MicroRNA-20a negatively regulates the growth and osteoclastogenesis of THP-1 cells by downregulating PPAR γ

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Abstract. The present study aimed to explore the mechanisms through which microRNA (miR)-20a may be involved in the differentiation of THP-1 human acute monocytic leukemia cells into osteoclasts. THP-1 cells were differentiated into macrophages (osteoclast precursors) and subsequently into osteoclast cells. The expression levels of miR-20a in THP-1 cells were significantly reduced in a time-dependent manner during phorbol-12-myristate-13-acetate (PMA), macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor- κ B ligand RANKL-induced osteoclastogenesis. Following transfection with a miR-20a mimics, the levels of miR-20a in PMA-treated THP-1 cells increased more than 40-fold as compared with expression in the control cells. In addition, the overexpression of miR-20a inhibited proliferation, initiated S phase cell cycle arrest and induced apoptosis of PMA-treated THP-1 cells. Additionally, miR-20a mimics treatment notably decreased the levels of tartrate-resistant acid phosphatase, nuclear factor of activated T-cells, cytoplasmic 1 and peroxisome proliferator-activated receptor γ (PPAR γ) during THP-1 cell further differentiation progress. In summary, miR-20a may negatively regulate the proliferation and osteoclastogenesis of THP-1 cells during its osteoclast differentiation progress by downregulating PPAR γ .

Introduction

THP-1 human acute monocytic leukemia cells can differentiate into macrophages (1), osteoclasts (2) and dendritic cells (3). THP-1 cells can also be differentiated from macrophages to osteoclasts using cytokines and exogenous hormones, such as macrophage colony-stimulating factor (M-CSF) and receptor

activator of nuclear factor- κ B ligand (RANKL) (2,4). M-CSF and RANKL are expressed in osteoblasts or activated T cells and are involved in osteoclastogenesis (5,6). The expression of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), a key transcription factor involved in osteoclastogenesis, is induced by RANKL (7). Tartrate-resistant acid phosphatase (TRAP) and cathepsin K are osteoclast-specific markers and are involved in the NFATc1 pathway (8). In addition, microRNA (miR)-218 negatively regulates osteoclast differentiation and bone remodeling by blocking the RANKL-induced p38 mitogen activated protein kinase/c-Fos/NFATc1 pathway (9).

MicroRNAs (miRNAs) are small non-coding RNAs, ~22 nucleotides in length, that are involved in post-transcriptional regulations (10). miRNAs regulate cell growth and differentiation of osteoblasts and osteoclasts from embryonic bone to skeletal maturation (11). Members of the miR-17-92 family, including miR-20a, are associated with monocytic differentiation and maturation by regulating the expression levels of acute myeloid leukemia-1 protein and M-CSF receptor (12). However, the expression and function of miR-20a during osteoclast differentiation remain elusive. miR-20a downregulates autophagy by targeting the 3' untranslated region (3'-UTR) of autophagy related 16-like 1 during hypoxia-induced osteoclast differentiation (13). miR-20a overexpression was also previously reported to suppress osteoclast differentiation and bone resorption by downregulating RANKL expression (14). Therefore, elucidation of the potential mechanism involving miR-20a during osteoclastogenesis is crucial.

The present study investigated the potential mechanisms of miR-20a during the differentiation of THP-1 cells into osteoclasts. The results may provide a new perspective to the understanding of miR-20a in osteoclast differentiation.

Materials and methods

Cell culture and differentiation induction. THP-1 human acute monocytic leukemia cells (cat. no. TIB-202; American Type Culture Collection, Manassas, VA, USA) were incubated in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C under

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5% CO₂ and saturation humidity. THP-1 cells in the logarithmic stage of growth were seeded (2×10^5 cells/well) into 6-well plates and co-cultured with 200 ng/ml phorbol-12-myristate-13-acetate (PMA; cat. no. 79346; Sigma-Aldrich; Merck KGaA), 25 ng/ml M-CSF (PeproTech, Inc., Rocky Hill NJ, USA) and 30 ng/ml RANKL (PeproTech, Inc.) to induce their differentiation into osteoclast-like cells, as previously described (2). Cells were cultured for 0, 24, 72 and 120 h, collected and stained by using an Acid Phosphatase, Leukocyte (TRAP) kit (cat. no. 387; Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. Subsequently, the TRAP-positive stained cells were photographed using a light microscopy (Nikon Corporation, Tokyo, Japan), and the number of TRAP-positive staining cells were counted and analyzed. Subsequently, the miR-20a expression levels were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) at 0, 24, 72 and 120 h, as described below.

Cell treatment and miR-20a mimics transfection. To investigate the influences of miR-20a on the process of monocyte differentiate into osteoclasts, THP-1 cells were co-cultured with 10 ng/ml PMA to induce their differentiation into macrophages (osteoclast precursors) at 37°C for three days (1). Subsequently, the THP-1 cell-derived macrophages were seeded (1×10^4 cells/well) into 96-well plates and transfected with 100 nM miR-20a mimics or 100 nM miRNA negative controls (miR-NC; GenePharma, Shanghai, China) at 37°C for 6 h using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's guidelines. Untransfected THP-1 cell-derived macrophages were used as a Blank group. At 24 h post-transfection, the transfection efficiency of miRNAs was assessed by determining the miR-20a expression levels by RT-qPCR, using the following primers: Forward, 5'-CCGCTCGTGAAA TGTTTAGG-3', reverse primer, 5'-ATGGAGCCTGGGACG AGA-3'. U6 (forward primer 5'-ATTGGAACGATACAG AGAAGATT-3'; reverse primer, 5'-GGAACGCTTCACGAA TTTG-3') was used as an internal control for normalization based on the study of Zhou *et al* (15). For RT-PCR, miRNAs was extracted from 2×10^6 cells by using a mirVana miRNA isolation kit (Applied Biosystems, Foster City, CA, USA) as per the manufacturer's protocol. The cDNA was synthesized by using the Prime Script RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China). The RT-PCR was performed using SYBR® PrimeScript® miRNA RT-PCR kit (Takara Biotechnology Co., Ltd.) on FTC-3000™ Real-time PCR Cycler (Funglyn Biotech Corp. Ltd., Toronto, ON, Canada). The parameters for RT-PCR were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 45 sec.

Cell proliferation assay. Following miR-20a mimics transfection, 2×10^5 cells were seeded into 6-well plates. Cell proliferation was examined at 0, 24, 48 and 72 h using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), following the manufacturer's protocol. Optical density (OD) was detected at 450 nm using a Benchmark microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The rate of cell proliferation was calculated using the following formula: $(OD_{\text{test}} - OD_{\text{blank}}) / (OD_{\text{control}} - OD_{\text{blank}}) \times 100$. All assays were performed in triplicates.

Cell cycle analysis. Following miR-20a mimic treatment for 48 h, cells were fixed overnight in ice-cold ethanol (at 4°C). The cells were washed with PBS and incubated with a 1 ml solution comprising 20 mg/ml propidium iodide (PI) and 10 U/ml RNaseA (cat. no. KGA214; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) for 30 min at room temperature. Cells were counted by Accuri C6 Fluorescence-activated cell sorting (BD Biosciences, Franklin Lakes, NJ, USA). Progression of the cell cycle was assayed using the ModFit LT 3.0 software (Verity Software House, Topsham, ME, USA).

Assessment of apoptosis. Following miR-20a mimic treatment, cells were collected at 48 h. Cell suspensions were prepared by centrifuging cell suspensions at $1,000 \times g$ for 5 min at room temperature. Cells from pellets were re-suspended in PBS and centrifuged at $1,000 \times g$ for 5 min under room temperature, and the supernatant was removed. Subsequently, cells were incubated with Annexin V-Fluorescein isothiocyanate (FITC)/PI, from the Annexin V-FITC/PI Double-staining Apoptosis Detection kit (cat. no. M3021-3; Molecular Biology and Chemical, Shanghai, China) for 15 min away from light, according to the manufacturer's protocol. Cells were analyzed immediately on a BD Accuri C6 flow cytometer equipped with BD Accuri C6 software (BD Biosciences; Becton-Dickinson and Company, Franklin Lakes, NJ, USA). Experiments were performed three times.

RT-qPCR. Transfected cells were further incubated with M-CSF (25 ng/ml) and RANKL (30 ng/ml) for 3 days (2). To assess the changes of TRAP, NFATc1 and peroxisome proliferator-activated receptor γ (PPAR γ) mRNA expression levels, 2×10^6 cells were collected and treated with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was used for RT-qPCR. The cDNA was synthesized by the Prime Script RT-PCR kit. SYBR-Green-based real time RT-PCR was performed using SYBR® Premix Ex Taq™ II (Takara Biotechnology Co., Ltd) on FTC-3000™ Real-time PCR Cycler. The parameters for RT-PCR were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 30 sec, and finally 72°C for 90 sec. Primers specific for TRAP, NFATc1, PPAR γ and GAPDH were synthesized as previously described (16) and as follows: TRAP forward, 5'-GGAGATCAGCTCCAAAGA GATG-3' and reverse primer, 5'-GGGCAGTCATGGGAG TTCAG-3'; NFATc1 forward, 5'-AGACCGTGTCCACCACCA GC-3' and reverse primer, 5'-CAGGATTCCGGCACAGTC AAT-3'; PPAR γ forward, 5'-TGGCCTCCTTGATGAATA-3' and reverse primer, 5'-GGCTTGATGACAGGTTGTC-3'; GAPDH forward, 5'-CATGAGAAGTATGACAACAGCCT-3' and reverse primer, 5'-AGTCCTTCCACGATACCAAGT-3'. GAPDH was used as an internal control and for the normalization of TRAP, NFATc1 and PPAR γ mRNA levels. Relative mRNA levels were calculated using the $2^{-\Delta\Delta C_q}$ method (17).

Western blotting. Cells in the Blank and miR-transfected groups were treated with M-CSF and RANKL for three days. Subsequently, the cells (5×10^6) were lysed using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Inc.). Cell lysates were centrifuged at $12,000 \times g$ for 1 min and 30 μg total proteins, the concentration determined

Figure 1. Expression levels of miR-20a during THP-1 cell osteoblast differentiation process. (A-D) THP-1 cells were treated with phorbol-12-myristate-13-acetate, macrophage colony-stimulating factor and receptor activator of nuclear factor- κ B ligand aiming to induce osteoclastogenesis. (A) Cells were stained for TRAP expression at 0, 24, 72 and 120 h post-treatment; images of TRAP-positive staining cells (with reddish/brown cytoplasm as indicated by arrows) differentiated from THP-1 cells; magnification, $\times 200$. (B) The percentage of TRAP-positive staining cells at different time-points from (A); *** $P < 0.001$ vs. 0 h. (C) Relative expression levels of miR-20a in differentiating THP-1 cells; *** $P < 0.001$ vs. 0 h; ### $P < 0.001$ vs. 24 h; ††† $P < 0.001$ vs. 72 h. (D) Relative expression levels of miR-20a in miR20a mimics-transfected THP-1 cells; *** $P < 0.001$ vs. Blank; ### $P < 0.001$ vs. miR-NC. miR, microRNA; NC, negative control; TRAP, tartrate-resistant acid phosphatase.

by BCA kit (cat. no. BCA1; Sigma-Aldrich; Merck KGaA) were separated by 8% SDS-PAGE, followed by transfer of separated proteins to polyvinylidene difluoride membranes. Membranes were blocked with Blocking Buffer (1X PBS; 0.1% Tween-20; 5% w/v bovine serum albumin (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature and incubated with antibodies against TRAP (1:100; cat. no. sc-28204; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), NFATc1 (1:100; cat. no. sc-13033; Santa Cruz Biotechnology, Inc.) or PPAR γ (1:100; cat. no. sc-7273; Santa Cruz Biotechnology, Inc.) at room temperature for 1.5 h. Subsequently, the goat anti-mouse IgG secondary antibody (1:10,000; cat. no. 115-035-003; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA), goat anti-Rabbit IgG secondary antibody (1:10,000; cat. no. 115-035-003; Jackson ImmunoResearch Laboratories Inc.) and rabbit anti-Goat IgG secondary antibody (1:10,000; cat. no. 33701ES60; Yeasen Corporation, Shanghai, China) was added correspondingly for incubation at 4°C overnight. Protein levels were normalized to those of GAPDH (1:200; cat. no. sc-47724; Santa Cruz Biotechnology, Inc.). Expression levels of protein in each sample were calculated using the Gel-Pro analyzer 3.0 software (Media Cybernetics, Inc., Rockville, MD).

Statistical analysis. All experiments were performed in triplicate. Data are expressed as mean $\pm$ standard deviation, and were analyzed using SPSS 19.0 software (IBM Corp., Armonk,

NY, USA). Student's t-test was used to analyze the statistical significance between two groups. Data from more >3 groups were analyzed by one-way or two-way analysis of variance, followed by Bonferroni's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-20a expression during THP-1 cell osteoblast differentiation. It was observed that the number of TRAP-positive staining cells was significantly increased and some of the THP-1 cells were induced into osteoblasts ($P < 0.001$; Fig. 1A and B). The TRAP-positive cells included both macrophages (osteoclast precursors) and osteoclasts. In addition, miR-20a expression levels were significantly increased at 24 h ($P < 0.001$) and 72 h ($P < 0.001$), but significantly decreased at 120 h ($P < 0.001$) compared with expression levels at 0 h (Fig. 1C). In addition, there was a time-dependent decrease in miR-20a expression levels from 24 to 120 h. These data demonstrated that miR-20a expression was decreased post-differentiation induction. Following transfection with miR-20a mimics into PMA treated THP-1 cells, miR-20a expression levels were significantly increased in cells of by ~ 40 -fold compared with the Blank and miR-NC-transfected groups ($P < 0.001$; Fig. 1D). Since the result in Fig. 1D proved that there was no significant difference between the Blank and miR-NC group, therefore, only the Blank group was used in Figs. 2 and 3.

Figure 2. miR-20a inhibits the growth of differentiated THP-1 cells. (A-D) Following miR-20a mimics transfection into differentiating THP-1 cells, proliferation, cell cycle and apoptosis were examined. (A) The relative change of cell proliferation was determined at 0, 24, 48 and 72 h post-transfection; ** $P < 0.01$ and *** $P < 0.001$ vs. the Blank. Data at 0 h was indicated as 1. (B) Images of cell cycle detection by flow cytometry. (C) The percentage of cell cycle distribution based on (B); * $P < 0.05$ and ** $P < 0.01$ vs. Blank. (D) Images of cell apoptosis by flow cytometry. (E) The percentage of apoptotic cells (Q2+Q4) based on (D). ** $P < 0.01$ vs. Blank. FITC, fluorescein isothiocyanate; miR, microRNA; PI, propidium iodide.

Figure 3. TRAP, NFATc1 and PPAR γ expression levels in THP-1 cell osteoblast differentiation. (A and B) THP-1 cells were transfected with miR-20a mimics, and were continually treated with M-CSF and RANKL for 3 days. (A) Relative mRNA expression levels of TRAP, NFATc1 and PPAR γ were examined by reverse transcription-quantitative polymerase chain reaction; *** $P < 0.001$ vs. Blank. (B) Relative protein expression levels of TRAP, NFATc1 and PPAR γ were examined by western blotting; expressions were normalized to GAPDH; * $P < 0.05$ and *** $P < 0.001$ vs. Blank. miR, microRNA; NFATc1, nuclear factor of activated T-cells, cytoplasmic 1; PPAR γ , peroxisome proliferator-activated receptor γ ; TRAP, tartrate-resistant acid phosphatase.

miR-20a inhibits the proliferation of PMA-treated THP-1 cells. Results of cell proliferation assays demonstrated that cell proliferation was markedly inhibited in the miR-20a mimics group compared with proliferation in the Blank group at 48 and 72 h ($P < 0.01$ and $P < 0.001$, respectively; Fig. 2A).

Furthermore, the percentage of cells in the S phase of the cell cycle was significantly decreased in the miR-20a mimics group compared with the Blank group ($P < 0.01$; Fig. 2B and C), which indicated that cell division was inactive following miR-20a mimics transfection. In addition, the total number of early and

late apoptotic cells was significantly higher in the miR-20a mimics group ($65.63 \pm 2.99\%$) compared with apoptotic cells in the Blank group ($54.3 \pm 2.17\%$; $P < 0.01$, Fig. 2D and E). These results demonstrated that miR-20a may inhibit the growth of PMA-treated THP-1 cells.

miR-20a negatively regulates osteoclastogenesis by decreasing PPAR γ during THP-1 cell differentiation process. As the master regulators of osteoclastogenesis, the mRNA expression levels of TRAP (18), NFATc1 (19) and PPAR γ (20) were significantly decreased during the THP-1 cell differentiation process in cells transfected with miR-20a mimics compare with cells in the Blank group ($P < 0.001$; Fig. 3A). In addition, results of western blot analysis confirmed that the protein levels of TRAP ($P < 0.05$), NFATc1 ($P < 0.001$) and PPAR γ ($P < 0.001$) were also significantly decreased in the miR-20a mimics group compare with the Blank group (Fig. 3B). These results indicated that miR-20a may regulate osteoclastogenesis by decreasing PPAR γ expression during THP-1 cell differentiation process.

Discussion

Results from the present study revealed that the expression levels of miR-20a in THP-1 cells decreased during PMA, M-CSF and RANKL-induced osteoclastogenesis. Following miR-20a mimics transfections, PMA-treated THP-1 cells exhibited weakened cell growth ability. In addition, TRAP, NFATc1 and PPAR γ levels were significantly decreased following upregulated miR-20a expression during the THP-1 cell differentiation progress. In summary, increased miR-20a expression negatively regulated the growth and osteoclastogenesis of THP-1 cells during its osteoclast differentiation progress by downregulating PPAR γ .

miRNAs not only positively regulate osteoblast differentiation, but also negatively regulate osteoclastogenesis (21-23). It has been reported that miR-20a promotes osteoblast differentiation through the activated bone morphogenetic protein (BMP)/Runx2 pathway *in vitro* by directly binding to the 3'-UTR of BMP-2 (24,25). However, another study demonstrated that miR-20a inhibited dexamethasone-induced osteoclast differentiation through the downregulation of RANKL expression (14), and miR-20a was reported to be downregulated during osteoclastogenesis owing to its decreased levels in mature osteoblasts (26). Therefore, the role of miR-20a regulation in osteoclastogenesis is controversial. In the present study, miR-20a was demonstrated to be downregulated in a time dependent manner during PMA, M-CSF and RANKL-induced osteoclastogenesis. Notably, miR-20a expression levels were significantly increased at 24 and 72 h when compared to 0 h, but significantly decreased at 120 h when compared to 0 h, which seemed to exist contradict but was true results. It was hypothesized that this may be associated with the differentiation state and degree of monocytes, which need to be investigated in the authors' future studies.

A previous study confirmed that miR-20a overexpression reduced the levels of osteoclast differentiation markers TRAP and NFATc1 in hypoxia-induced osteoclast differentiation (13). In addition, it was previously reported that miR-20a inhibited NFATc1 activity (27).

The nuclear transcription factor PPAR γ is a subtype of the PPAR family and is involved in cell differentiation, growth and apoptosis (28). Human PPAR γ was reported to be a target gene of miR-20a and a component of the BMP/Runx2 pathway, and is involved in osteoblast differentiation in human mesenchymal stem cells (25). Additionally, mouse miR-20a suppressed the differentiation of adipocyte progenitor cells by inducing the expression of PPAR γ in bone marrow stem cells (29). During the differentiation of bone marrow stem cells to osteoblasts, naringin promoted the differentiation of osteoblasts through the overexpression of miR-20a, which negatively regulated expression levels of PPAR γ (30). In the present study, the upregulation of miR-20a negatively regulated osteoclast differentiation of THP-1 cells by decreasing PPAR γ . However, whether miR-20a directly targeted PPAR γ during THP-1 osteoblast differentiation is still unclear and requires further investigation.

A previous study demonstrated that the regulation of miR-181a induces osteoclast apoptosis (31), and the overexpression of miR-20a was reported to inhibit hepatocellular carcinoma cell proliferation by regulating cell cycle and apoptosis (32). miR-20a downregulated proliferation-associated target genes to modulate cell cycle progression and to downregulate E2F transcription factors in order to control the G1/S transition as well as WEE1 involved in the G2/M transition (33). In the present study, miR-20a overexpression inhibited cell proliferation, and induced S phase cell cycle arrest and cell apoptosis of PMA treated THP-1 cells.

In conclusion, miR-20a may negatively regulate the growth and osteoclastogenesis of THP-1 cells during osteoclast differentiation by downregulating PPAR γ expression, which indicated a role for miR-20a in osteoclast differentiation and also provided a new direction for the investigation of pathological processes involved in osteoclast differentiation.

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Availability of data and materials

The datasets used/and or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HW conceived and designed the research, acquired the data and drafted the manuscript. YS performed the statistical analysis and revised the manuscript for important intellectual contents.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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