

# Antioxidative fullerol promotes osteogenesis of human adipose-derived stem cells

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**Abstract:** Antioxidants were implicated as potential reagents to enhance osteogenesis, and nano-fullerenes have been demonstrated to have a great antioxidative capacity by both in vitro and in vivo experiments. In this study, we assessed the impact of a polyhydroxylated fullerene, fullerol, on the osteogenic differentiation of human adipose-derived stem cells (ADSCs). Fullerol was not toxic against human ADSCs at concentrations up to 10  $\mu$ M. At a concentration of 1  $\mu$ M, fullerol reduced cellular reactive oxygen species after a 5-day incubation either in the presence or in the absence of osteogenic media. Pretreatment of fullerol for 7 days increased the osteogenic potential of human ADSCs. Furthermore, when incubated together with osteogenic medium, fullerol promoted osteogenic differentiation in a dose-dependent manner. Finally, fullerol proved to promote expression of FoxO1, a major functional isoform of forkhead box O transcription factors that defend against reactive oxygen species in bone. Although further clarification of related mechanisms is required, the findings may help further development of a novel approach for bone repair, using combined treatment of nano-fullerol with ADSCs.

**Keywords:** polyhydroxylated fullerene, bone repair, reactive oxygen species, forkhead box protein O1

## Introduction

Traumatic injuries and pathological conditions, such as osteoporosis, osteonecrosis, and bone tumors, can lead to bone fractures that do not heal through endogenous mechanisms. These difficult fractures in individuals are a serious threat to the health of the workforce and to the economy. In 2008, a survey showed that 110 million adults in the US reported disabling musculoskeletal conditions, and a World Health Organization report showed that 50% of the 9 million osteoporotic fractures that had occurred worldwide had happened in the Americas and Europe.<sup>1,2</sup> It is estimated that of the 7.9 million fractures sustained each year in the United States, 5% to 20% result in delayed or impaired healing.<sup>2</sup> Therefore, there is a need for effective, low cost, and innovative approaches of bone repair for musculoskeletal injuries and disease.

Application of stem cells represents one of the most valued advances in bone tissue engineering, which offers promise for bone repair.<sup>3</sup> Mesenchymal stem cells (MSCs) have the ability to differentiate along various lineages of mesenchymal origin, including chondrocyte, osteoblast, and adipocyte lineages, depending upon the biological environment. Moreover, MSCs can be obtained from multiple adult tissues, such as bone marrow, trabecular bone, articular cartilage, muscle, and adipose.<sup>4</sup> Retrieval of adipose tissue involves a minimally invasive procedure that can be easily performed in outpatient clinics, and the yield of adipose-derived stem cells (ADSCs) is about  $5 \times 10^3$  cells per gram of tissue, which is 100-fold higher than the number of stem cells from bone marrow.<sup>5</sup> Therefore, adipose tissue may be a better source of stem cells for potential clinical use. Although the efficacy of ADSCs to enhance bone repair

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has not yet been investigated in clinical trials, ADSCs have been reported to enhance bone healing in various animal models.<sup>6–11</sup>

While combined delivery of ADSCs and growth factors has been reported to enhance osteogenesis *in vivo*,<sup>9,10,12</sup> contradictory reports have been published that raise a serious concern about the efficacy of this approach. For example, ADSCs do not respond to various bone morphogenetic proteins (BMPs) *in vitro*.<sup>13,14</sup> In addition, a recent report demonstrated that delivery of human ADSCs and BMP-2 had no advantage over delivery of BMP-2 alone to enhance fracture healing in nude rats.<sup>15</sup> The obvious limitations of growth factors due to their short half-life and high production cost should be considered as well. Therefore, alternatives to growth factors are needed to enhance the osteogenesis of ADSCs.

Recently Yamada et al<sup>16</sup> demonstrated by *in vitro* and *in vivo* experiments that N-acetylcysteine, an antioxidative small molecule, could enhance the osteogenesis of rat bone marrow stromal cell and bone regeneration. We hypothesize that an antioxidant would favor osteogenic differentiation in ADSCs as well. Here, we investigated an extremely powerful antioxidant, polyhydroxylated fullerene (fullerol),<sup>17–19</sup> and report for the first time that antioxidative fullerol enhanced osteogenesis and reduced reactive oxygen species (ROS) in human ADSCs, as well as increased expression of FoxO1, a major isoform of forkhead box O transcription factors that defend against ROS and promote osteoblast differentiation in bone.<sup>20,21</sup>

## Materials and Methods

### Cell culture

Human ADSCs were purchased from a commercial source (Lonza, Basel, Switzerland) and were characterized for osteogenic assays in our previous paper.<sup>22</sup> The cells were maintained in basal medium that was Dulbecco's Modified Eagle's Medium (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA), 50 mg/mL ascorbic acid (AA), 100 IU/mL penicillin G, and 100 mg/mL streptomycin in a humidified atmosphere of 5% carbon dioxide at 37°C. To induce osteogenic differentiation, 10 mM  $\beta$ -glycerophosphate (GP) and 10<sup>-7</sup> M dexamethasone (DEX) were supplemented to basal medium, and the medium was designated as osteogenic medium. Fullerol (M.E.R. Co., Tucson, AZ, USA) was added when cells became 70%–80% confluent before (pretreatment) or at the time (cotreatment) of induction. All experiments were conducted with cells at passage 8 and a starting cell density of 5,000 cells/cm<sup>2</sup>.

### Lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays

Both LDH (Biovision Inc., Milpitas, CA, USA) and MTS (Promega Corporation, Fitchburg, WI, USA) colorimetric kits were used for cell toxicity and proliferative assays, respectively, by following the enclosed instructions in each product. For the LDH assay, cytotoxicity of 100% was set to the positive control where cells were treated by 1% Triton X-100, a detergent leading to a complete release of the LDH enzyme from the cells. In the MTS assay, cells produced a colorful substance, in which the optical density value reflecting the cell number was measured at 490 nm. Cells were treated with different doses of fullerol (0–10  $\mu$ M) for 6 hours and 48 hours, for LDH and MTS assays, respectively.

### ROS detection

The reagent CM-H<sub>2</sub>DCF-DA (5-[and-6]-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester) is a derivative of 2,7-dichlorodihydrofluorescein diacetate (DCF-DA), but with an additional thiol reactive chloromethyl group, which helps the transportation of the compound through cell membrane. The acetyl group is removed by intracellular esterase, producing DCF-DA, which can be retained within cells. The dye DCF-DA itself is nonfluorescent until cellular oxidation, at which point it becomes fluorescent. In the present experiments, ROS detection was performed by a commercial kit for DCF-DA assay (Invitrogen, Eugene, OR, USA), according to the manufacturer's manual. Briefly, after treatment, cells were rinsed with Hank's Balanced Salt Solution twice and stained with 20  $\mu$ M DCF-DA in Hank's Balanced Salt Solution for 30 minutes at 37°C, and the relative fluorescence units were read on a fluorescence plate reader with an excitation wavelength of 488 nm and an emission wavelength of 515 nm. The signal was then normalized by the deoxyribonucleic acid (DNA) content measured by a fluorescent dye ethidium homodimer-1 with an excitation wavelength of 528 nm and an emission wavelength of 617 nm.

### Alizarin red staining

Mineralization was quantified by alizarin red staining using a standardized procedure in our laboratory.<sup>22</sup> Briefly, the cells were stained with 40 mM alizarin red (pH =4.0) for 20 minutes, while the excess dye was washed away with water. After the picture of the whole plate was taken by a common digital camera, the dye bound to the deposited minerals in

each well was extracted by 10% (volume/volume [v/v]) acetic acid. The resultant solution was then neutralized by 10% (v/v)  $\text{NH}_4\text{OH}$  and transferred to a 96-well plate. The optical density at 405 nm was measured on a microplate reader.

## Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total ribonucleic acid (RNA) was isolated from cells using the RNeasy® Mini kit (Qiagen, Valencia, CA, USA), and complementary DNA (cDNA) was synthesized with the Reverse Transcription System kit (Promega, Madison, WI, USA) following the manufacturers' instructions. Real-time PCR was performed with the QuantiTect® SYBR Green PCR master mix (Qiagen, Valencia, CA, USA). The threshold cycle value was calculated from amplification plots. Data were analyzed using the  $2^{-\Delta\Delta\text{CT}}$  method with 18s ribosomal RNA (rRNA) serving as the reference.<sup>23</sup> Gene expression was normalized to the control group in each experiment and represented as fold of change. The target genes including *runx-related transcription factor 2 (Runx2)*, *osteocalcin (OCN)*, *alkaline phosphatase (ALP)*, *peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ )*, and *leptin (LEP)*, as well as their primer sequences and product sizes are shown in Table 1.

## Western blots

The cells (approximately 500,000) from one well of a 6-well plate were lysed with sodium dodecyl sulfate (SDS) sample buffer, without bromophenol blue and consisting of 125 mM Tris-HCl pH 6.8, 150 mM  $\beta$ -mercaptoethanol, 1% SDS, 20% glycerol, 1X protease inhibitor cocktail (Santa Cruz Biotechnology Inc., Dallas, TX, USA) and 1 mM phenylmethylsulfonyl fluoride (Santa Cruz Biotechnology Inc.). The lysates were homogenized by ultrasonic

homogenizer and centrifuged at 20,000 rpm for 20 minutes at 4°C to remove cell debris. Clear supernatants were transferred to a precooled fresh tube and immediately placed on ice. The protein concentration of the cell lysates was determined using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). One hundred  $\mu\text{g}$  of total proteins were run on 5%–12% SDS-polyacrylamide gels at constant current of 80 V and electro-transferred to nitrocellulose membranes (Thermo Scientific, Waltham, MA, USA) at constant voltage of 10 V overnight. Membranes were blocked with 5% bovine serum albumin in Tris buffered saline with Tween solution (50 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20) for 1 hour at room temperature, washed, and incubated overnight at 4°C in 5% bovine serum albumin in Tris buffered saline with Tween solution containing specific antibodies including anti-OCN (Santa Cruz Biotechnology Inc.), anti-Runx2 (Cell Signaling, Danvers, MA, USA), anti-superoxide dismutase (SOD) 2 (Santa Cruz Biotechnology Inc.), anti-FoxO1a (Abcam, Cambridge, MA, USA), anti- $\beta$ -actin (Sigma-Aldrich Co., St Louis, MO, USA) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling) for 1 hour at room temperature followed by chemiluminescent substrate for horseradish peroxidase antibody and enhancer solution (Thermo Scientific) mixed in a 1:1 ratio. The membranes were incubated in the dark with autoradiography films (Genesee Scientific, San Diego, CA, USA), and the films were developed to visualize the bands. Quantifying band density: The grayscale images (300–400 dpi) on films were scanned using a flatbed scanner. Densitometry was carried out in Photoshop (Adobe Systems, Inc., San Jose, CA, USA). Using the magic wand tool from the tool palette, the area of each band was selected, and the mean histogram was recorded and then charted. All Western blots were conducted in duplicates.

**Table 1** Target genes, primer sequences, and product sizes for quantitative real-time polymerase chain reaction

Molecule	Primer sequences	Size of product (bp)
<i>Runx-related transcription factor 2 (Runx2)</i>	5'-AGATGATGACACTGCCACCTCTG-3' (sense) 5'-GGGATGAAATGCTTGGAAGTGC-3' (antisense)	125
<i>Alkaline phosphatase (ALP)</i>	5'-ACCATTCCCACGTCTTCACATTTG-3' (sense) 5'-AGACATTCTCTCGTTACCGCC-3' (antisense)	162
<i>Osteocalcin (OCN)</i>	5'-CAGCGAGGTAGTGAAGAGAC-3' (sense) 5'-TGAAAGCCGATGTGGTCAG-3' (antisense)	144
<i>Peroxisome proliferator-activated receptor gamma (PPAR<math>\gamma</math>)</i>	5'-CCTGAAACTTCAAGAGTACCA-3' (sense) 5'-TCATGAGGCTTATTGTAGAGC-3' (antisense)	96
<i>Leptin (LEP)</i>	5'-GTGCGGATTCTTGTGGCTTT-3' (sense) 5'-GGAATGAAGTCCAAACCGGTG-3' (antisense)	174
<i>18s</i>	5'-GTGACCAGTTCACTCTTGGT-3' (antisense) 5'-GAATCGAACCTGATCCCCGTC-3' (antisense)	99

**Abbreviation:** bp, base pair.

## Data analysis

Data from the analyses of ROS, mineralization and gene expression were expressed as mean  $\pm$  standard deviation (SD). Statistical evaluation was performed with two-tailed Student's *t*-test using Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA) to determine the difference between two groups, with a *P*-value of 0.05 or less considered as a significant difference.

## Results

### Fullerol displays no toxicity against human ADSCs

Figure 1A displayed the results from the assay of cytotoxicity in human ADSCs, represented by LDH release from cells. Cytotoxic activities of control and fullerol groups of various doses (0.1, 0.3, 1, 3, and 10  $\mu$ M) for 6 hours were all less than 10%, and no significant difference was found between the control and any fullerol group. Figure 1B was obtained from the proliferative assay for cells treated with the same set of fullerol doses for 48 hours. Again, there were no significant changes in the cell number between the control and any fullerol group.

### Fullerol decreases intracellular ROS level during osteogenic induction of human ADSCs

Fullerol at two doses (low = 0.1  $\mu$ M and high = 1  $\mu$ M) was further tested for the ROS scavenging activity in human ADSCs during a 5-day culture. The results are shown in Figure 2. With basal medium, treatment of both high and low doses significantly reduced the intracellular ROS level. The ROS levels in all concentrations of osteogenic medium were significantly lower than that in the basal medium

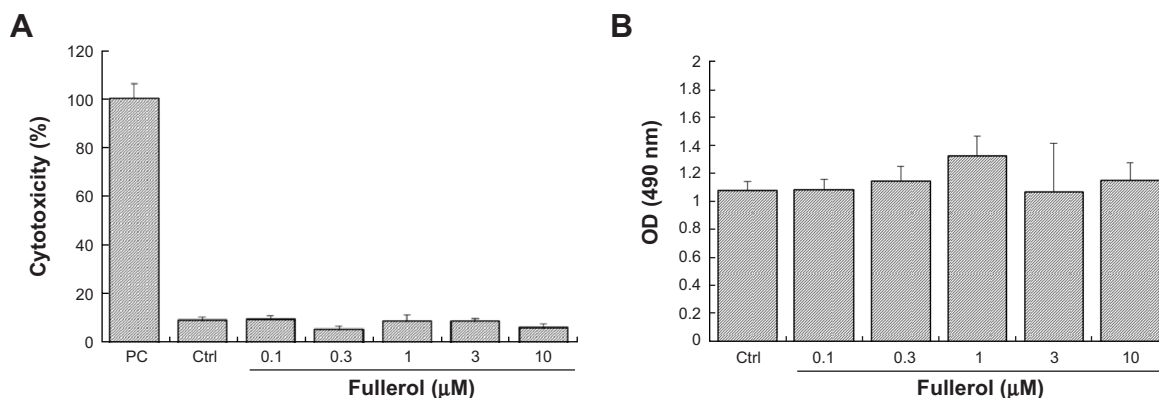
control group. Furthermore, in the presence of osteogenic medium, the high dose group, but not the low dose group, had an ROS level that was significantly lower than the control group.

### Pretreatment with fullerol increases osteogenic potential of human ADSCs

We then investigated whether fullerol pretreatment was able to enhance the osteogenic potential of human ADSCs while it reduced intracellular ROS level. Osteogenesis was assessed by analyses of both the mineralization and gene expression of osteogenic markers. Quantitative RT-PCR analysis showed that cells treated with an osteogenic medium lacking AA (ie, basal medium containing GP and DEX) and fullerol for 7 days had a significantly higher expression of *Runx2*, *OCN*, and *ALP* compared with that of cells treated with the AA-free osteogenic medium alone (Figure 3A). Importantly, after incubation in a complete osteogenic medium for a further 2 weeks, cells with fullerol pretreatment exhibited much more mineralization by alizarin red staining (Figure 3B).

### Fullerol promotes osteogenic differentiation of human ADSCs

The effects of fullerol on osteogenic differentiation of human ADSCs were determined independently as well as in combination with osteogenic medium. Treatment of fullerol at the high dose (1  $\mu$ M) for 13 days was determined via alizarin red staining to significantly enhance the mineralization (Figure 4A and 4B). Accordingly, expression of osteogenic markers was significantly elevated in the presence of high dose fullerol by quantitative RT-PCR analysis (*ALP* and *OCN*, Figure 4C) and Western blot analysis (*OCN*, Figure 4D). Interestingly,

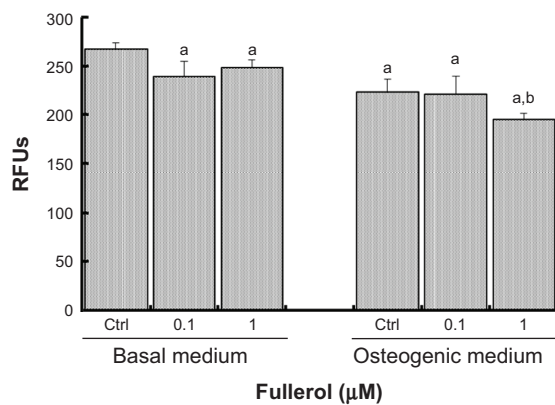


**Figure 1** Assays for cell survival and cell proliferation in human ADSCs treated with different doses of fullerol (10, 3, 1, 0.3, 0.1, and 0  $\mu$ M) (*n*=4).

**Notes:** (A) LDH assays with 6-hour treatment of fullerol. (B) MTS assays with 48-hour treatment of fullerol.

**Abbreviations:** ADSCs, adipose-derived stem cells; Ctrl, control group without any treatment; LDH, lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; OD, optical density; PC, positive group for LDH assay with treatment of cells by 1% Triton X-100 to release the highest intracellular LDH into culture medium.





**Figure 2** Fullerol reduced ROS production in human ADSCs cultured in basal medium or osteogenic medium in the presence or in the absence of fullerol (0.1 and 1  $\mu$ M) for 5 days (n=5).

**Notes:** Intracellular ROS were monitored by a fluorescent dye DCF-DA, and the RFUs were recorded on a fluorescence plate reader. Letter a denotes  $P < 0.05$  versus Ctrl in basal medium. Letter b denotes  $P < 0.05$  versus Ctrl in osteogenic medium.

**Abbreviations:** ADSCs, adipose-derived stem cells; Ctrl, control; DCF-DA, 2,2-dichlorodihydrofluorescein diacetate; RFUs, relative fluorescence units; ROS, reactive oxygen species.

fullerol simultaneously inhibited gene expression of adipogenic markers PPAR $\gamma$  and LEP (Figure 4C).

## Fullerol augments expression of FoxO1 gene expression in human ADSCs

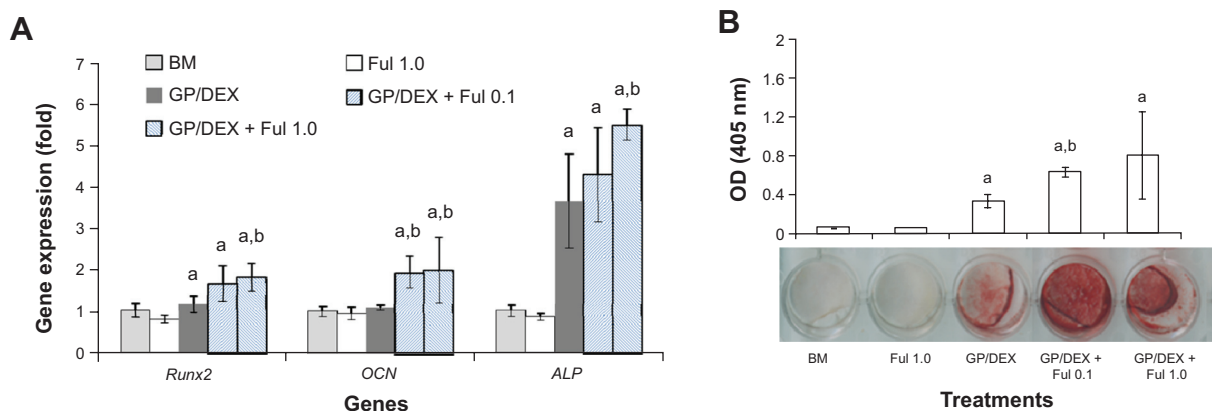
In an attempt to study the possible mechanistic pathway underlying promotion of osteogenesis by fullerol in human ADSCs, we investigated expression of an important transcription factor FoxO1 and its target genes *Runx2* and *SOD2* by Western blot analysis. FoxO1 level was relatively low in basal medium and robustly elevated after osteogenic induction. Fullerol (1  $\mu$ M) caused an additive increase in FoxO1 in the presence of osteogenic medium (Figure 5A). In line with its stimulation

of FoxO1 expression, fullerol also augmented expression of *Runx2* and *SOD2* (Figure 5B).

## Discussion

In the current study, we investigated the effects of antioxidative nano-fullerol on the osteogenic capacity of primary cultures of human ADSCs by conventional experimental techniques for cell biology and biochemistry, including cell survival and proliferative assays, intracellular ROS measurement, calcium deposit detection, real-time RT-PCR, and Western blotting. The outcomes are encouraging in the following areas: (1) fullerol was nontoxic toward human ADSCs at doses up to 10  $\mu$ M, (2) fullerol was an ROS scavenger for human ADSCs, (3) pretreatment or cotreatment of fullerol enhanced osteogenic capacity of human ADSCs in culture, and (4) fullerol stimulated gene expression of *FoxO1* and its target genes *Runx2* and *SOD2*.

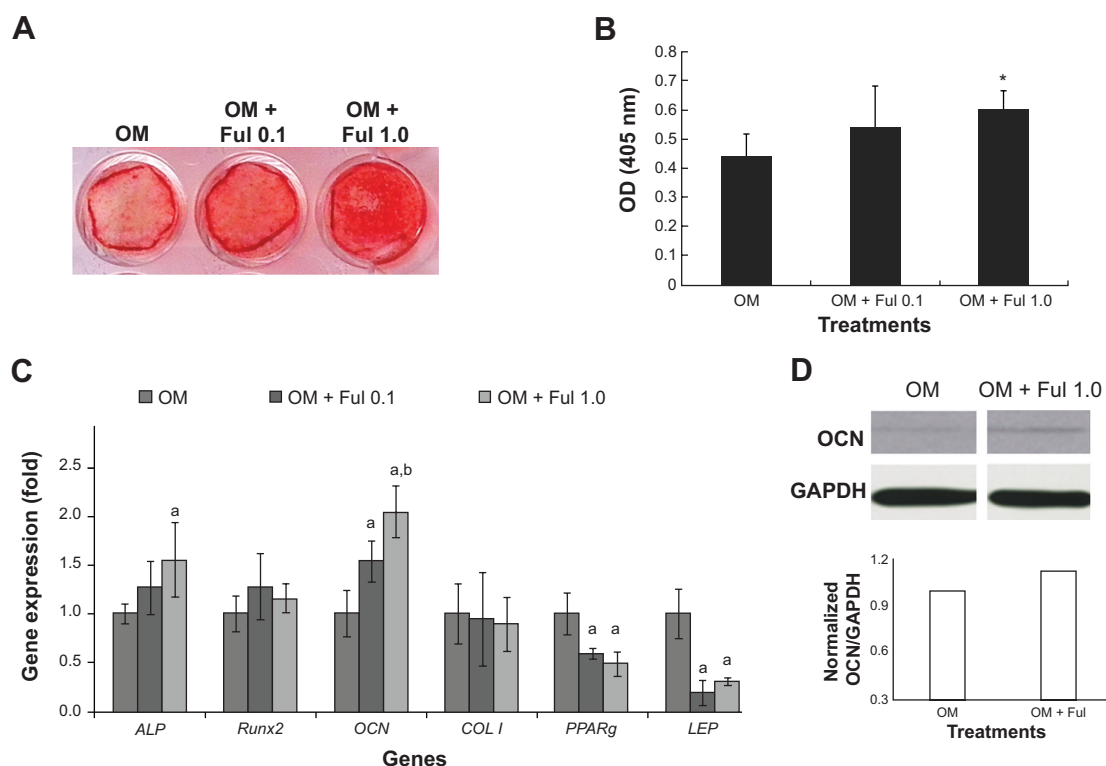
Fullerene has a unique structure, composed of 60 carbon atoms that form a hollow sphere about 1 nanometer in diameter.<sup>24</sup> Capable of being reversibly reduced by up to six electrons and with as many as 34 methyl radicals added to a  $C_{60}$  sphere, fullerenes (fullerene  $C_{60}$  and its derivatives) have been characterized as “radical sponges” and are reported to have antioxidant activity several hundred-fold higher than other antioxidants.<sup>25</sup> This was supported by our previous findings that fullerol was more powerful than a conventional antioxidant, glutathione, in inhibiting expression of adipogenic markers and stimulating expression of osteogenic markers in a mouse bone marrow stromal cell line, D1 cells.<sup>26</sup> A variety of different fullerenes have been studied for their biomedical applications, and they have taken on an important role in the research areas of both nanobiotechnology and nanomedicine.<sup>27</sup> Furthermore, due to its simple, straightforward preparation and its robust



**Figure 3** Fullerol pretreatment increased osteogenic potential of human ADSCs.

**Notes:** Cells were pretreated with fullerol (1.0  $\mu$ M or 0.1  $\mu$ M) for 7 days, followed by osteogenic induction for 14 days (n=4). (A) Gene expression of osteogenic markers, *Runx2*, *OCN*, and *ALP* by real-time RT-PCR at day 7, using 18s as internal control. (B) Alizarin red staining at day 21. Letters a and b denote  $P < 0.05$  versus BM and GP/DEX group, respectively.

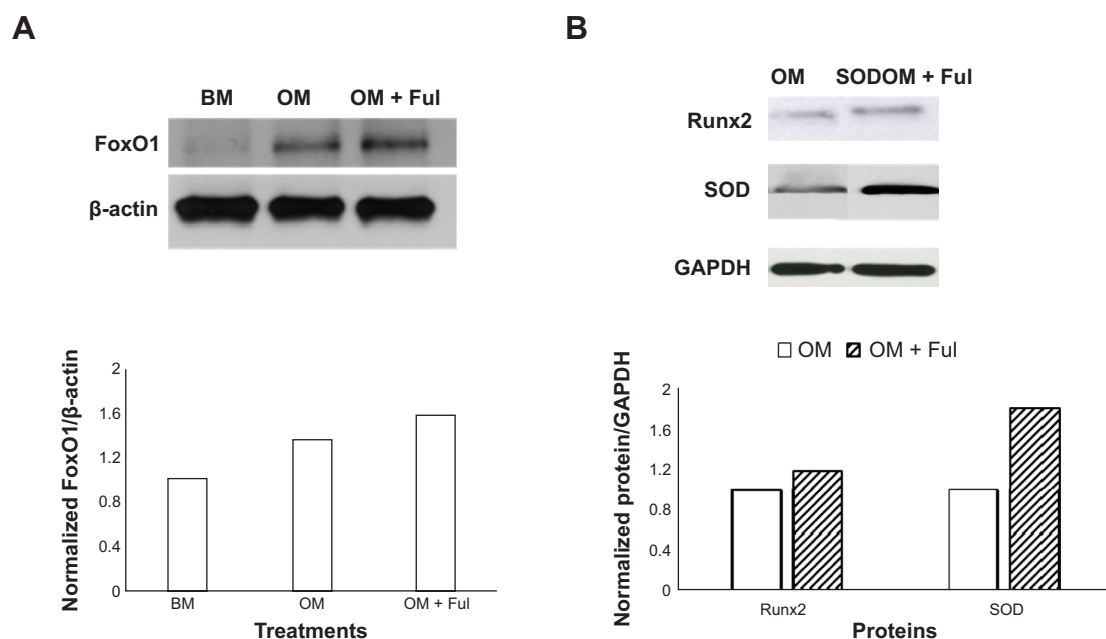
**Abbreviations:** ADSCs, adipose-derived stem cells; ALP, alkaline phosphatase; BM, basal medium; Ful, fullerol; GP/DEX, glycerophosphate/dexamethasone; OCN, osteocalcin; OD, optical density; *Runx2*, runt-related transcription factor 2.



**Figure 4** Osteogenic differentiation in human ADSCs treated with different doses of fullerol (0, 0.1, and 1.0  $\mu$ M) for 13 days ( $n=4$ ).

**Notes:** (A and B) Alizarin red staining showed that fullerol increased mineralization (A: images and B: quantification); (C) Quantitative real-time RT-PCR revealed that fullerol stimulated expression of osteogenic genes *ALP* and *OCN* and inhibited expression of adipogenic genes *PPARγ* and *LEP*. Letters a and b denote  $P < 0.05$  versus OM and OM + Ful 0.1 group, respectively. (D) Western blots demonstrated that fullerol elevated cellular *OCN* protein level.

**Abbreviations:** ADSCs, adipose-derived stem cells; *ALP*, alkaline phosphatase; BM, basal medium; *COL I*, type I collagen; Ful, fullerol; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; GP/DEX, glycerophosphate/dexamethasone; *LEP*, leptin; *OCN*, osteocalcin; OD, optical density; OM, osteogenic medium; *PPARγ*, peroxisome proliferator-activated receptor gamma; RT-PCR, reverse transcription-polymerase chain reaction; *Runx2*, runt-related transcription factor 2.



**Figure 5** Fullerol promoted gene expression of FoxO1.

**Notes:** This transcription factor's targets, *Runx2* and *SOD2*, were analyzed by Western blots. Human ADSCs were treated with OM alone or together with 1  $\mu$ M fullerol (OM + Ful) for 7 (A) or 13 (B) days.

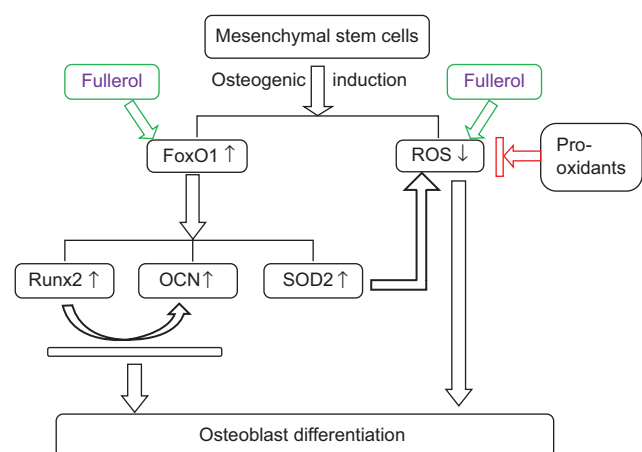
**Abbreviations:** ADSCs, adipose-derived stem cells; BM, basal medium; FoxO1, forkhead box protein O1; Ful, fullerol; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase; OD, optical density; OM, osteogenic medium; *Runx2*, runt-related transcription factor 2; SOD: superoxide dismutase.

water solubility,<sup>19,28–30</sup> fullerol has been considered as a candidate with priority for the biomedical purpose of fullerenes.<sup>31</sup> Our present data showed that fullerol served as an antioxidant in human ADSCs without cytotoxicity at doses up to 10  $\mu$ M (Figures 1 and 2), and this is in agreement with a majority of previous reports with regards to other tissues and cell types.<sup>17–19,28,32–39</sup>

It has been shown that ROS inhibit osteogenesis and bone formation. Mody et al<sup>40</sup> reported that three oxidants, hydrogen peroxide ( $H_2O_2$ ), xanthine, and xanthine oxidase, and minimally oxidized low-density lipoproteins inhibited osteoblastic differentiation of MC3T3-E1 mouse bone preosteoblast cell line and M2-10B4 mouse marrow stromal cell line, while increasing intracellular oxidative stress. Chen et al<sup>41</sup> observed a dramatic decrease of ROS as a consequence of upregulation of SOD2 and catalase upon osteogenic induction of human bone marrow-derived mesenchymal stem cells (BM-MSCs), while exogenous  $H_2O_2$  retarded this osteogenic differentiation. Barbagallo et al<sup>42</sup> revealed that increased expression of a major redox regulating gene, heme oxygenase-1, augmented osteogenesis in human BM-MSCs. Yamada et al<sup>16</sup> showed that antioxidant N-acetylcysteine was beneficial to osteogenesis of BM-MSCs and bone regeneration in rats. Particularly, Reid et al<sup>43</sup> demonstrated that in human ADSCs, ROS were upregulated and downregulated during osteogenesis and adipogenesis, respectively. Therefore, we questioned if an antioxidant would be beneficial to osteogenic differentiation of human ADSCs. In the present work, we confirmed that osteogenic medium induced downregulation of ROS, and fullerol had an additive effect (Figure 2). Furthermore, either pretreatment or cotreatment of antioxidative fullerol promoted osteogenesis of human ADSCs in the presence of osteogenic induction (Figures 3 and 4). It is also demonstrated by an ongoing research project in our laboratory that fullerol has a similar impact on osteogenesis of mesenchymal stem cells from bone marrow. On the other hand, osteogenesis and adipogenesis are regarded to be closely correlated with the capacity of the progenitor cells during bone regeneration, and many reagents have been demonstrated to have opposite impacts on osteogenesis and adipogenesis.<sup>44–48</sup> In this regard, we and others have recently reported that fullerol could inhibit the differentiation of marrow stromal cells and OP9 preadipocytes into adipocytes due to its scavenging activity against intracellular ROS.<sup>26,49,50</sup> Taken together, these findings highlighted fullerol as a novel pharmaceutical candidate for the purpose of producing augmented bone regeneration. To this end, it is necessary to perform in vivo studies using fullerol-treated

ADSCs (for example, subcutaneous implantation) for bone regeneration.

FoxO1 belongs to the winged helix/forkhead family of transcription factors that have been shown to regulate expression of many genes involved in many cell processes, including cell proliferation, survival, differentiation, and redox balance.<sup>51</sup> In particular, several recent studies revealed that FoxO1 is able to promote osteoblast differentiation of the progenitor cells.<sup>20,21,52–54</sup> However, its role in regulating osteogenic differentiation of ADSCs has not been reported yet. The data obtained from the present Western blot analysis showed that in human ADSCs, expression of FoxO1 was upregulated after addition of the osteogenic medium, and more importantly, cotreatment of fullerol with the osteogenic medium further augmented expression of this transcript factor and its significant targets *Runx2*, *OCN*, and *SOD2* (Figure 5). Teixeira et al<sup>21</sup> performed osteogenic induction using BMP-2 in mouse mesenchymal cells C3H10T1/2 and revealed that FoxO1 expression increased along with the osteogenic markers *Runx2* and *ALP* during osteogenic differentiation. This is consistent with our present data (Figure 5) with regard to osteogenesis of human ADSCs in the presence of an induction medium containing AA, DEX, and GP, although Yang et al<sup>55</sup> reported a negative role of FoxO1 in regulation of *Runx2* and osteocalcin expression. Based on this preliminary data, we proposed a possible mechanism by which fullerol may exert its modulatory effect on osteoblast differentiation of mesenchymal stem cells through increasing FoxO1 and decreasing ROS (Figure 6). Additionally, SOD2



**Figure 6** Schematic illustration of putative pathways for the enhancement of osteoblast differentiation of mesenchymal stem cells by fullerol.

**Notes:** Fullerol increases osteogenic markers *Runx2* and *OCN*, and an antioxidative enzyme *SOD2* through *FoxO1*. Meanwhile, fullerol supports osteoblast differentiation by the means of reducing the intracellular ROS level both directly and indirectly.

**Abbreviations:** ↑, increase; ↓ decrease; *OCN*, osteocalcin; *ROS*, reactive oxygen species; *Runx2*, runt-related transcription factor 2; *SOD2*, superoxide dismutase 2.

is hypothesized to play a role in diminishing ROS by fullerol, adding a new idea to the current ones on how fullerol exhibits its antioxidative activity within cells.<sup>56,57</sup> Emphasis should be placed on the fact that the proposed mechanism is currently at its early stage and needs to be further verified by more detailed experimental evidence.

## Conclusion

Our findings provided preliminary evidence that nano-fullerols were able to enhance the osteogenesis of human ADSCs. This finding might offer potential opportunity for the use of nano-fullerol as alternatives to growth factors, serving as positive regulators of human ADSCs for the tissue engineering-based repair of bone fractures and defects.

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## Disclosure

All authors have no conflict of interest to declare.

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