Abstract. The function of microRNA-130a in development and progression of osteoarthritis was determined. In osteoarthritis patients, the serum levels of microRNA-130a were decreased, compared with normal group. Overexpression of microRNA-130a increased cell proliferation and decreased apoptosis of chondrocytes, and downregulation of microRNA-130a also decreased cell proliferation and induced apoptosis in chondrocytes. Downregulation of microRNA-130a promoted Bax and caspase-3/9 protein expression, increased inflammation divisors and suppressed the PTEN/PI3K/Akt signaling pathway. PTEN inhibitor, VO-Ohpic trihydrate increased the destructive effect of microRNA-130a on cell proliferation of chondrocytes. PI3K inhibitor, wortmannin also increased the destructive effect of microRNA-130a on osteoarthritis. In conclusion, microRNA-130a is an important regulator of osteoarthritis in chondrocytes through PTEN/PI3K/Akt signaling pathway.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease that is associated with high morbidity and high disability rate. Pathogenesis of RA has not been illuminated yet (1), thus, exploring the pathophysiological process of RA synovial proliferation is an urgent problem to be solved (2). The pathological manifestations of RA include recurrent attacks of inflammation, excessive proliferation and dysfunction of synovial tissues, invasion and destruction of articular cartilage and bone by synoviocytes and vascular cells (3). At the initial stage of RA, inflammatory cells aggregate to the articular cavity under the action of inflammatory mediators and chemokines, and generate inflammatory response targeting the synovial membrane (4). Macrophage-like synoviocytes and fibroblast-like synoviocytes will produce a large amount of inflammatory cytokines and matrix degrading enzymes under incentive conditions, maintain and promote the persistence of inflammatory response, promote angiogenesis, and induce destruction of joint tissue (5).

It is known that autoimmune disorder can induce RA, but the cause of such disturbance remains unclear (6). microRNA, which is most promising in research in this direction, is an important component in the cellular and genetic regulatory network (7). It is a kind of endogenous and tiny non-coding single-strand RNA molecule with the average length of 22 nucleotides, which has become the hot research direction in immunology at present as a result of its diversified species and important roles (8). It plays an important role in processes such as hematopoiesis, proliferation, apoptosis, tissue differentiation and organ differentiation (9). In addition, it is suggested in a recent study to be involved in regulating body immune system (9). Currently, increasing literature illustrates the huge potential of microRNA at fundamental and clinical levels, especially as the therapeutic targets, inserted molecules and biomarkers (9). It is been known that the functions of microRNA in animals are not restricted to the regulation of cell proliferation and differentiation, lipid conversion, and hormone secretion regulation. More importantly, it can prevent disturbance of normal immune function (8). Jiang and Wang (10) indicated that histone deacetylase 3 was involved in ankylosing spondylitis via miR-130a and enhancement of tumor necrosis factor-1α (TNF-1α) expression. Li et al (11) showed that miR-130a played an important role in regulating the expression of TNF-α in osteoarthritis.

Akt, which is overexpressed in RA synoviocytes, can not be detected in normal synoviocytes (12). Addition of Akt in RA synoviocytes cultured in vitro endows cells with the ability to act against apoptosis, suggesting that the PI3K/Akt signal pathway, which is highly active in RA, may be involved in the course of RA (12). PTEN is an important negative regulatory factor in PI3K/Akt signal pathway, which can downregulate the activities of pathway as well as its downstream factors at multiple levels (13). This characteristic has been verified in
numerous studies on tumor (13). Research on RA suggests that PTEN may also be involved in activating the pathway (13). Expression quantity of PTEN is lower than normal level in lining layer of affected joints, but it is normal in the sub-lining layer and can hardly be detected in invasive RA synoviocytes, indicating certain association between PTEN function and the highly activated PI3K/Akt in RA (14). Therefore, expressing PTEN protein in the pathway, targeting Akt mRNA sequence, upregulating PTEN or downregulating Akt can inhibit the pathway activity, which thus inhibits FLS proliferation, promotes its apoptosis and delays injury in affected joints (15). In this study, we sought to determine how microRNA-130a regulates osteoarthritis.

Materials and methods

**Patients.** The serum levels of patients with osteoarthritis (n=6, male, 55.45±5.23) and healthy volunteers (n=6, male, 50.56±7.62) were obtained from the department of Orthopaedics, Shanghai Tongji Hospital at the time of total knee replacement surgery. Ten milliliters of peripheral blood was collected and centrifugated at 2,000 x g for 10 min at 4˚C. Serum samples were collected and saved at -80˚C. This study was carried out in accordance with the approved guidelines of Shanghai Tongji Hospital and was approved by the Ethics Committee of Shanghai Tongji Hospital.

**RT-PCR.** Total RNA was isolated from serum samples and cell samples using TRIzol regent, and samples were treated with DNase I (both from Invitrogen, USA). cDNA was performed using oligo(dT)20 and Superscript II reverse transcriptase (both from Invitrogen, USA). Real-time PCR was performed using a StepOnePlus Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with an ABI 7500 quantitative PCR instrument. The primer sequences were as follows: U6 forward, 5'-CTCgCTTCggCAgCACA-3' and reverse, 5'-ACgCTTCAorthopaedics, Shanghai Tongji hospital at the time of total knee replacement surgery. Ten milliliters of peripheral blood was collected and centrifugated at 2,000 x g for 10 min at 4˚C. Serum samples were collected and saved at -80˚C. This study was carried out in accordance with the approved guidelines of Shanghai Tongji Hospital and was approved by the Ethics Committee of Shanghai Tongji Hospital.

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**Cell isolation, culture conditions, and cell transfection.** The use of animals was approved by the Ethics Committee of Shanghai Tongji Hospital. Male SD rats (220-250 g, 8-9 weeks) were obtained from Shanghai Slick Experimental Animal Co., Ltd. (Shanghai, China) and housed at 22-23˚C, 55-60% humidity, 7:00-19:00. The cartilage tissues were acquired, washed with phosphate-buffered saline (PBS), sterilized with 75%-ethanol alcohol and cut into pieces using micro-scissors. The tissues were digested with 0.25% Trypsin-EDTA for 30 min on ice, then digested with collagenase II (both from Invitrogen) for 4 h on ice and then filtered using 200-mesh sieve. Chondrocytes were cultured with Dulbecco's modified Eagle's medium (DMEM) with high-dose (4.5 g/l) glucose, 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin at 37˚C with 5% CO2. The microRNA-130a mimics, microRNA-130a inhibitors and their negative controls (NC) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). MicroRNA-130a inhibitors and VO-Ohpic trihydrate (10 nM, PTEN inhibitor) or wortmannin (2 nM, PI3K inhibitor) were added into cells for 48 h.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.** After transfection or inhibitor treatment, 10 µl MTT (5 mg/ml; Invitrogen) was added into cultured medium for 4 h at 37˚C. After the removal of culture medium, dimethyl sulfoxide (150 µl; Invitrogen) was added to each well, leaving the cells at room temperature in the dark for 20 min, and absorbance at 570 nm.

**Apoptosis assay.** After inhibitor treatment, cells were washed three times with PBS and resuspend using buffer (BD Biosciences, San Jose, CA, USA). Cells was stained using the 5 µl of Annexin V-FITC and 5 µl of PI double (BD Biosciences) for 15 min in the dark at room temperature. Flow cytometry (FACSCanto™) was used to measure apoptosis rate, and analyzed using CellQuest Pro software (both from BD Biosciences). Then enzyme-linked immunosorbent assay (ELISA) assay was performed. After inhibitor treatment, supernatant of all the cells was collected and used to measure interleukin-1β (IL-1β), IL-6 and IL-18 levels using ELISA kits (Nanjing Jiancheng Biology Engineering Institute, Nanjing, China). Plates were read using a Multiskan Go Microplate Spectrophotometer (Thermo Fisher Scientific) at 450 nm.

**Western blot analysis.** After transfection, cell was lysed in lysis buffer on ice for 30 min, followed by a 12,000 rpm centrifugation at 4˚C for 10 min. The amount of BCA detection reagent needed was calculated according to protein content. Proteins were resolved on an 8-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% nonfat milk in Tris-buffered saline and were incubated with primary antibodies against: Bax (sc-6236,1:500), caspase-3 (sc-98785, 1:500), caspase-9 (sc-8355, 1:500), PTEN (sc-6817-R, 1:500), PI3K (sc-7174, 1:500), p-Akt (sc-33437, 1:500) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sc-36714, 1:2,000) at 4˚C overnight. After washing with TBST, membranes were detected using HRP-conjugated secondary anti-rabbit antibodies (7074, 1:5,000; Cell Signaling Technology, Inc.) and The band was visualized with an enhanced chemiluminescence kit and analyzed using AlphaEaseFC 4.0 software.

**Statistical analysis.** Results are expressed as the mean ± SD. Data were analyzed between two groups using the Student's t-test, while among more than two groups by the One-way ANOVA method. P<0.05 provided evidence of significant differences.

**Results**

The serum levels of microRNA-130a in osteoarthritis patients. To identify the function of microRNA-130a in development and progression of osteoarthritis, microRNA-130a expression was surveyed by RT-PCR. As shown in Fig. 1, the serum levels of microRNA-130a were decreased, compared with normal group.
Overexpression of microRNA-130a increased cell proliferation and decreased apoptosis of chondrocytes. Interestingly, we used microRNA-130a mimics to increase microRNA-130a expression for the function of microRNA-130a on osteoarthritis. Fig. 2 showed that microRNA-130a mimics effectively increased microRNA-130a expression. Overexpression of microRNA-130a increased cell proliferation and decreased apoptosis of chondrocytes (Fig. 2).

Downregulation of microRNA-130a also decreased cell proliferation and induced apoptosis in chondrocytes. Next, we also used anti-microRNA-130a mimics to decrease microRNA-130a expression for the function of microRNA-130a on osteoarthritis. Similar to the results of Fig. 3, anti-microRNA-130a mimics effectively decreased microRNA-130a expression, however, downregulation of microRNA-130a also decreased cell proliferation and induced apoptosis in chondrocytes (Fig. 3).

Downregulation of microRNA-130a promoted Bax and caspase-3/9 protein expression in chondrocytes. We tested the hypothesis that the downregulation of microRNA-130a on anticancer effects in osteoarthritis. Notably, downregulation of microRNA-130a significantly promoted Bax and caspase-3/9 protein expression in chondrocytes (Fig. 4).

Downregulation of microRNA-130a increases inflammation divisors in chondrocytes. To directly test the hypothesis that osteoarthritis-induced inflammation divisors is a down-stream target of microRNA-130a, IL-1β, IL-6 and IL-18 levels were measured in this study. There was a significant increases of IL-1β, IL-6 and IL-18 levels in chondrocytes by microRNA-130a downregulation, compared with negative control group (Fig. 5).

Downregulation of microRNA-130a increased PTEN/PI3K/Akt signaling pathway in chondrocytes. We investigated which of PTEN/PI3K/Akt signaling pathways regulates the function of microRNA-130a on osteoarthritis. Compared with negative control group, the downregulation of microRNA-130a significantly increased PTEN protein expression and suppressed PI3K and p-Akt protein expression in chondrocytes (Fig. 6). Summarily, osteoarthritic patients have alterations in microRNA-130a expression, and microRNA-130a may regulate osteoarthritis.

PTEN inhibitor decreased the destructive effect of microRNA-130a on PTEN/PI3K/Akt signaling pathway of chondrocytes. In contrast, we used VO-Ohpic trihydrate, PTEN inhibitor, was used to inhibit PTEN expression in chondrocytes after microRNA-130a downregulation. PTEN inhibitor significantly suppressed PTEN protein expression, and induced PI3K and p-Akt protein expression chondrocytes after microRNA-130a downregulation (Fig. 7).
assessed whether PTEN participates in the function of microRNA-130a on osteoarthritis. PTEN inhibitor significantly promoted cell proliferation, decreased apoptosis, and suppressed Bax and caspase-3/9 protein expression in chondrocytes after microRNA-130a downregulation (Fig. 8).

**PTEN inhibitor decreases the destructive effect of microRNA-130a on inflammation divisors of chondrocytes.** We analyzed whether PTEN participates in the function of microRNA-130a on inflammation divisors of osteoarthritis. As showed in Fig. 9, PTEN inhibitor significantly reduced IL-1β, IL-6 and IL-18 levels in chondrocytes after microRNA-130a downregulation. Furthermore, PTEN regulates microRNA-130a effect on osteoarthritis.

**Wortmannin increases the destructive effect of microRNA-130a on PI3K/Akt signaling pathway of chondrocytes.** We further clarified the molecular mechanism underlying the suppressive effect of microRNA-130a on osteoarthritis. PI3K inhibitor, wortmannin, significantly suppressed PI3K and p-Akt protein expression in chondrocytes after microRNA-130a downregulation (Fig. 10).

**Wortmannin increases the destructive effect of microRNA-130a on cell proliferation of chondrocytes.** In order to determine the function of PI3K on microRNA-130a in osteoarthritis, we revealed cell proliferation of chondrocytes. As showed in Fig. 11, the inhibition of PI3K significantly decreased cell proliferation, increased apoptosis, and induced cell death.
Bax and caspase-3/9 protein expression in chondrocytes after microRNA-130a downregulation.

Wortmannin increases the destructive effect of microRNA‑130a on inflammation divisors of chondrocytes. We then examined the inhibition of PI3K on the function of microRNA‑130a on IL-1β, IL-6 and IL-18 levels. Interestingly, the inhibition of PI3K significantly reduced IL-1β, IL-6 and IL-18 levels in chondrocytes after microRNA-130a downregulation (Fig. 12). Taken together, PI3K/Akt signaling pathway participates in the function of microRNA‑130a on inflammation in osteoarthritis.

Discussion

Present study generally focuses on the molecular mechanisms by which RA initiates and develops; for instance, expression, activity and function of disease-related factors under specific tissue, cell and physiopathological conditions, as well as function, regulation and signal communication of relevant signal pathways (16). Illuminating the mechanism that drives abnormal activities or functions of cytokines, signal pathways and cells is an important component in research on RA (17). As is commonly suggested at present, RA is induced by imbalance of cellular and molecular network, which gives rise to abnormal expression and activities of cytokines [such as TNF-α, IL-1, IL-6 and nuclear factor-κB (NF-κB)], proteins and enzyme systems (like FAK, shC and MMPS) (18). In this study, we found that the serum levels of microRNA-130a were decreased. Overexpression of microRNA-130a increased cell proliferation and decreased apoptosis of chondrocytes, and downregulation of microRNA-130a also decreased cell

Figure 5. Downregulation of microRNA-130a increases inflammation divisors in chondrocytes. Interleukin-1β (IL-1β) (A), IL-6 (B) and IL-18 (C) levels. "p<0.01 compared with negative group. Ne-pcDNA3.1, negative group; anti-miR-130a, anti-microRNA-130a mimics group.

Figure 6. Downregulation of microRNA-130a suppresses PTEN/PI3K/Akt signaling pathway in chondrocytes. Downregulation of microRNA-130a suppressed PTEN, PI3K and p-Akt protein expression using western blotting (A) and statistical analysis for PTEN (B), PI3K (C) and p-Akt (D) protein expression in chondrocytes. "p<0.01 compared with negative group. Ne-pcDNA3.1, negative group; anti-miR-130a, anti-microRNA-130a mimics group.
proliferation and induced apoptosis in chondrocytes. Zumbrennen-Bullough et al suggested that microRNA-130a is upregulated suppresses hepcidin synthesis, and thereby promotes iron availability through BMP and ALK2 (19).
Activation of PI3K signal transduction pathway has been recently recognized to be one of the important mechanisms for anti-apoptosis of cells and induction of abnormal proliferation. PI3K/Akt signal pathway is closely associated with inflammation-associated cytokines, such as TNF-α, IL-1, IL-6 and NF-κB, and plays an important role in pathological process (20). PI3K can be activated by multiple extracellular stimuli, such as cytokines, growth factors, T-cell antigen, small G-protein, thrombin, cytoplasmic protein tyrosine kinase, as well as other physical and chemical factors (21). In addition, it can induce extensive biological effects through PI3K/Akt pathway, and regulate multiple cell functions, such as apoptosis, proliferation, metabolism, growth and transformation, membrane transport, secretion and chemotaxis. Moreover, it plays an important role in the pathogeneses of inflammation, tumor, metabolic and cardiovascular diseases (17). Our data suggest that downregulation of microRNA-130a increased inflammation divisors in chondrocytes. Akt is an effector of PI3K/Akt signaling pathway located in the important convergence point of multiple upstream signal activities (15). Akt is a serine/threonine protein kinase, the activation of which will induce the interaction between downstream phosphorylation cascade reaction and target proteins (22). On the contrary, it participates in the regulation
of multiple biological effects, such as cell growth and survival, proliferation and apoptosis, carbohydrate metabolism, gene transcription, neovascularization, cell migration and movement, as well as cell cycle regulation (22). This study extends
these observations suggesting that the downregulation of microRNA-130a significantly suppressed the PTEN/PI3K/Akt signaling pathway in chondrocytes.

Akt can also indirectly act on proteins or cytokines such as Bax, p53 and NF-κB, thus affecting cell survival, growth and proliferation (12). Akt plays a vital role of blocking PI3K/Akt pathway activity through targeting Akt, thereby offering possibility to treat PI3K/Akt signal activation-related diseases (23). Our data suggest that wortmannin also increased the destructive effect of microRNA-130a on inhibition of cell proliferation and apoptosis of chondrocytes through PI3K/Akt signaling. Lu et al (24) reported that microRNA-130a attenuated cardiac dysfunction and remodeling after myocardial infarction via activation of PI3K/Akt signaling via suppression of PTEN expression. This study shows that microRNA-130a regulates PI3K/Akt signaling in osteoarthritis model in vitro.

PTEN is a tumor suppressor gene possessing dual-phosphorylase activities, which is a natural inhibitor of PI3K/Akt signal pathway (14). Downregulated PTEN expression and afuaction can frequently be seen in multiple tumors (14). PTEN downregulates PI3K/Akt pathway, and inhibits a series of downstream anti-apoptosis, proliferation and invasion related signals through the dephosphorylation of PI3K (15). Furthermore, PTEN can reduce the phosphorylation levels of multiple key survival kinases, thus promoting apoptosis and inhibiting proliferation as well as infiltration (15). PTEN reduces key protein activity through dephosphorylation, and blocks multiple signal pathways that promote tumor growth, invasion, metastasis and anti-apoptosis (25). It is pointed out in literature that PTEN gene mutation or methylation cannot be detected in RA synovioocytes, but PTEN has significantly lowered activity than normal level (25). In our assays, we found that PTEN inhibitor, decreased the destructive effect of microRNA-130a on cell proliferation and apoptosis of chondrocytes. Song et al indicated that miR-130a alleviates coronary artery endothelial cell injury through downregulating PTEN and activating PI3K/Akt/eNOS signaling pathway (26). This study extends these observations suggesting that miR-130a/PTEN/PI3K/Akt signaling pathway participated in osteoarthritis apoptosis.

In conclusion, this study found that microRNA-130a targets PTEN and PI3K/Akt signal to regulate osteoarthritis-induced apoptosis and inflammation, which mediates the establishment and development of osteoarthritis. These results provide insights into the mechanism underlying directed differentiation of osteoarthritis.

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Availability of data and material

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors’ contributions

YY designed the study. YZ, SX, EH, HZ, BL and CS performed the experiments. YY and YZ analyzed the data. YY wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was carried out in accordance with the approved guidelines of Shanghai Tongji Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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