Brief Report

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Osteogenic differentiation of human ligament fibroblasts induced by conditioned medium of osteoclast-like cells

Fangcang Yu, Yazhou Cui, Xiaoyan Zhou, Xiumei Zhang, Jinxiang Han*

Shandong Academy of Medical Sciences, Shandong Medical Biotechnological Center, Key Laboratory for Biotech Drugs of the Ministry of Health, Ji'nan, China.

Summary **Osteoclasts secrete factors that may promote mesenchymal stem cell mineralization *in vitro*. Fibroblasts are the most common cells in connective tissue and are involved in the process of exotic ossification in many diseases such as ankylosing spondylitis. The purpose of this study was to investigate whether osteoclast-like cells would induce the osteogenic differentiation of fibroblasts *in vitro*. In the present study, osteoclast-like cells (OLCs) were generated by $CD14^+$ cells from human peripheral blood. Fibroblasts were primarily cultured from spinal ligaments. After treatment with conditioned medium of OLCs, the level of alkaline phosphatase (ALP) and mineralization of fibroblasts increased significantly. cDNA microarray analysis identified a set of differentially expressed mRNA associated with signal transduction, cell differentiation, and bone formation, and microarray analysis of microRNA expression profiles revealed a group of microRNAs, including hsa-miR-20a, hsa-miR-300, hsa-miR-185, hsa-miR-30d, hsa-miR-320a, hsa-miR-130b, hsa-miR-33a, hsa-miR-155, and hsa-miR-222, that were significantly down-regulated. These microRNAs were predicted to have an inhibitory effect on genes associated with osteogenic differentiation, such as BMP2, Osteocalcin, and Runx2. The current results suggest that osteoclasts might induce the osteogenic differentiation of fibroblasts *in vitro* and that miRNA may play an important role in regulation of the cell-cell interaction between osteoclasts and fibroblasts.**

Keywords: Mineralization, cell-cell interaction, mRNA, microRNA, microarray

1. Introduction

The osteoclast (OC) is a tissue-specific macrophage polykaryon derived from the monocyte/macrophage precursor cells at or near the bone surface (*1*). Fibroblasts are the most common cells of the connective tissue and are derived from primitive mesenchyme (*2*,*3*). Fibroblasts are mainly responsible for the production and turnover of extracellular matrix (ECM), which is rich in collagen and other macromolecules, and play an important role in trauma repair and pathologic ectopic ossification in some diseases, such as ankylosing spondylitis and systemic sclerosis (*3*,*4*). The mechanisms of osteogenic differentiation of fibroblasts and ossification have not been clarified. Fibroblasts and osteoblasts are from the same lineage (*5*). The widely accepted view is that osteoclasts can promote the differentiation of osteoblasts. Conditioned medium derived from human osteoclasts induces preosteoblasts to form bone-like nodules (*6*). Gene array analysis, in combination with loss-of-function studies, revealed that mature osteoclasts secrete Wnt10b, sphingosine 1-phosphate, and bone morphogenetic protein-6 (BMP6) and might promote osteoblast mineralization (*7*). Thus, osteoclasts may play a key role in the process of inducing heterotopic ossification mediated by fibroblasts. The objective of the present study was to evaluate the effect that osteoclasts have on inducing the osteogenic differentiation of fibroblasts and initially investigate the mechanism by which this occurs.

**Address correspondence to:*
Dr. Jinxiang Han, Shandong Academy of Medical Sciences, Jing-shi Road No. 18877, Ji'nan 250062, Shandong, China.
e-mail: samshjx@sina.com

2. Materials and Methods

2.1. *Primary culture of ligament fibroblasts*

Interspinous ligaments were obtained with the informed consent of patients with lumbar spondylolisthesis. Fibroblasts were primarily cultured from ligaments with the collagen collagenase digestion method using collagenase I (Sigma-Aldrich China, Shanghai, China). Fibroblasts were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) (HiClone, Logan, Utah, USA) containing 10% Fetal Bovine Serum (FBS) (Invitrogen, Carlsbad, California, USA), 100 U/mL penicillin (Invitrogen, Carlsbad, California, USA), and 100 μg/mL streptomycin (Invitrogen, Carlsbad, California, USA) and were incubated in a humidified atmosphere (37°C, 5% CO_2). The culture medium was changed after 24 h and every other day thereafter.

2.2. *Generating osteoclast-like cells from peripheral blood*

Peripheral blood was drawn from normal volunteers and supplemented with the anticoagulant ethylenediam inetetraacetic acid (EDTA). $CD14^+$ cells were isolated by Ficoll-Paque (GE Healthcare, Salt Lake City, Utah, USA) density gradient centrifugation followed by isolation with anti-CD14 immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). This was done in accordance with the manufacturer's instructions. The isolated $CD14^+$ cells were seeded in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Five × 10^5/well $CD14^+$ cells were added in 24-well containing either bovine bone slices (8 mm × 8 mm × 0.2 mm, sterilized by ultraviolet radiation) or glass cover slips (8 mm × 8 mm) and incubated at 37°C with 5% CO_2 in a humidified incubator. The medium was supplemented with 25 ng/mL macrophage colony-stimulating factor (M-CSF) (PeproTech, Rocky Hill, New Jersey, USA) and 30 ng/mL receptor activator of nuclear factor kappa-B ligand (RANKL) (PeproTech, Rocky Hill, New Jersey, USA). Cultures were incubated and culture medium containing the factors was replenished every 3 d. After 21 d of culturing, the characteristics of osteoclasts were identified. Using the same method, more osteoclasts were cultured in a 25 cm^2 cell culture flasks (without bovine bone slice or glass cover slips) in order to harvest conditioned medium.

Observation of morphological characteristics: An inverted phase contrast microscope was used to observe the characteristics of multinucleate cells. Tartrate-resistant acid phosphatase (TRAP) staining: Histochemical staining for TRAP, a marker of osteoclasts, was carried out with an Acid Phosphatase, Leukocyte (TRAP) Kit (Sigma-Aldrich China, Shanghai, China). Assessment of bone resorption function: Bovine bone slices with cultured OLCs were subjected to fixation, dehydration, isoamyl acetate replacement, routine critical point drying, and plating platinum. Cells were then observed and photographs were taken with a Hitachi S570 scanning electron microscope.

2.3. *Conditioned medium of osteoclast-like cells treatment*

After OLCs were cultured for 21 d, addition of M-CSF and RANKL to medium was halted and the medium was refreshed every 2 d. The conditioned medium was collected and centrifuged to remove cells that were suspended in the medium. Fibroblasts were conditioned in OLC-conditioned medium and medium was refreshed every 2 d. Fibroblasts were cultured in normal medium (DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, without M-CSF and RNAKL) as a control.

2.4. *Assessment of fibroblast mineralization*

After fixation with 95% alcohol, a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Nantong Jiangsu, China) was used to detect the expression of ALP. Cultures were fixed and stained for mineralization with Alizarin red. Alizarin red was eluted with 10% cetylpyridinium chloride, and levels were quantified in comparison to a standard curve. To obtain a normal distribution and equal variance between the two test groups (four wells of OLC-conditioned medium (OCMT) fibroblasts and four wells of control fibroblasts), all data were $\log_{10}$-transformed. When only two groups were compared, an unpaired Student's *t*-test was used to test significance.

2.5. *Microarray detection of changes in the mRNA and miRNA expression profiles of fibroblasts*

2.5.1. *mRNA microarray*

Total RNA from each sample was quantified using the NanoDrop 1000 and RNA integrity was assessed using standard denaturing agarose gel electrophoresis. About 5 μg of total RNA from each sample was used for labeling and array hybridization in the following steps: 1) Reverse transcription with an Invitrogen Superscript ds-cDNA synthesis kit; 2) ds-cDNA labeling with a NimbleGen one-color DNA labeling kit; 3) Array hybridization using the NimbleGen Hybridization System and then washing with the NimbleGen wash buffer kit; 4) Array scanning using the Axon GenePix 4000B microarray scanner (Molecular Devices Corporation). Scanned images (TIFF format) were then imported into NimbleScan software (version 2.5) for grid alignment and expression data analysis.

2.5.2. *miRNA microarray*

Total RNA are harvested using TRIzol (Invitrogen, Carlsbad, California, USA) and an RNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. After RNA measurement using the NanoDrop instrument, the samples were labeled using the miRCURY™Hy3™/Hy5™ Power labeling kit and hybridized on the miRCURY™ LNA Array (v.14.0). The samples were hybridized on a hybridization station following the scheme outlined in sample submission. Scanning was performed with the Axon GenePix 4000B microarray scanner. GenePix pro V6.0 was used to read the raw intensity of the image.

2.5.3. *Data analysis*

Expression data were normalized through quantile normalization and the Robust Multichip Average (RMA) algorithm included in the NimbleScan software. The Probe level files and Gene level files were generated after normalization. The 2 gene level files were imported into Agilent GeneSpring GX software (version 11.5) for further analysis. Genes with values greater than or equal to the lower cut-off (50.0) in all samples ("All Target Values") were chosen for data analysis. Differentially expressed genes were identified through Fold Change filtering. Pathway analysis and GO Analysis were performed to reveal the biological functions of this subset of differentially expressed genes. Finally, hierarchical clustering was performed to provide distinguishable gene expression profiling among samples. microRNA targets were predicted by three programs: miRanda, PicTar, and Target-Scan. The overlap of the results predicted by three programs served as the acceptable target genes of microRNA.

3. Results and Discussion

3.1. *Primary cultured fibroblasts and OLCs with characteristics of osteoclasts*

Fibroblasts were generated by spinal ligaments (Figure 1A). After 21 d of induction of M-CSF and RANKL, osteoclast-like cells had the characteristics of osteoclasts, such as a large volume, irregular shape, pseudopodium and multinucleation (Figure 1B), and high TRAP positivity (Figure 1C). Under SEM observation, osteoclast-like cells were found to have bone resorption ability since there were absorption lacunae below the osteoclasts (Figure 1D).

3.2. *Osteogenic differentiation of fibroblasts induced by OLC-conditioned medium*

After 21 d of induction with OLC-conditioned medium, the ALP positivity of fibroblasts was approximately 40% (Figure 2B). In contrast, the ALP

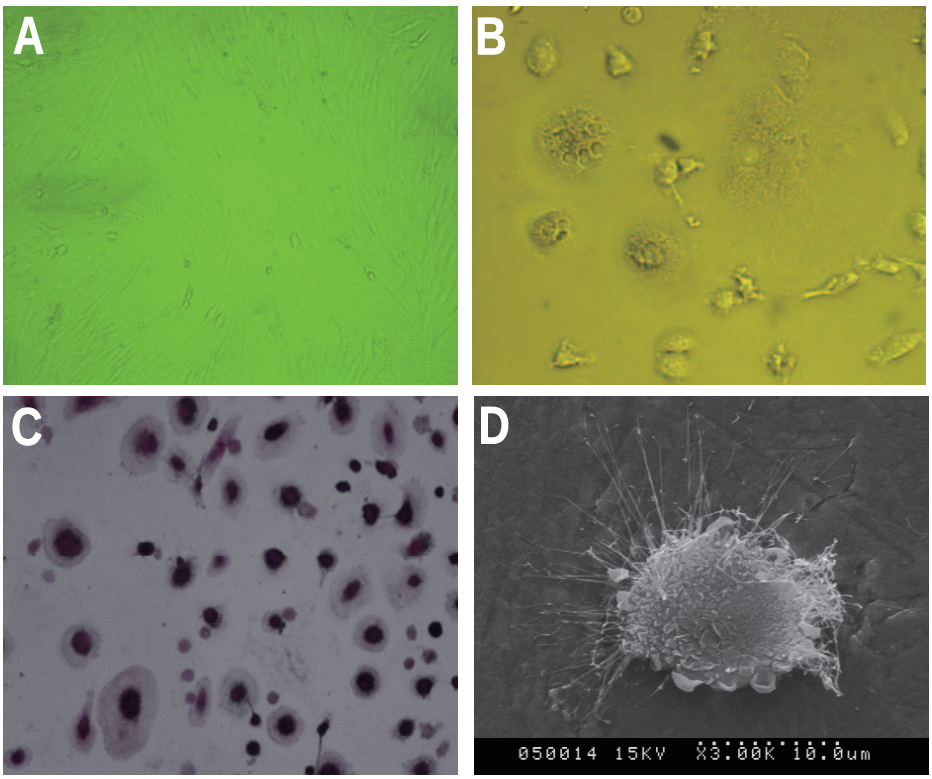

Figure 1. Primary cultured ligament fibroblasts and osteoclast-like cells generated by peripheral blood mononuclear cells. **(A)** Fibroblasts generated by ligaments; **(B)** Multinuclear osteoclast-like cells; **(C)** TRAP staining of OLC; **(D)** Bone resorption of OLC.

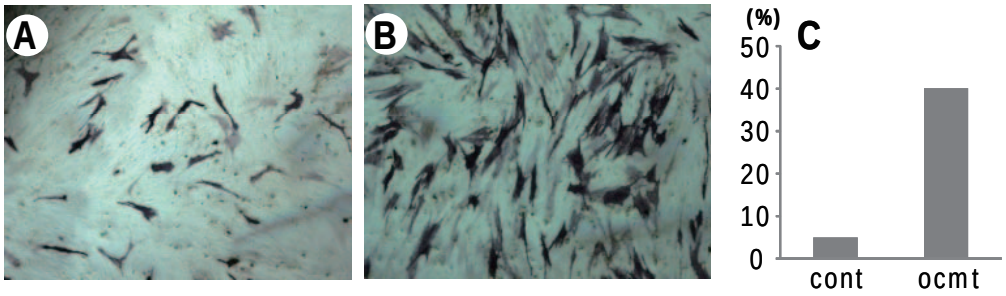

Figure 2. Results of Alkaline Phosphatase Color Development. (A) Control; **(B)** Fibroblasts treated with OLC-conditioned medium; **(C)** Comparison of ALP positivity of the control and fibroblasts treated with OLC-conditioned medium. $p < 0.05$.

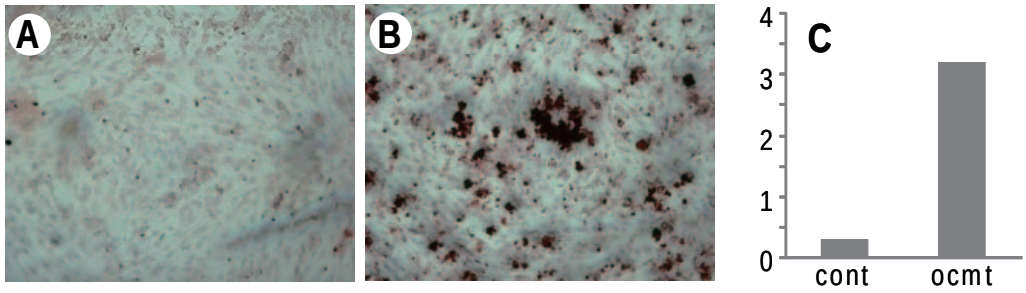

Figure 3. Effect of osteoclast-conditioned media on fibroblast mineralization. (A) Control; **(B)** Fibroblasts treated with OLC-conditioned medium; **(C)** Comparison of the mineralization of control and fibroblasts treated with OLC-conditioned medium. $p < 0.05$.

positivity of the control group was only 5% (Figure 2A). Conditioned media from osteoclast-like cells significantly increased mineralization of fibroblast cultures (Figure 3).

3.3. *mRNA microarray data analysis*

Compared to fibroblasts cultured in normal medium, fibroblasts treated with OCMT had 522 genes up-regulated more than 2-fold and 415 genes down-regulated more than 2-fold. The scatterplot is a visualization of the variation between chips (Figure 4). GO analysis revealed that the differentially expressed genes were mainly involved in bioprocesses like response to stimulus, signal transduction, cell activation, myeloid cell differentiation, calcium-mediated signaling, cell adhesion, and immune response. The cellular components of differentially expressed genes were mainly distributed in MHC protein complex, plasmolemma, the extracellular region, extracellular space, collagen, and the like. The molecular function of differentially expressed genes was mainly related to MHC class II receptor activity, protein complex binding, signal transducer activity, chemokine activity, and collagen binding. Pathway analysis revealed that differentially expressed genes mainly participated in pathways, such as cell adhesion molecules, leukocyte transendothelial migration chemokine signaling pathways, cytokine-cytokine receptor interaction, and Toll-like receptor signaling pathways.

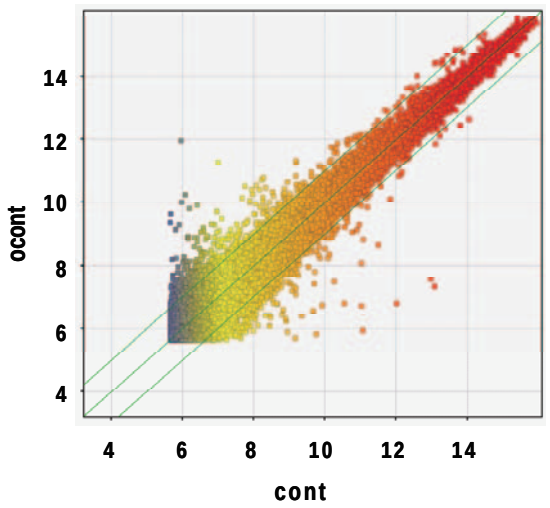

Figure 4. Scatter-plot for ocmt-cont (mRNA). Scatter-plot to assess the variation (or reproducibility) between mRNA chips. The axes of the scatter-plot are the averaged normalized signal values of the samples in each group (log_2 scaled). The green lines are Fold Change Lines (The default fold change value given is 2.0). Genes above the top green line and below the bottom green are 2-fold change genes.

3.4. *miRNA microarray data analysis*

Compared to fibroblasts cultured in normal medium, fibroblasts treated with OCMT had 28 miRNAs up-regulated more than 2-fold and 59 miRNAs down-regulated more than 2-fold. Target genes prediction software analysis revealed that among the down-regulated miRNAs the target genes of miR-20a and hsa-miR-300 included BMP2. Osteocalcin (bone

gamma-carboxyglutamate protein, BGLAP) was one of the target genes of hsa-miR-185 while hsa-miR-30d, hsa-miR-320a, hsa-miR-20a, hsa-miR-130b, hsa-miR-33a, hsa-miR-155, and hsa-miR-222 all targeted Runx2. These genes are all related to osteogenic differentiation.

Mature osteoclasts secreted products that promote fibroblast mineralization and caused a series of changes in mRNA and miRNA expression profiles of fibroblasts. Through cytokine-cytokine receptor interaction and regulation of signaling pathways, the products secreted by osteoclast-like cells may affect the metabolism and differentiation of fibroblasts. The current microarray analysis suggests that the Toll-like receptor signaling pathway may play an important role in these processes. A recent study has found that the Toll-like receptor is associated with osteogenic differentiation of stromal cells (*8*).

No previous reports have described the effects of osteoclasts on fibroblasts, but studies have found that fibroblasts can induce differentiation of precursor cells into mature osteoclasts. Bloemen and colleagues (*9*) found that gene expression of intercellular adhesion molecule-1 (ICAM-1) and osteoclastogenesis-related genes (RANKL, RANK, TNF-a, and IL-1b) was highly up-regulated in co-cultures with fibroblasts. The current study has shown that conditioned medium of osteoclast-like cells can influence the adhesion molecule pathway of fibroblasts. Hypoxia-induced ID-2 may contribute to joint destruction in RA patients by promoting synovial fibroblast-dependent osteoclastogenesis (*10*). Moreover, oestrogen can inhibit osteoclast formation induced by periodontal ligament fibroblasts (*11*). The current study suggests that there is interaction between osteoclasts and fibroblasts. Fibroblasts can induce osteoclastogenesis and osteoclasts induce osteogenic differentiation of fibroblasts. These findings will help to understand the pathomechanism of ectopic ossification mediated by fibroblasts.

miRNAs are small noncoding RNAs that significantly regulate the translation of protein coding genes in higher organisms (*12-14*). These small RNAs (approximately 22 nucleotides) are involved in almost every biological process, including early development, lineage commitment, growth and differentiation, cell death, and metabolic control (*12-16*). Each miRNA targets an average of 100-200 genes by binding preferentially to their 3' UTRs by means of partial sequence complementarity (*17*). miRNAs engage in a wide diversity of biological processes by coordinating with transcription factors, and this kind of cross-layer coregulation may have greater specificity than intra-layer coregulation (*18*). The current study has suggested that microRNA plays an important role in the osteogenesis of mesenchymal stem cells (*19*). Changes in microRNA expression profiles were examined in the current study during the process of osteogenic differentiation induced by OLC-conditioned medium. This study also found that microRNAs such as hsa-miR-20a, hsa-miR-300, hsa-miR-185, hsa-miR-30d, hsa-miR-320a, hsa-miR-130b, hsa-miR-33a, hsa-miR-155 and hsa-miR-222 may play a key role by regulating the expression of BMP2, Osteocalcin, Runx2, in the process of osteogenic differentiation.

The present study investigated the effects of osteoclast-like cells on induction of fibroblast mineralization without direct cell-to-cell contact. This study also identified a set of mRNA and miRNA differentially regulated during fibroblast mineralization. However, the factors secreted by osteoclast-like cells that influence signal transduction and that regulate expression miRNA and their target genes remain unclear. More research is needed to determine the precise mechanism of these processes.

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