

Original Article

Involvement of miR-145 in osteoarthritis pathogenesis via inducing OPG expression in osteoclasts

Yiming Ma, Liang Yan, Haibo Xu, Yong Tao, Sheng Chen, Jianhua Zhu, Lei Xie

Department of Orthopaedics, Second Hospital of Shaoxing, Shaoxing, Zhejiang 312000, China

Received September 22, 2016; Accepted October 28, 2016; Epub February 15, 2017; Published February 28, 2017

Abstract: Osteoarthritis is featured as bone degeneration with unclear pathogenesis. Current opinions agree that OA is related with over-proliferation or differentiation of osteoclasts and impeding of apoptosis. MiR-145 could affect proliferation, differentiation and apoptosis of cells via mediating osteoprotegerin (OPG), whose role in osteoclasts has not been studied. Therefore, this study observed the effect of miR-145 on OPG level in osteoclasts, in addition to its functional mechanism. Osteoclasts were obtained by RAW164.7 induction, and were transfected with designed miR-145 inhibitor. CCK-8 assay was employed to observe its effect on proliferative activity in osteoclasts. Real-time PCR was used to test mRNA level of OPG, while Western blotting was used to test protein level of OPG, cyclin B1, cyclin D3, Bax, and caspas3. The effect of miR-145 inhibitor on OPG and these proliferative/apoptotic molecules were analyzed. Compared to control group, miR-145 inhibitor significantly decreased cell proliferation and elevated cell apoptosis level. Real-time PCR and Western blotting showed remarkably increased OPG expression in miR-145 inhibitor transfected osteoclasts. The knockdown of miR-145 can inhibit cell proliferation/differentiation, induce cell apoptosis, limit over-absorption of bones, and eventually alleviate OA via up-regulating OPG expression.

Keywords: Osteoarthritis, osteoclasts, MicroRNA, osteoprotegerin

Introduction

Osteoarthritis (OA) is one common articular injury, and frequently accompanied with severe destruction of bones and cartilages. OA has become the major reason causing pains in muscles and bones and immobility, but still lacks effective treatment approach due to complicated pathogenesis mechanism [1, 2]. Recent findings showed that both joint degeneration and bone absorption preceded inflammatory response [3, 4]. Therefore, the finding of related factors affecting bone absorption can retard disease progression. Osteoprotegerin (OPG)/nuclear factor (NF)- κ B receptor activating factor (RANK)/NF- κ B receptor activating factor ligand (RANKL) system is an important molecular pathway mediating bone reconstruction. RANKL on osteoblasts can bind with RANK on osteoclasts, leading to production and maturation acceleration. Meanwhile OPG produced from osteoclasts can inhibit their over-generation via RANKL binding. Therefore, the balance between OPG and RANKL plays an important

role in patho-physiological process in bone biogenesis [5, 6]. Some studies showed that exogenous OPG significantly induced apoptosis of osteoclasts, thus inhibiting bone absorption, although its regulatory mechanism is still unclear [7-9]. Therefore, the finding of endogenous pathway of OPG up-regulation can help to improve the dynamic balance of bone absorption, alleviate bone degeneration, eventually decreasing OA.

MiRNA is one group of RNA sequence with 22-24 nt with highly cross-species conserved regions. It can regulate the expression of endogenous proteins via degrading mRNA of target proteins or inhibiting the translation process [10-12]. Both biochips (GSE17785) and bioinformatics studies showed that miR-145 in chondrocytes can regulate TNFRSF11B to inhibit its expression. The expression of OPG can further lead to apoptosis of osteoclasts. Moreover, miR-145 has also been found to be significantly down-regulated in primary knee joint OA [13]. However, whether miR-145 was

MiR-145 in OA pathogenesis

correlated with OPG in osteoclasts, or whether it can regulate proliferation or apoptosis of osteoclasts, remained unclear.

This study used osteoclasts induced by RAW164.7, and transfected these cells with miR-145 inhibitor. The effect on proliferation and apoptosis of osteoclasts was observed, along with mRNA or protein quantification of OPG and other proliferation/apoptosis related molecules. We further investigated if miR-145 inhibitor could suppress osteoclast proliferation and induce their apoptosis, eventually decreasing bone reabsorption, via up-regulating OPG expression. This study will provide evidences for targeted treatment of OA.

Materials and methods

Cell line

Mouse RAW264.7 cell line (KCB 200603YJ) was purchased from ATCC (US).

Design and synthesis of miR-145 primers

Primers were designed by Toyobo (China), along with the synthesis of inhibitor (anti-miR-145).

Reagents and instruments

DMEM culture medium was purchased from Life technologies (US). Fetal bovine serum (FBS) was purchased from Gibco (US). RANKL was purchased from Sigma (US). TRAP staining kit was purchased from Kaiji Bio (China). CCK-8 test kit was purchased from Toyobo (Japan). mRNA primer of OPG and Lipofectamine 3000 were purchased from Invitrogen (US). Total RNA extraction, reverse transcription and PCR kits were purchased from Toyobo (China). OPG polyclonal antibody was purchased from Abcam (US). Polyclonal antibody for cyclin B1, cyclin D3, bax, caspase-3 and beta-actin were purchased from Sanying Biotech (China). Instruments included: CO₂ incubation (ThermoFisher, US); Microplate reader (ThermoFisher, US); UV spectrometry (Tianmei, China); Mini-Protean II vertical electrophoresis (Biorad, US); Gel imaging system (UVP, US); Real-time quantitative PCR (Applied Biosystem, US).

Cell culture

RAW264.7 cells were cultured in high-glucose DMEM medium containing 10% FBS, and were

kept in a 37°C incubation with 5% CO₂ for cell passage and further induction.

Induction of osteoclasts

Original medium was removed and replaced by complete DMEM medium (10% FBS, 10 U/ml penicillin, 100 U/ml streptomycin, and 0.01 mol/L HEPES buffer) containing 100 ng/ml RANKL. Cells were kept in a 37°C incubation with 5% CO₂ induction, with the changing of medium every 3 days. Osteoclasts were observed after 6-8 days, with TRAP staining for the examination of cell induction.

Grouping and cell transfection

Cells were transfected following manual instruction of Toyobo (China). One day before transfection, osteoclasts were seeded into 6-well plate at 1×10^5 per well, which had 2 ml serum- and antibiotic-free high-glucose DMEM medium. All cells were assigned into control, miR-NC and anti-miR-145 groups. When cells reached 60%-70% confluence, 5 μ L Lipofectamine 3000 was diluted in 150 μ L DMEM, while 5 μ L anti-miR-145 was also diluted in 150 μ L DMEM. Both mixtures were incubated at room temperature for 5 min to form inhibitor-transfection reagent complex. Such complex was added into cells with culture medium (1.5 mL per well) for 36-48 h for the following experiments.

CCK-8 assay

Osteoclasts were seeded into 96-well plate, and divided into control, miR-NC and anti-miR-145 groups (N = 6 each). After 36-48 h transfection, CCK-8 reaction buffer was added for 24 h incubation. OD values at 450 nm were then tested by a microplate reader to calculate survival rate of all cells.

Total RNA extraction, reverse transcription and real-time PCR

At 48 h after transfection, cells were extracted for total RNA following the manual instruction of test kit (Toyobo, China). RNA concentration was quantified and performed in reverse transcription in a 20 μ L system under the program: 37°C 15 min, 50°C 5 min and 98°C 5 min. Real-time PCR was performed under the following conditions: 95°C 10 min, followed by 40 cycles of 95°C 15 s and 60°C 1 min. Relative expression was used as the criteria. Primer

Table 1. PCR primer sequences and product lengths of target genes

Gene		Primer sequence (5'-3')
OPG	Up stream	5'-AATGAGACGTCATCTTCTAAAGCA-3'
	Down stream	5'-TCCATGAAGCTACGAAGCTGCTTG-3'
GAPDH	Up stream	5'-TGGTATCGTGGAAGGACTCATGAC-3'
	Down stream	5'-ATGCCAGTGAGCTCCCGTTCAGC-3'

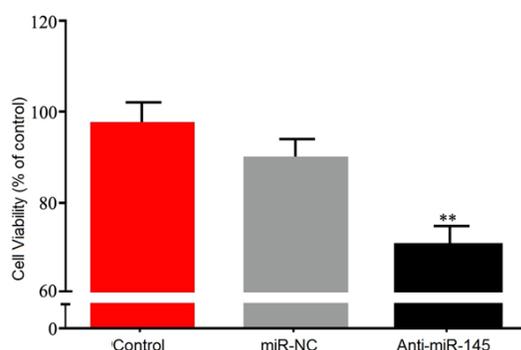


Figure 1. Effects of anti-miR-145 on proliferative activity of osteoclasts. **, P<0.01 compared to control group.

sequences and amplification product lengths were shown in **Table 1**.

Western blotting assay

Cells after transfection were mixed with lysis buffer and centrifuged at 12,000 g under 4°C for 15 min following homogenization. Protein concentration was determined by BCA method. After separation in 12% SDS-PAGE, proteins were transferred to PVDF membrane, which was blocked in 5% defatted milk powders at 37°C for 2 h. Rabbit anti-human OPG (1:2,000 dilution), cyclin B1, cyclin D3, bax, caspase-3 and β-actin polyclonal antibody (all in 1:1,000 dilution) were added for 4°C overnight incubation. Following TBST rinsing for three times (10 min each), goat anti-rabbit secondary antibody was added for 1 h vortex, followed by ECL chromogenic development. Quantity One software was used to analyze the image. Relative protein content was calculated as the ratio of gray values of target protein bands against that of β-actin.

Statistical methods

SPSS17.0 software was used for statistical processing of all data, which were presented as

mean ± standard deviation (SD). All data were processed in one-way analysis of variance (ANOVA). A statistical significance was defined when P<0.05.

Results

Effects of miR-145 knockdown on proliferative activity of osteoclasts

CCK-8 results showed significantly decreased survival rate of miR-145 inhibitor treated cells (70.51 ± 4.07%) compared to the control group (97.53 ± 4.63%) or miR-NC group (89.85 ± 4.02%, **Figure 1**).

Effects of miR-145 knockdown on mRNA/protein expression level of OPG

Real-time PCR results showed significantly elevated mRNA OPG level in miR-145 inhibitor treated cells compared to control or miR-NC group. Western blotting results also showed about 3-fold increase of OPG level in miR-145 inhibitor treated cells compared to control or miR-NC group (**Figure 2**).

Effect of miR-145 knockdown on proliferation of osteoclasts proliferation and apoptotic protein expression

In osteoclasts, the transfection of miR-145 inhibitor significantly depressed the expressions of cell cycle protein cyclin B1 and cyclin D3, while pro-apoptotic protein bax and caspase-3 were remarkably up-regulated (P<0.05 in all cases, **Figure 3**).

Discussion

OA is featured with bone destruction and degeneration, with complicated pathogenesis mechanism which is under the regulation of multiple factors [2, 14]. Recent studies revealed the critical regulatory role of OPG/RANK/RANKL system in physiological process of bone formation and absorption [15, 16]. OPG can bind to RANKL to inhibit its activation with RANK, thus alleviating the formation of osteoclasts and inducing their apoptosis, eventually inhibiting bone reabsorption [16]. The production of OPG is under the effect of various inflammatory factors. Parathyroid hormone and glucocorticoid can inhibit OPG synthesis [17, 18]. Studies showed rapidly accelerated formation of osteoclasts in OPG gene knockout mice,

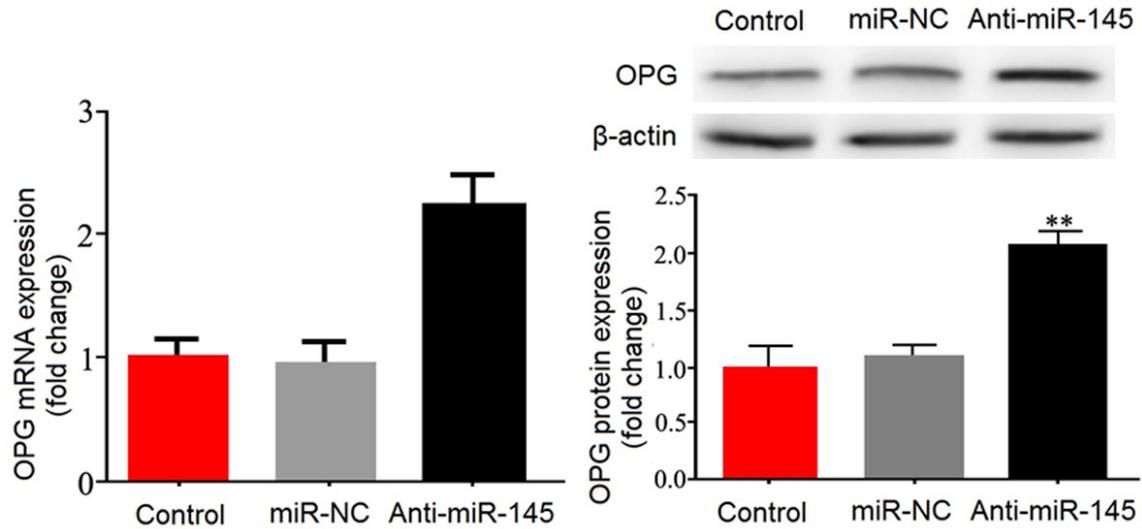


Figure 2. Effect of anti-miR-145 on OPG expression. **, P<0.01 compared to control group.

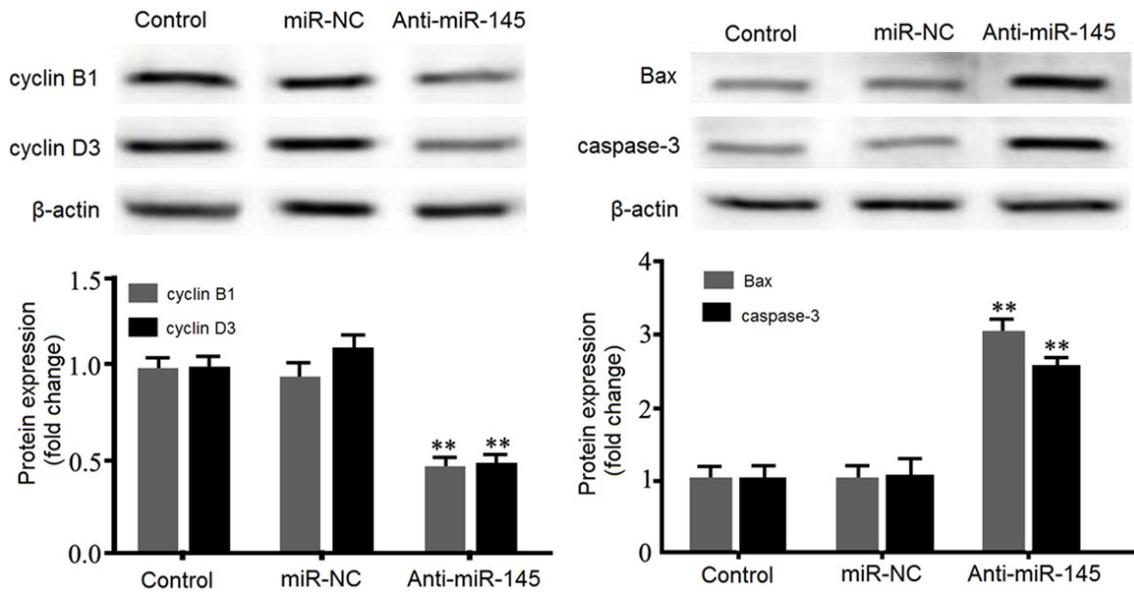


Figure 3. Effect of anti-miR-145 on cyclin B1, cyclin D3, bax and caspase-3 expressions in osteoclasts. **, P<0.01 compared to control group.

while OPG-over expression mice showed severe osteopetrosis with decreased osteoclast number [19-21]. Moreover, OPG can also inhibit osteoclast activity in RANKL-independent manner, as shown by significant inhibition on osteoclast differentiation, maturation and apoptosis, thus retarding the progression of OA [22].

MiRNA is one endogenous non-coding small RNA molecule. It can degrade target gene mRNA or inhibit target protein translation via

binding onto 3'UTR of mRNA [10]. Large amounts of miRNA with abnormal expression existed in bone joint disease and imbalance of cartilage homeostasis. These miRNA can protect cartilage tissues via functioning on cartilage activity related genes [23, 24]. Therefore, the screening of miRNA marker with abnormal expression in OA, coupled with targeted regulation, may become one novel treatment for OA. Recent studies revealed the existence of miR-145 in cytoplasm of chondrocytes, with signifi-

cantly lower expression in OA patients compared to normal knee joints tissues, with lower expression in more advanced diseases [25]. MiR-145 could affect IL-1 β induced ECM precipitation via regulating its target protein Smad3, thus mediating disease progression of OA [25, 26]. MiRNA has multiple functional targets. In a diabetic model, miR-145 can affect proliferation-apoptosis homeostasis of mononuclear macrophage via targeted regulation on OPG, eventually alleviating induced inflammatory response [27]. Currently, the major challenge of OA is multi-target and multi-effects inherent in the complicated pathogenesis. Therefore, as one endogenous small molecule, miRNA can function at multiple targets, thus having promising clinical futures.

Results of this study showed that in RAW264.7 induced osteoclasts, the transfection of miR-145 inhibitor significantly elevated mRNA and protein levels of OPG, compared to un-transfected or miR-NC group, accompanied with depressed expression of proliferation marker proteins cyclin B1 and cyclin D3, and elevated pro-apoptotic molecule caspase-3 expression. Therefore, we proposed that in osteoclasts, the knockdown of miR-145 can eliminate its inhibitory effect on OPG expression, further suppressing induced proliferation and expression of apoptotic proteins, eventually inhibiting proliferation and differentiation of osteoclasts and inducing cell apoptosis.

To explore the effect of miR-145 on proliferative activity of osteoclasts, CCK-8 assay was performed and found significantly higher survival rate of osteoclasts in miR-145 inhibitor transfected cells compared to un-transfected or miR-NC group. These results confirmed our initial hypothesis that the knockdown of miR-145 could up-regulate OPG expression, further inhibit osteoclast proliferation and induce their apoptosis. These biological processes had a synergistic effect on the alleviation of over-absorption of bone by osteoclasts, thus decreasing OA injury.

This study replenished evidences of the important role of miR-145 in regulating osteoclast proliferation and apoptosis, demonstrating that miR-145 could regulate OPG transcription, and further affects the expression level of induced proliferation and apoptosis-related proteins, eventually alleviating pathogenesis

and progression of OA. This study provides functional targets of molecular treatment of OA, and demonstrating functional mechanism. However, the precise mechanism of miR-145 in regulating OPG and its protective effects on OA model are still unclear, and require further studies.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yiming Ma, Department of Orthopaedics, Second Hospital of Shaoxing, No. 123 of Yanan Road, Yuecheng District, Shaoxing, Zhejiang 312000, China. Tel: +86-0575-88055555; Fax: +86-0575-88055555; E-mail: jijunzhaozxc@sina.com

References

- [1] Hunter DJ and Ferreira ML. Osteoarthritis: Yet another death knell for paracetamol in OA. *Nat Rev Rheumatol* 2016; 12: 320-321.
- [2] Loeser RF, Collins JA and Diekmann BO. Ageing and the pathogenesis of osteoarthritis. *Nat Rev Rheumatol* 2016; 12: 412-420.
- [3] Duarte JH. Osteoarthritis: alendronate treatment improves pathology in animal model of OA by blocking osteoclastic bone resorption. *Nat Rev Rheumatol* 2014; 10: 446.
- [4] Yao Z, Xing L and Boyce BF. NF-kappaB p100 limits TNF-induced bone resorption in mice by a TRAF3-dependent mechanism. *J Clin Invest* 2009; 119: 3024-3034.
- [5] Kearns AE, Khosla S and Kostenuik PJ. Receptor activator of nuclear factor kappaB ligand and osteoprotegerin regulation of bone remodeling in health and disease. *Endocr Rev* 2008; 29: 155-192.
- [6] Tyrovolas JB. The "Mechanostat Theory" of Frost and the OPG/RANKL/RANK System. *J Cell Biochem* 2015; 116: 2724-2729.
- [7] Shimizu S, Asou Y, Itoh S, Chung UI, Kawaguchi H, Shinomiya K and Muneta T. Prevention of cartilage destruction with intraarticular osteoclastogenesis inhibitory factor/osteoprotegerin in a murine model of osteoarthritis. *Arthritis Rheum* 2007; 56: 3358-3365.
- [8] Liu W, Xu C, Zhao H, Xia P, Song R, Gu J, Liu X, Bian J, Yuan Y and Liu Z. Osteoprotegerin Induces Apoptosis of Osteoclasts and Osteoclast Precursor Cells via the Fas/Fas Ligand Pathway. *PLoS One* 2015; 10: e0142519.
- [9] Ren H, Ren H, Li X, Yu D, Mu S, Chen Z and Fu Q. Effects of intermedin on proliferation, apoptosis and the expression of OPG/RANKL/M-CSF in the MC3T3-E1 osteoblast cell line. *Mol Med Rep* 2015; 12: 6711-6717.

MiR-145 in OA pathogenesis

- [10] Ross SA and Davis CD. The emerging role of microRNAs and nutrition in modulating health and disease. *Annu Rev Nutr* 2014; 34: 305-336.
- [11] Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009; 136: 215-233.
- [12] Ambros V. The functions of animal microRNAs. *Nature* 2004; 431: 350-355.
- [13] Martinez-Sanchez A, Dudek KA and Murphy CL. Regulation of human chondrocyte function through direct inhibition of cartilage master regulator SOX9 by microRNA-145 (miRNA-145). *J Biol Chem* 2012; 287: 916-924.
- [14] Nalesso G, Thomas BL, Sherwood JC, Yu J, Addimanda O, Eldridge SE, Thorup AS, Dale L, Schett G, Zwerina J, Eltawil N, Pitzalis C and Dell'Accio F. WNT16 antagonises excessive canonical WNT activation and protects cartilage in osteoarthritis. *Ann Rheum Dis* 2016; [Epub ahead of print].
- [15] Wang Z, Ding L, Zhang S, Jiang T, Yang Y and Li R. Effects of icariin on the regulation of the OPG-RANKL-RANK system are mediated through the MAPK pathways in IL-1beta-stimulated human SW1353 chondrosarcoma cells. *Int J Mol Med* 2014; 34: 1720-1726.
- [16] Corrado A, Neve A, Macchiarola A, Gaudio A, Marucci A and Cantatore FP. RANKL/OPG ratio and DKK-1 expression in primary osteoblastic cultures from osteoarthritic and osteoporotic subjects. *J Rheumatol* 2013; 40: 684-694.
- [17] Shimizu M, Noda H, Joyashiki E, Nakagawa C, Asanuma K, Hayasaka A, Kato M, Nanami M, Inada M, Miyaura C and Tamura T. The Optimal Duration of PTH(1-34) Infusion Is One Hour per Day to Increase Bone Mass in Rats. *Biol Pharm Bull* 2016; 39: 625-630.
- [18] Pacifici R. T cells, osteoblasts, and osteocytes: interacting lineages key for the bone anabolic and catabolic activities of parathyroid hormone. *Ann N Y Acad Sci* 2016; 1364: 11-24.
- [19] Li XF, Xue CC, Zhao YJ, Cheng SD, Zhao DF, Liang QQ, Chen L, Wang Q, Lu S, Shi Q, Wang YJ and Shu B. Deletion of Opg leads to increased neovascularization and expression of inflammatory cytokines in the lumbar intervertebral disc of mice. *Spine (Phila Pa 1976)* 2016; [Epub ahead of print].
- [20] Mizuno M, Miyazawa K, Tabuchi M, Tanaka M, Yoshizako M, Minamoto C, Torii Y, Tamaoka Y, Kawatani M, Osada H, Maeda H and Goto S. Reveromycin A Administration Prevents Alveolar Bone Loss in Osteoprotegerin Knockout Mice with Periodontal Disease. *Sci Rep* 2015; 5: 16510.
- [21] Shimamura M, Nakagami H, Osako MK, Kurinami H, Koriyama H, Zhengda P, Tomioka H, Tenma A, Wakayama K and Morishita R. OPG/RANKL/RANK axis is a critical inflammatory signaling system in ischemic brain in mice. *Proc Natl Acad Sci U S A* 2014; 111: 8191-8196.
- [22] Ndip A, Wilkinson FL, Jude EB, Boulton AJ and Alexander MY. RANKL-OPG and RAGE modulation in vascular calcification and diabetes: novel targets for therapy. *Diabetologia* 2014; 57: 2251-2260.
- [23] Jones SW, Watkins G, Le Good N, Roberts S, Murphy CL, Brockbank SM, Needham MR, Read SJ and Newham P. The identification of differentially expressed microRNA in osteoarthritic tissue that modulate the production of TNF-alpha and MMP13. *Osteoarthritis Cartilage* 2009; 17: 464-472.
- [24] Iliopoulos D, Malizos KN, Oikonomou P and Tsezou A. Integrative microRNA and proteomic approaches identify novel osteoarthritis genes and their collaborative metabolic and inflammatory networks. *PLoS One* 2008; 3: e3740.
- [25] Yang B, Kang X, Xing Y, Dou C, Kang F, Li J, Quan Y and Dong S. Effect of microRNA-145 on IL-1beta-induced cartilage degradation in human chondrocytes. *FEBS Lett* 2014; 588: 2344-2352.
- [26] Zhu HY, Li C, Zheng Z, Zhou Q, Guan H, Su LL, Han JT, Zhu XX, Wang SY, Li J and Hu DH. Peroxisome proliferator-activated receptor-gamma (PPAR-gamma) agonist inhibits collagen synthesis in human hypertrophic scar fibroblasts by targeting Smad3 via miR-145. *Biochem Biophys Res Commun* 2015; 459: 49-53.
- [27] Chakraborty C, Doss CG, Bandyopadhyay S and Agoramoorthy G. Influence of miRNA in insulin signaling pathway and insulin resistance: micro-molecules with a major role in type-2 diabetes. *Wiley Interdiscip Rev RNA* 2014; 5: 697-712.