM), ascorbate 2-phosphate (50 μM), β-glycerophosphate (10 mM) in α-MEM.

Extraction of total RNA, RT-PCR and real-time PCR

Expression of Osx, Runx2, Bmp-2, Col-1, OPN and OCN at transcriptional levels was investigated utilizing real-time PCR assay. Initially, we extracted total RNA using Simply P total Tissue/cell RNA extraction kit (BioFlux, Hangzhou, China). cDNA synthesis was performed using PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio, Tokyo, Japan) according to manufacturer's protocol. Agarose electrophoresis was applied to test total RNA and cDNA of each sample according to the method outlined in Molecular Cloning: A Laboratory Manual (2001, 3rd edition). RT-PCR kit (Tiangen, Beijing, China) was used to amplify cDNA samples; primers are listed in Table 1. Then, expression of certain genes was quantified in real-time PCR using ABI PRISM 7300 Sequence Detection system (Applied Biosystems, Foster City, CA, USA) with the program containing 95 °C, 10 min; 40 cycles of 95 °C, 15 s; 60 °C, 1 min. A melting curve was generated with each reaction to test primer dimmer formation/ false priming. Then relative expression of PCR products was verified by double standard curve analysis. Results of real-time PCR were normalized by GAPDH for comparison of target gene transcription levels in different samples.

Data statistical analysis

All experiments were repeated at least three times with similar results obtained. Differences within groups in all assays were tested by ANOVA and Dunnett's *t*-test was conducted to analyse effectiveness of different quercetin concentrations. Differences were considered statistically significant at $P < 0.05$. All statistical analyses were implemented with SPSS 19.0 software (SPSS Inc., Chicago IL, USA).

Results

mASC characterization

To characterize cells we isolated from fat tissue, we performed immunofluorescence staining to test expression of a number of charcteristic CD markers. As shown in Fig. 1, our cells were positive for α-SMA, CD44, CD146, and negative for CD90; this is a typical phenotype for ASCs.

MTT results

Effects of the range of concentrations of quercetin on ASC proliferation for 1–5 days are shown in Fig. 2. It was observed that cell number was significantly higher in the 100 μM group than in controls on day 1 ($P < 0.05$). Number of cells was significantly higher in OM group on day 1 compared to the 10 μM-treated group ($P < 0.05$). However, there was no significant difference between the groups on day 2. Quercetin (10 μM) significantly enhanced mASC proliferation after 3 days incubation compared to the other 4 groups (controls, OM group 50 and 100 μM group) ($P < 0.05$). In the 50 μM group on day 3, numbers were significantly higher compared to controls ($P < 0.05$). On day 4, cell numbers in quercetin-treated groups and control groups were not significantly different. On day 5, both 10 and 50 μM groups revealed a significant effect compared to the control group ($P < 0.05$). In the group treated with quercetin at 100 μM and OM, cell numbers were significantly higher than all other groups ($P < 0.05$). Overall, none of the quercetin-treated groups consistently exhibited any effect of enhancing mASC proliferation.

Table 1. Primer sequences of target genes and housekeeping genes for RT-PCR assay

	Sequence (5′→3′)	Length of products	GenBank number
GAPDH	F: GACGGCCGCATCTTCTTGTGC R: TGCAAATGGCAGCCCTGGTGA	192	NM_008084.2
Osx	F: GTCCTATGGCGGGGAGGACTGG R: TGGCAGCTGCAAGCTCTCTGTA	173	NM_130458.3
Runx2	F: CCGAACTGGTCCGCACCGAC R: CTTGAAGGCCACGGGCAGGG	199	NM_009820.4
BMP2	F: ACCCCCAGCAAGGACGTCGT R: TGGAAGCTGCGCACGGTGTT	168	NM_007553.2
Col-1	F: CCACCCCAGCCGCAAAGAGTC R: GTCATCGCACACAGCCGTGC	198	NM_007742.3
OCN	F: AGCAGCTTGGCCCAGACCTA R: TGAGGCTCCAAGGTAGCGCC	164	NM_001032298
OPN	F: GTGGTGATCTAGTGGTGCCAAGAGT R: AGGCACCGGCCATGTGGCTAT	157	NM_009263

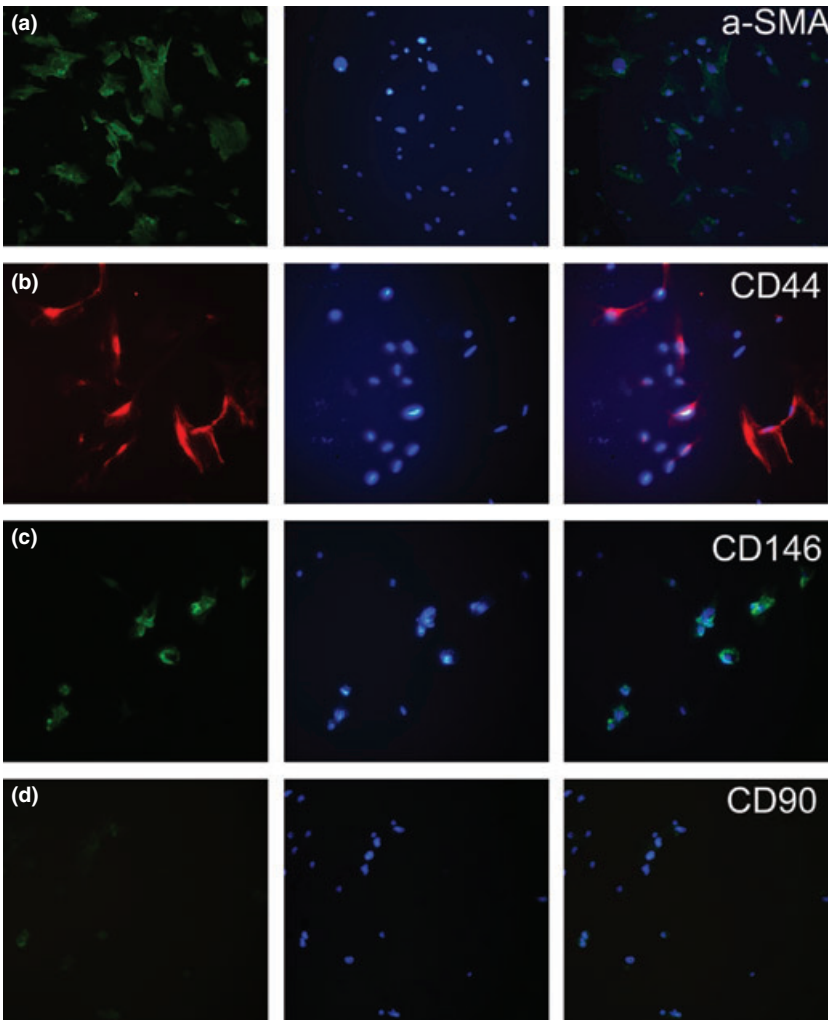

Figure 1. Immunofluorescence staining of CD markers on mouse adipose stem cells revealed expressions of α-SMA (a), CD44 (b), CD146 (c), and absence of CD90 (d).

Quercetin promoted Osx, Runx2, Bmp-2, Col, OPN and OCN transcription after osteogenic induction

After 3, 7 and 11 days, quercetin incubation plus osteogenic differentiation, Osx, Runx2, BMP2, Col I, OPN and OCN mRNAs were examined by real-time PCR (Fig. 3).

Osx transcription. mRNA levels of Osx in the three quercetin groups (100 μM, 50 μM and 10 μM) were significantly higher than that in control groups on day 3 ($P < 0.05$), and that in 100 μM quercetin groups was significantly higher than in OM groups ($P < 0.05$). However, mRNA level of Osx in 100 μM quercetin groups was significantly lower than the 10 μM and OM quercetin groups on day 7 ($P < 0.05$). Additionally, transcript levels of *OSX* gene coding in 50 μM groups were similar than the 100 μM quercetin in groups on the same day. On day 11, this was significantly higher in 100 μM quercetin groups than in the other three groups (10 μM, OM and controls) ($P < 0.05$). Also, it was found to be higher in the 50 μM group compared to the 10 μM group on day 11 ($P < 0.05$) (Fig. 3).

Runx2 transcription. Runx2 transcript levels in 50 μM quercetin groups were significantly higher than in the other four groups (50 μM, 10 μM, OM and controls) on day 3 ($P < 0.05$), and on day 11, 100 μM quercetin groups were significantly higher compared to the three quercetin-treated groups (10 μM, OM and controls) ($P < 0.05$). Additionally, transcript levels of the gene coding for Runx-2 in 10 μM quercetin-treated groups were significantly higher compared to controls on day 3 ($P < 0.05$). However, it was the opposite between the two groups on day 11 ($P < 0.05$). On day 7, there were no differences between the groups (Fig. 3).

BMP-2 transcription. On day 3, mRNA levels of Bmp-2 in the four groups (100 μM, 50 μM, 10 μM and OM)

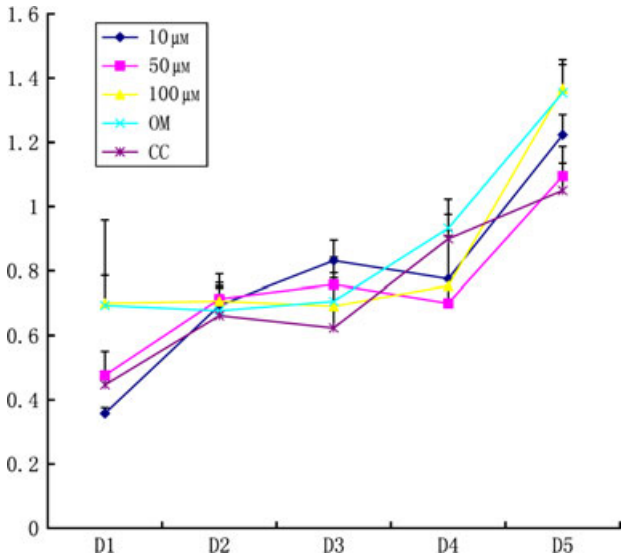

Figure 2. Effects of various concentrations of quercetin on proliferation of mouse adipose stem cells. 10, 50 and 100 μM are concentrations of quercetin in the media. OM, osteogenic medium; CC, control group cultured in basal medium.

were significantly higher compared to control groups ($P < 0.05$). It was also significantly higher in the 50 μM-treated groups compared to the other three groups (100 μM, 10 μM, OM) on day 3 ($P < 0.05$). Transcript levels of the three quercetin-treated groups (100 μM, 50 μM and 10 μM) on day 7 were significantly higher compared to OM and controls ($P < 0.05$). Moreover, in the 10 μM group, it was significantly higher than in 100 μM- and 50 μM-treated groups on day 11 ($P < 0.05$) (Fig. 3).

Col-I transcription. In the 50 μM quercetin groups, Col-I transcription was significantly higher compared to the other four groups (100 μM, 10 μM, 0 μM and controls) on day 3 ($P < 0.05$). Transcript levels of Col-I in both the OM groups and controls were significantly higher compared to all three groups (100 μM, 50 μM and 10 μM) on day 7 ($P < 0.05$). Additionally, in the 100 μM groups, it was significantly higher compared to 50 and 10 μM groups on day 7. There were no differences between groups on day 11 ($P < 0.05$) (Fig. 3).

OPN transcription. mRNA levels of OPN in 10 μM quercetin-treated groups suggested a reduction compared to the other groups (100 μM, 50 μM and OM) on day 3 ($P < 0.05$). At the same time, expression of *OPN* gene in 50 μM groups was significantly higher compared to OM groups on day 3 ($P < 0.05$). In addition, both in 100 μM and 50 μM groups, values on day 3 were significantly higher compared to controls ($P < 0.05$). However, it was significantly higher in OM groups than in the other three quercetin-treated ones (100 μM, 50 μM and 10 μM), and transcript levels of OPN of 100 μM groups and the controls were significantly higher compared to 10 μM groups on day 7 ($P < 0.05$). Additionally, 50 μM and 10 μM groups were significantly higher compared to 100 μM after 11 days incubation ($P < 0.05$). Moreover, results were similar to those of controls (Fig. 3).

OCN transcription. On day 3, there was no difference about the OCN transcription level among the groups. On day 7, expression of *OCN* gene in the three quercetin-treated groups (100 μM, 50 μM and 10 μM) was significantly lower compared to controls ($P < 0.05$). Both 100 μM and 10 μM groups were significantly lower than 50 μM and OM groups, respectively ($P < 0.05$), and it was significantly lower in 10 μM groups compared to 100 μM groups on day 7 ($P < 0.05$). However, in the 10 μM quercetin-treated groups, mRNA levels were significantly higher compared to the other three groups (50 μM, 0 μM and controls) on day 11 ($P < 0.05$) (Fig. 3).

Discussion

Adipose stem cells isolated with current protocols include a mixture of several cell types. Besides actual stem cells or progenitors, fibroblasts or other cells may also be propagated *in vitro*. It may be difficult to evaluate results of ASC studies due to lack of enrichment of stem cells or progenitors by cell markers. Recent studies have shown that a combination of markers is useful for identifying stem cells or progenitors in heterogenic cell populations. As reported, typical ASCs should express markers such as α-SMA, CD44 and CD146, but not CD90 (27–30). The cells we isolated from mouse fat pads exhibited the same phenotype as mentioned above.

MTT assay, as a tetrazolium reduction-based methodology for viable cells, has also been thoroughly used to assess mitochondrial dehydrogenase activity and measure cell viability (31). Data concerning influence of quercetin on mASC growth have not been demonstrated in previous studies. Accordingly, as indicated by our data, quercetin has no clear potential of any time-dependent effect on enhancment or reduction of mASC proliferation under the experimental conditions we tested. Results showed that our three concentrations were inactive on mASC proliferation and no optimal concentration was found in our experimental setup.

During the process of osteogenic differentiation of mesenchymal progenitor cells, a group of osteogenic genes is expressed in a characteristic pattern. In studies

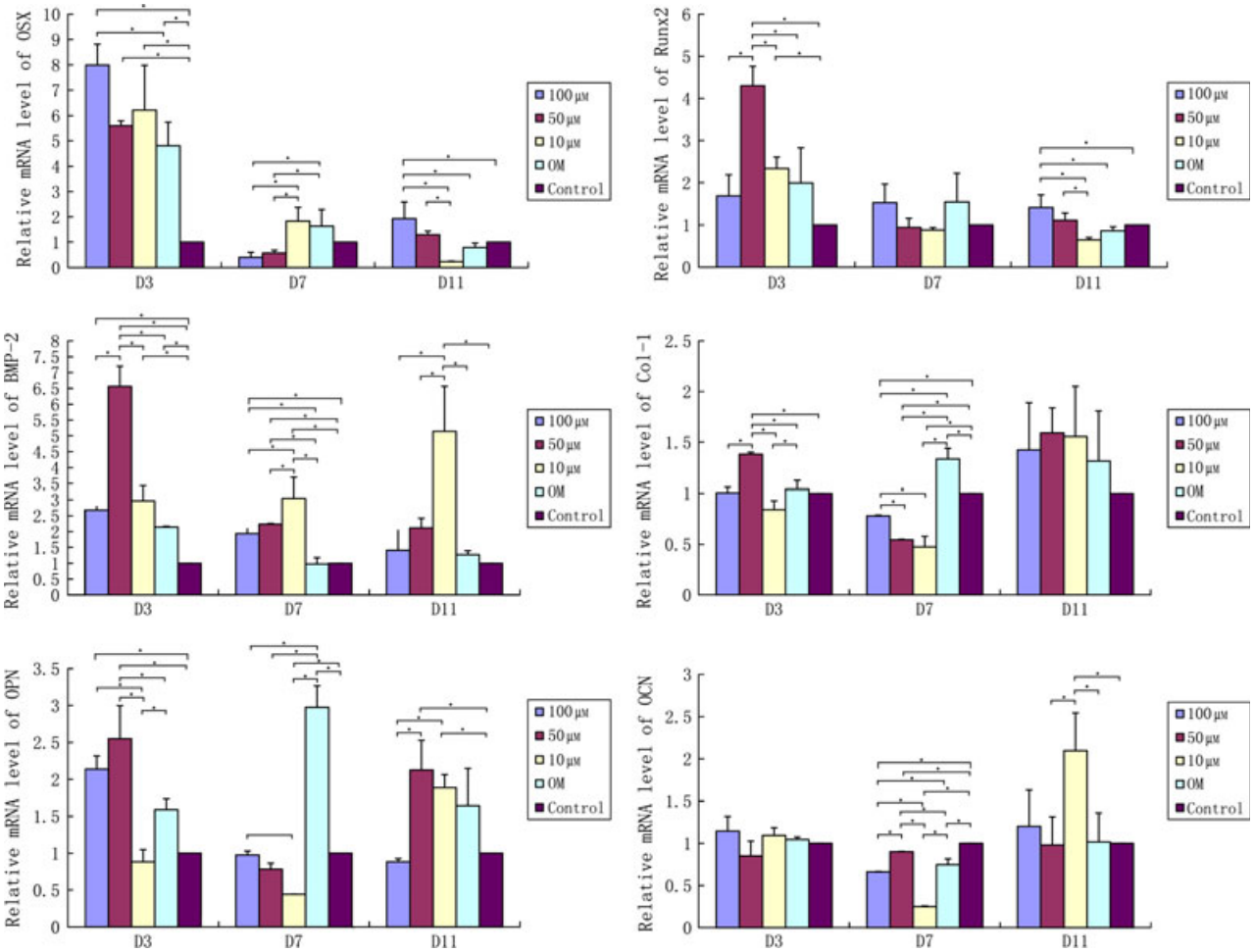

Figure 3. Quantitative RT-PCR analysis of Osx, Runx2, BMP-2, Col-I, OPN and OCN at different time points. Quercetin promoted Osx, Runx2, BMP-2, Col-I, OPN and OCN transcription in mouse adipose stem cells compared to controls after osteogenic induction. #*Represent significant differences between control and treated groups, respectively ($P < 0.05$ by ANOVA).

of testing osteogenic potential of stem cells under certain stimuli, these genes are usually evaluated to determine whether the stimuli are effective. In general, both *Osx* and *Runx2* are essential transcription factors for early stages of osteogenic differentiation from mesenchymal cells (32). BMP-2 is also considered as an early marker, while Col-I and OCN are late markers, concomitant with the process of mineralization. OPN appears twice, that is, during proliferation and later in stages of differentiation (33). Osx, located downstream of Runx2 (32), plays an important role in bone tissue formation (32) and is highly specific to osteoblasts (32). Runx2, is a master regulator of osteogenesis (34,35), is up-regulated early in osteoblast differentiation (36) and activates expression of many further downstream osteoblast genes, such as *OCN* and *Col1-a1* (37,38). As a key mediator of bone development, BMP-2 stimulates osteogenic differentiation and plays an important role in initiating and regulating bone formation and healing (39–44). Similarly, Col-I belongs to the main structural component of bone matrix and is expressed from initial stages of osteoblast differentiation. A further marker gene of bone differentiation is *OPN* which promotes remodelling of bone (45,46). As a non-collagenous matrix protein, OPN is important in stabilizing the matrix and has an active effect on reconstructing bone mineralization tissue (47,48). OCN is another non-collagenous protein, which is present at the late differentiation stage, possibly for regulating matrix mineralization (49).

Although several results were not significant, our outcome by PCR indicated an increased trend in expression of genes consistent with previous studies. In general, our data demonstrated that quercetin promoted transcription of mASC *Osx, Runx2, BMP-2, Col-1, OPN* and *OCN* after osteogenic induction. Inductive effects

were more prominent on early markers of osteogenesis such as BMP-2, Osx and Runx2 as summarized in Fig. 4.

Wattel *et al.* (26) showed that quercetin was potentially able to inhibit resorption of osteoclasts *in vitro* with reduced bone resorption in a time- and dose-dependent manner, at concentrations ranging from 0.1 to 100 μM. The study suggested that antioxidant properties partially explained the inhibitory effect on bone resorption. Moreover, it was indicated that 0.25% rutin (a glycoside containing quercetin) inhibited femoral trabecular bone loss induced by oestrogen deficiency to make bone resorption slower and increase osteoblast activity (25). Our research showed that 10 and 50 μM partially promoted RNA levels of the related mASC genes after osteogenic induction, which was in accord with previous results. However, effects of quercetin at certain concentrations were not identified. Accordingly, the results of this study have shown that there were no more quercetin-treated groups of different concentrations; this was due to design limitations here. Exact concentration of quercetin is very important for osteoblast differentiation of mASCs. Thus, in future research, the range of concentrations will be expanded based on this work. In addition, multiple parallel experiments need to be performed to optimize concentration of quercetin for enhancing osteogenic differentiation of mASCs. Prouillet *et al*. (24) found that quercetin significantly increased alkaline phosphatase activity and stimulated osteoblast activity of MG-63-cultured human osteoblasts. It has been suggested that flavonol derivatives may represent a new pharmacological tool for treatment of osteoporosis. Thus, the capacity of quercetin must be advanced to improve it for increasing osteogenic differentiation of mASCs; this could bring about a more fulfilled future to treat diseases such as osteoporosis.

In conclusion, our data have demonstrated that quercetin was not active on mASC proliferation, but generally enhanced their osteogenesis by up-regulating gene expression of osteoblast markers such as *Osx, Runx2, BMP-2, Col-1, OPN* and *OCN*.

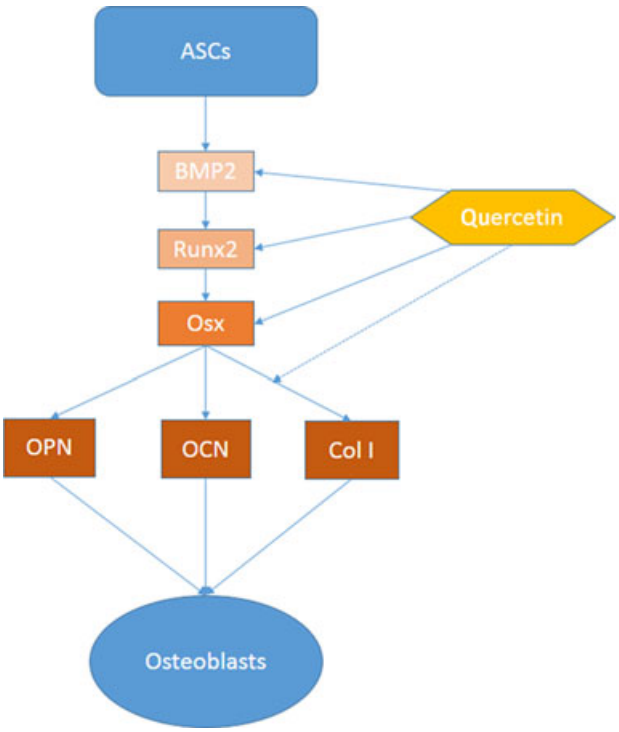

Figure 4. Schematics of hierarchy of gene expressions during differentiation from adipose stem cells to osteoblasts. Dominant effects of quercetin are indicated by solid arrows, while minor effects are indicated by dashed arrows.

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Competing interests: The authors have declared that no competing interests exist.

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