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Original Article

Silver nanoparticles promote osteogenic differentiation of mouse embryonic fibroblasts *in vitro*

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Abstract: Objective: This study investigated if silver nanoparticles (AgNps) could promote the proliferation and osteogenic differentiation of mouse embryonic fibroblasts. Methods: Mouse embryonic fibroblasts were divided into two groups: Group 1 cells were cultured in DMEM/F12 medium and Group 2 cells were cultured in osteogenic medium. Both groups were then treated with 16, 32, or 100 μM AgNps. Fibroblast proliferation and viability were measured using BrdU and MTT methods at varying time points. Alizarin red staining and alkaline phosphatase (ALP) activity were measured to observe fibroblast differentiation into osteoblasts. Proteomics (cytokine array) was used to detect 111 different cytokines during differentiation. Results: AgNps stimulated proliferation of mouse embryonic fibroblasts at a concentration of 16 μM. Marked enhancement of calcium mineralization was observed in cells cultured with AgNps compared with cells cultured without AgNps. Group 2 cells displayed nodules around the center where the cell density was high. ALP activity of mouse embryonic fibroblasts cultured in osteogenic medium increased during the whole culture period. Addition of AgNps at concentrations of 32 μM and 100 μM induced higher ALP activity at days 7 and 14. Proteomic array results show that low density lipoprotein receptor (LDL-R) and proprotein convertase subtilisin/kexin type 9 (PCSK-9) were significantly increased, while osteoprotegerin (OPG) was significantly reduced in medium containing 16 μM AgNPs. Conclusion: AgNps could promote differentiation of mouse embryonic fibroblasts into osteoblastic cells. LDL-R and PCSK-9, as well as OPG, may play a critical role in this process.

Keywords: Silver nanoparticles, mouse embryonic fibroblast, osteogenic differentiation

Introduction

Along with the development of nanoscience, many nanotechniques and nanomaterials have been applied in biomedical research and clinical practice. In this respect, our research has focused for many years on the application of silver nanoparticles (AgNps) in wound healing [1-8]. In our previous experiments, we demonstrated that AgNps could suppress fibroblast proliferation at the wound site and subsequently drive myofibroblast differentiation [1]. Furthermore, our group found that AgNps could promote osteogenic differentiation of mesenchymal stem cells and improve bone fracture healing [6]. Notably, mesenchymal stem cells and mouse embryonic fibroblasts share common characteristics including plastic adherence, similar morphological surface markers, and multipotency [9]. Collectively, these results laid a foundation for the possibility of fibroblasts differentiating into osteoblasts. From the view of developmental biology, mesenchymal stem cells and fibroblasts both develop from the mesoderm, therefore it is possible that AgNps also promote the proliferation and osteogenic differentiation of fibroblasts.

BALB/3T3 is a mouse embryonic fibroblast cell line used only for commercial and research purposes. Because embryonic fibroblasts behave much like wound fibroblasts, we chose this type

of fibroblast for the current study. Chitosan hydrogels loaded with two growth factors (platelet-derived growth factor BB and bone morphogenetic protein 2) could reportedly induce osteogenic differentiation of 3T3 fibroblast cells [10]. In addition, bone morphogenetic protein 9 and hypoxia-inducible factor 1*α* were shown to be critical regulators of osteogenic and angiogenic differentiation of mouse embryonic fibroblasts [11]. However, no evidence of metal nanoparticles promoting osteogenic differentiation of fibroblasts has been reported. Based on this background and the recent concept of direct cell reprogramming, we hypothesized that AgNPs could promote fibroblast transdifferentiation into osteoblasts in this study. Our findings provide a glimpse of a new approach for wound healing management in the future.

Materials and methods

Preparation and synthesis of AgNps

AgNps were synthesized by borohydride reduction of $AgNO_3$ in the presence of citrate as a stabilizing agent, as previously described [7]. Briefly, $NaBH_4$ (50 mg) was added to a vigorously stirred solution containing $AgNO_3$ (16 mg in 1 L) and sodium citrate (0.7 mM) at room temperature. The reaction was allowed to proceed for 4 h. A golden yellow solution was formed, showing an absorption peak at 400 nm. The solution was further concentrated to 100 mL by rotary evaporator. Poly (vinylpyrrolidone) (M.W. 10,000) was added to a final concentration of 1 mM to stabilize AgNp. The AgNps prepared are spherical with an average diameter of 9 nm, as examined by transmission electron microscopy.

Mouse embryonic fibroblast culture

BALB/3T3 clone A31 (ATCC®CCL-163™), a mouse embryonic fibroblast cell line, was purchased from American Type Culture Collection (USA). Fibroblasts were expanded in Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12) (Gibco) with 10% fetal bovine serum (Gibco) at 37°C and 5% CO_2 in a humidified atmosphere. The culture medium was changed every 3-4 days, and cells were passaged to new flask upon reaching 90% confluence. Fibroblasts between passages 12-14 were used for this study.

Fibroblasts were divided into two groups: Group 1 cells were cultured in DMEM/F12 medium only and Group 2 cells were cultured in osteogenic medium (Stempro Osteogenesis Differentiation Kit, Gibco) to induce osteoblastic differentiation [12]. Both groups of cells were seeded on culture plates. AgNps at varying concentrations (16, 32, and 100 μM) were subsequently added to the wells of both groups. In each well, 10 random visual fields (100 ×) were chosen to calculate the outgrowth cell number.

Fibroblast proliferation assay

Fibroblasts (1.2×10^4 cells/mL) were cultured with different concentration of AgNps in 96-well plates. Cell proliferation was measured at 24 h and 48 h using a 5-bromo-2'-deoxyuridine (BrdU) cell proliferation assay kit (Cell Signaling Technology, USA) according to the manufacturer's protocol. Optical density (OD) values of samples were tested at 450 nm using a microplate reader (Multiskan FC, Type: 357; Thermo Fisher Scientific).

Fibroblast viability assay

Fibroblast proliferation and viability were measured with a 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide (MTT) cell proliferation assay kit (Trevigen, USA), according to the manufacturer's instructions, with an initial cell density of 1.2×10^4 cells/mL on 96-well plates.

Fibroblast differentiation and staining

Mineralization of cells, indicating the formation of bone, was measured by Alizarin red staining (ARS). Both groups of fibroblasts were seeded in 48-well plates at a density of 2.4×10^4 cells/well and cultured with or without AgNps for 21 days. On days 7, 14, and 21, cells were fixed with 4% paraformaldehyde for 10 min at room temperature and washed with phosphate-buffered saline (PBS) twice. Next, cells were stained with 2% ARS solution (Sigma-Aldrich, USA) (pH 4.1-4.3) for 20 min and washed with ddH_2O. Finally, cells were observed under light microscope. In addition, supernatants were moved to 96-well plates and 200 μL of 10% cetylpyridinium chloride was added. OD values were recorded at wavelength of 570 nm.

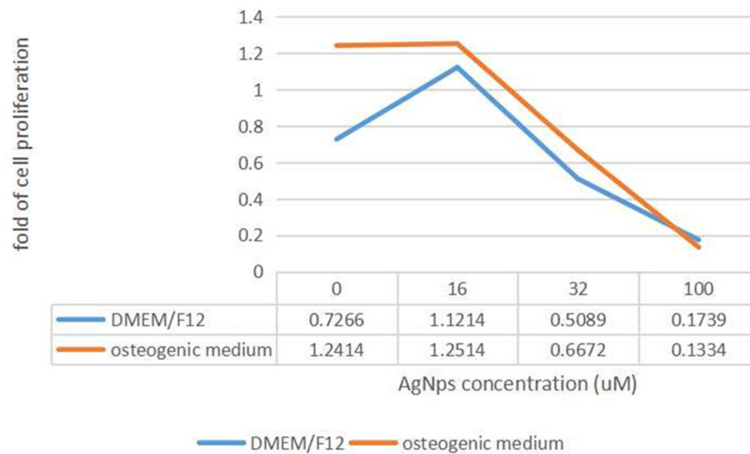

Figure 1. Both DMEM/F12 and osteogenic medium with AgNps at the concentration of 16 μM induced an enhancement of BrdU incorporation relative to those of other concentrations at the 24 h culture.

Statistical analyses

All data were analyzed using SPSS 22.0 (IBM, USA). Data with continuous variables are represented as the mean ± standard deviation (SD). Statistical analyses were performed using independent two-tailed Student's t-test. Values of $P < 0.05$ were considered statistically significant.

Results

Silver nanoparticles increase mouse embryonic fibroblast proliferation

To examine whether AgNps affected the proliferation of mouse embryonic fibroblasts, cells were cultured at a density of 1.2×10^4 cells/mL with varying concentrations of AgNps for 24 h. Cells were labeled with BrdU to quantify cells in the S phase of the cell cycle. As shown in **Figure 1**, 16 μM AgNps enhanced BrdU incorporation in both DMEM/F12 and osteogenic medium groups. In contrast, AgNps at higher concentrations of 32 μM and 100 μM had no effect on BrdU incorporation of fibroblasts. Taken together, these results suggest that AgNps stimulate mouse embryonic fibroblast proliferation only within a specific range of concentrations.

Effect of silver nanoparticles on mouse embryonic fibroblast viability

An MTT assay was performed to test the effect of AgNps on mouse embryonic fibroblast viability. Increased cell viability was observed with 100 μM AgNps in both DMEM/F12 and osteogenic medium at 24 h and 48 h. However, we did not observe any significant change in cell viability with concentrations of 16 μM or 32 μM AgNps (**Figure 2**).

AgNps promote osteoblast differentiation of mouse embryonic fibroblasts

We next investigated if AgNps influence differentiation of mouse embryonic fibroblasts into osteoblasts. ARS of calcium depositions on day 14 show marked enhancement in culture with

Alkaline phosphatase (ALP) activity

ALP is a homodimeric protein enzyme conducive to bone formation. In the present study, ALP activity of cell culture supernatants (initial fibroblast seeding density of 1.4×10^4 cells/mL in 24-well plates) was measured using a Mouse Bone Alkaline Phosphatase ELISA Kit (BlueGene, China) on days 7, 14, 21, according to the manufacturer's instructions.

Proteomic array

A proteomic array was performed using a Proteome Profiler Mouse XL Cytokine Array Kit (Bio-techne, USA) on day 14 fibroblasts (seeding density of 1.2×10^4 cells/mL) cultured in medium with or without AgNps. The antibody array included 111 different capture antibodies for the parallel determination of relative levels of selected soluble mouse cytokines. Four-hundred microliters of culture supernatants were added onto the array membrane and incubated overnight at 4°C on a rocking platform shaker (Mini biotRocker 3D, Taiwan). Subsequently, 2 mL of IRDye 800CW Streptavidin (1:2000 dilution; LI-COR, USA) was added to each well and incubated for 30 min at room temperature on the rocking platform shaker. Next, membranes were scanned and images were collected with an Odyssey Infrared Imaging System CLX-0983 (LI-COR Biosciences). Analysis was conducted with LI-COR Image Studio™ Lite software.

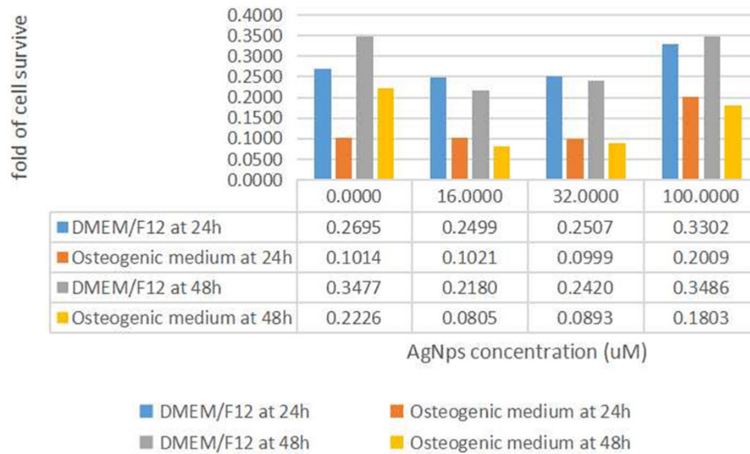

Figure 2. The results showed that increase in cell viability was observed at AgNps concentration of 100 μM either in DMEM/F12 or in osteogenic medium at the time point of both 24 h and 48 h. However, the AgNps concentration of 16 μM and 32 μM did not change the cell viability significantly in different medium at various time points.

16 μM and 100 μM AgNps (**Figure 3**). However, the morphology of fibroblasts cultured in osteogenic medium with 32 μM AgNps was different from all other conditions on days 7 and 14. The osteogenic group displayed nodules around the center where the cell density is high. Furthermore, the OD values of cell culture supernatants showed similar results (**Figure 4**).

To further confirm the osteoblastic induction effect of AgNps on mouse embryonic fibroblasts, ALP enzyme activity of cells cultured with or without AgNps was assayed (n = 3). When mouse embryonic fibroblasts were cultured in osteogenic medium, ALP activity increased. However, the addition of AgNps at concentrations of 32 μM and 100 μM induced even higher ALP activity at day 7 and day 14 (**Figure 5**). In contrast, no significant increase of ALP activity was observed in mouse embryonic fibroblasts cultured in DMEM/F12. Taken together, these results indicate that AgNps could further promote the differentiation of mouse embryonic fibroblasts towards an osteoblast lineage in the presence of osteogenic medium.

Key cytokines and chemokines promoted by AgNps during osteogenic differentiation

Certain marker morphogenic proteins and transcription factors may be involved in the effects observed when fibroblasts are exposed to AgNps. To identify these target proteins, a proteomic array was performed. The relative expression levels of 111 soluble mouse proteins were detected among samples with a proteome array. The results show that compared with the other groups, low density lipoprotein receptor (LDL-R) and proprotein convertase subtilisin/kexin type 9 (PCSK-9) were significantly increased, and osteoprotegerin (OPG) was reduced in osteogenic medium after treatment with 16 μM AgNPs. In addition, insulin-like growth factor binding protein (IGFBP)-2 and IGFBP-6 were increased, although these levels did not reach statistical significance (**Figure 6**).

Discussion

The ability of fibroblasts to be induced into other cell types *in vitro*, including myofibroblasts, osteocytes, chondrocytes, neurons, vascular endothelial cells, cardiomyocytes, hepatocytes, brown adipocytes, and pancreatic beta-cells, has been established for more than 15 years [13-20]. Furthermore, fibroblast differentiation into bone cells has been recognized as an effective method for bone formation [21]. In this study, we show that AgNps could promote the differentiation of mouse embryonic fibroblasts into osteoblasts *in vitro*. Using BrdU and MTT to assess the proliferation and viability of fibroblasts, we observed that the effect of AgNps on proliferation was concentration-dependent. Moreover, only the highest concentration (100 μM) of AgNps increased the viability of fibroblasts. This result is consistent with the conclusions of other studies that AgNps exert cytotoxic effects on cells at high concentrations (above 5 mg/mL, equivalent to 46 μM) but induce cell activation (i.e., proliferation, cytokines release, and chemotaxis) at non-toxic concentrations (2.5 mg/mL, equivalent to 23 μM) [6, 22]. Nonetheless, the mechanism underlying the effects of AgNps on fibroblasts is still not fully understood.

To evaluate the osteogenic differentiation of fibroblasts, the ALP activity of cells was assessed. In addition, samples were stained with ARS to evaluate mineralization levels. ALP,

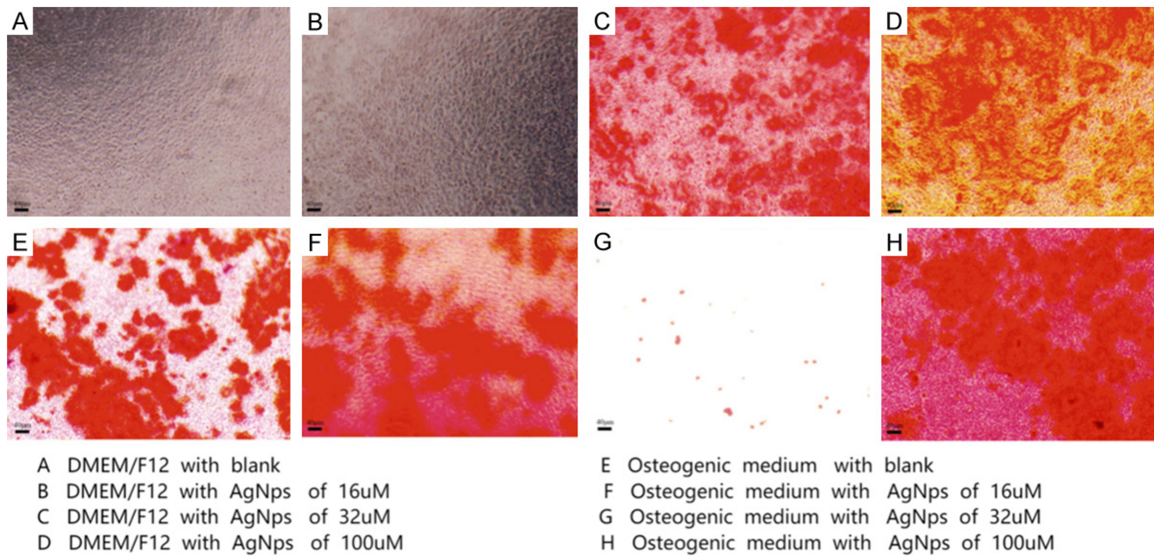

Figure 3. Marked enhancement of calcium mineralization was observed in the culture with 16 μM and 100 μM of AgNps as compared to that without AgNps at day 14 (100 ×).

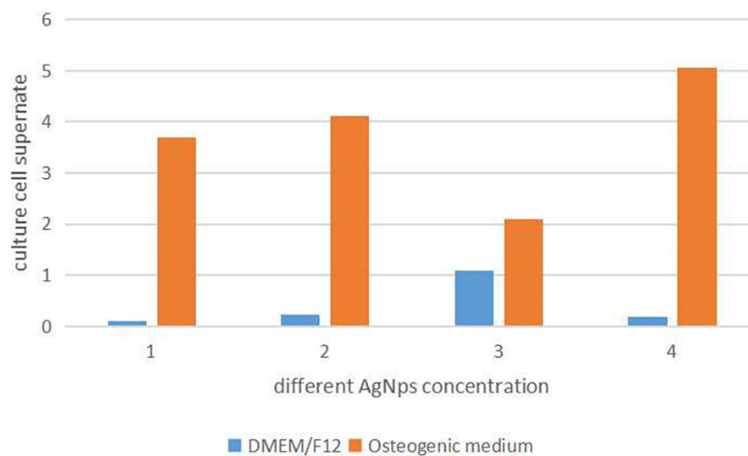

Figure 4. Marked enhancement on Day 14 of the OD value of cell culture supernatant was observed in the culture with 16 μM and 100 μM of AgNps as compared to that without AgNps. 1, blank for control; 2, with AgNps of 16 μM; 3, with AgNps of 32 μM; 4, with AgNps of 100 μM.

a prevalent membrane-bound glycoprotein, promotes the hydrolysis of phosphate monoesters at basic pH values and is expressed in several cell types, including osteoblasts, osteoclasts, and bone marrow stromal cells. ALP activity on the cell membrane is considered an essential marker for bone cell proliferation and differentiation [22, 23]. Here, we observed significantly higher levels of ALP activity at days 7 and 14 in mouse embryonic fibroblasts cultured with AgNps. Previously, it was reported that induction of ALP activity on cells exposed to AgNps was more than other metal nanoparticles [24]. Additionally, one of the distinctive characteristics of osteogenic cells is their ability to form mineralized calcium and phosphate deposits. ARS, used to stain inorganic calcium depositions and mineralized bone nodule formations, has been used extensively to study and quantify bone differentiation in cell systems. ARS in our experiments corroborated our ALP results.

In most differentiation processes, multiple cytokines operate in a large network, whereby the action of one cytokine is regulated by the presence or absence of other cytokines. Therefore, to observe phenotypic responses to AgNps and explore their potential mechanisms, we investigated the underlying protein molecular processes responsible. Our results show that PCSK-9 and LDL-R levels were significantly increased in osteogenic medium containing 16 μM AgNPs. PCSK-9, which belongs to a family of serine proproteinases termed proprotein

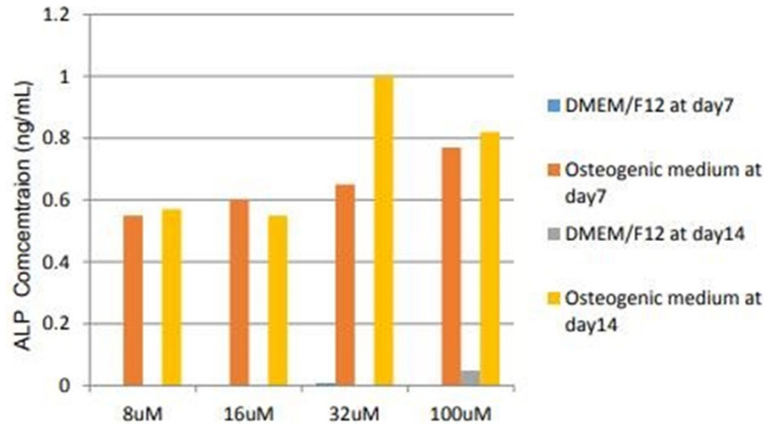

Figure 5. No significant increase of the ALP activity of mouse embryo fibroblasts cultured in DMEM/F12 was observed at day 7 and day 14, which indicated that mouse embryo fibroblasts did not undergo spontaneous osteoblast differentiation in the normal control medium. In contrast, ALP activity of mouse embryo fibroblasts cultured in the osteogenic medium increased at whole period. Addition of AgNps at the concentrations of 32 μM and 100 μM induced a little higher ALP activity at day 7 and day 14.

convertases, typically binds to LDL-R on the surface of hepatocytes and brings the receptor complex to the endosome for degradation. Therefore, PCSK-9 could cause a down-regulation of LDL-R at the cell surface, leading to increased circulating levels of low-density lipoprotein cholesterol [25]. Importantly, recent evidence confirms that adipocytes and osteoblasts originate from a common mesenchymal precursor; thus, an inverse reciprocal relationship sometimes exists between osteogenesis and adipogenesis, with factors simultaneously stimulating one of these processes while inhibiting the other [26].

In contrast, the level of OPG was significantly reduced in osteogenic medium containing 16 μM AgNPs. OPG, a secretory glycoprotein of the tumor necrosis factor receptor family, is highly expressed in adult bone, lung, heart, kidney, and placenta. The role of OPG in pathological aspects of bone diseases and periodontal disease has been well established. OPG therapy has been used to reduce bone resorption and enhance osseous healing. Indeed, therapeutic strategies have been based on the potent inhibitory action of OPG on osteoblast differentiation and function, as it acts as a soluble decoy receptor for RANKL [27-29]. In our future experiments, OPG and RANKL levels should be measured.

We also observed a mild increase in certain protein markers, such as IGFBP-2 and IGFBP-6, in osteogenic medium of cells treated with AgNPs. There are six highly conserved IGFBPs (IGFBP1-6), a class of secreted proteins exerting multiple functions in serum and most extracellular. IGFBPs can bind to their own receptors or translocate into the interior compartments of cells, where they may execute insulin-like growth factor-independent actions. Interestingly, most IGFBPs regulate important biological processes for cell proliferation and differentiation. IGFBP-2 has identified roles in tissue homeostasis and embryo development, as well as in stromal wound healing. IGFBP-6 can regulate cell proliferation, migration, angiogenesis, apoptosis, and fibrosis progression. Accordingly, some researchers propose that these proteins are major players at the crossroads of tissue repair, inflammation, and fibrosis [30, 31]. Further studies will elucidate their possible and complex role in regulation of osteogenic differentiation. Despite the aforementioned findings, how AgNPs interact at the molecular level during promotion of fibroblast differentiation into osteoblasts remains to be determined. Proteome analysis is currently underway in our research group to attempt to unravel this mystery and to identify key factors involved. Sometimes fibroblasts and osteoblasts concomitantly express the same cytokine receptors, such as integrin receptors, which can modulate critical cellular processes including adhesion, migration, survival, extracellular matrix organization, and differentiation. These processes are physiologically related to the growth, development, and tissue homeostasis, and may even be significant in pathological conditions, for instance, organ fibrosis [32].

There are some limitations and shortcomings of our study. First, additional *in vivo* studies are necessary to determine if the osteoblastic activities of cells differentiated from fibroblasts

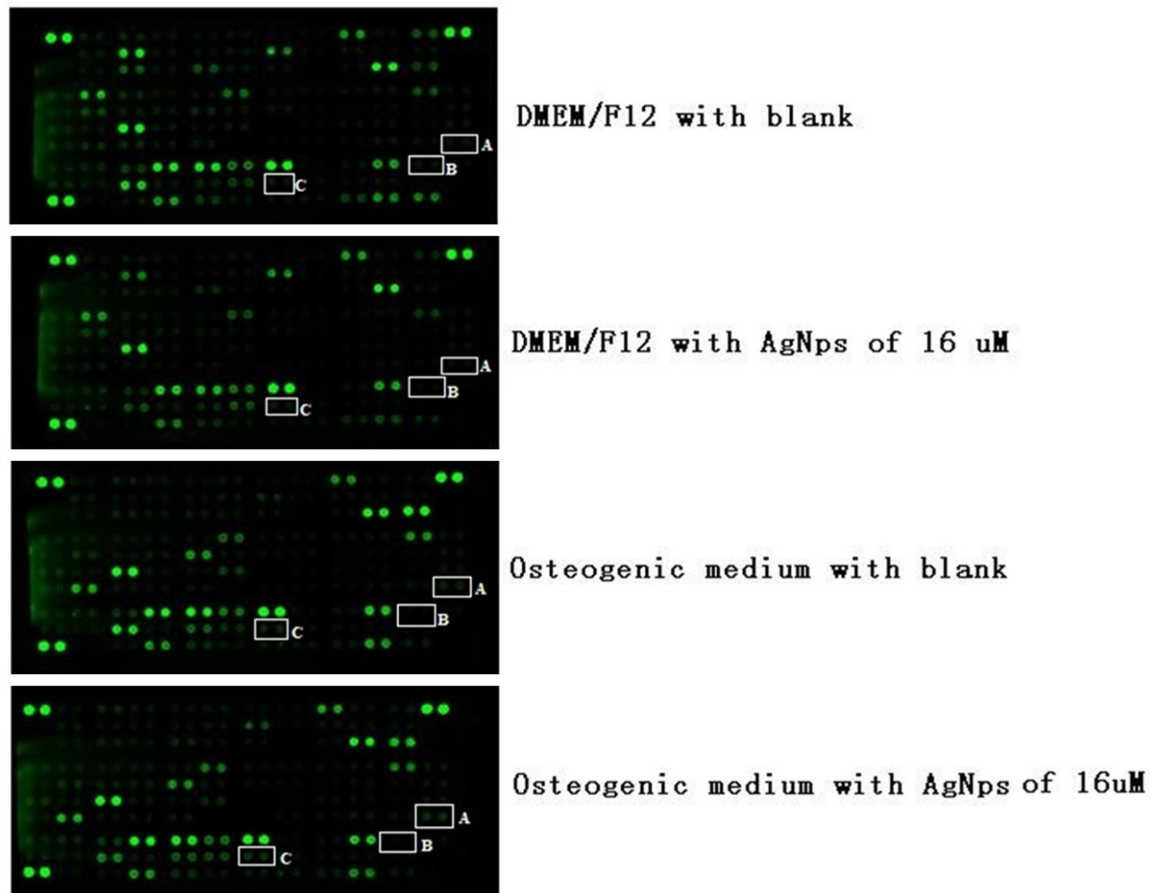

Figure 6. The results showed LDL-R and PCSK-9 were significantly increasing while Osteoprotegerin reducing in the osteogenic medium containing 16 μM AgNPs, compare with those in other groups. In addition, IGFBP-2 and IGFBP-6 also increased a little but not so significant in the osteogenic medium containing AgNPs group. A: LDL-R; B: Osteoprotegerin; C: PCSK9.

are sufficient to induce bone tissue repair. In addition, the best concentration of AgNps for proper *in vivo* treatment should be sought and the canonical signaling pathway (such as OPG/RANKL and Wnt/β-Catenin) that balances reciprocal regulation between osteoblastogenesis and adipogenesis for osteogenesis regulation should be unraveled [33].

In conclusion, our study reveals the proliferative and osteogenic differentiation induction effects of AgNPs on mouse embryonic fibroblasts. We show here that nanoscale metals can elicit differential effects toward distinct cell types. A previous study also showed that AgNps markedly promote fibroblast proliferation and mediate wound healing [34]. This is an important issue because the ability to manipulate fibroblasts as "stem-like" cells would open an effective gate in terms of tissue regeneration in the future [35-38]. Although the mechanism is not yet clearly understood, it gives insight into the possible use of AgNPs in promoting osteogenic differentiation and treatment for bone fractures. Overall, the results of these studies suggest that fibroblasts possess osteogenic transdifferentiation capability and might be a useful alternate source of cells for bone repair. This study leads surgeons one step closer to the clinical application of fascinating AgNps.

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Disclosure of conflict of interest

None.

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