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MicroRNA-23a Modulates Tumor Necrosis Factor-Alpha-Induced Osteoblasts Apoptosis by Directly Targeting Fas

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ABSTRACT

Tumor necrosis factor (TNF)-alpha is a key cytokine regulator of bone and mediates inflammatory bone loss. The molecular signaling that regulates bone loss downstream of TNF-alpha is poorly defined. Recent studies implicated an important role of microRNAs (miRNAs) in TNF-alpha-mediated bone metabolism, including osteoblasts differentiation, osteoclasts differentiation and apoptosis. However, there are very few studies on the complex regulation of miRNAs during TNF-alpha-induced osteoblasts apoptosis. In the present study, the clonal murine osteoblastic cell line, MC3T3-E1, was used. We screened for differentially expressed miRNAs during TNF-alpha induced MC3T3-E1 cell apoptosis and identified microRNA-23a as a potential inhibitor of apoptosis. To delineate the role of microRNA-23a in apoptosis, we respectively silenced and overexpressed microRNA-23a in MC3T3-E1 cells. We found that microRNA-23a depletion significantly enhances TNF-alpha-induced MC3T3-E1 cell apoptosis and over-expressing microRNA-23a remarkably attenuates this phenomenon. Mechanistic studies showed that microRNA-23a inhibits Fas expression through a microRNA-23a-binding site within the 3′-untranslational region of Fas. The post-transcriptional repression of Fas was further confirmed by luciferase reporter assay. These results showed that microRNA-23a, an important protecting factor, plays a significant role in the process of TNF-alpha induced MC3T3-E1 cell apoptosis, by regulating Fas expression. J. Cell. Biochem. 114: 2738–2745, 2013.

KEY WORDS: TNF-alpha; microRNA-23a; MC3T3-E1; APOPTOSIS; Fas

Tumor necrosis factor (TNF)-alpha is a vital cytokine, which is a major player in pathological bone loss caused by chronic inflammation and many other diseases, such as postmenopausal osteoporosis [Jurisic et al., 2008a,b; David and Schett, 2010; Mucci et al., 2012]. Local or systemic increase in the concentration of TNF-alpha leads to complex deregulation of bone metabolism that not only favors bone destruction by increasing osteoclasts differentiation and activation, but also inhibits bone formation by blocking differentiation and activity of osteoblasts as well as inducing their apoptosis [David and Schett, 2010]. It has been well established by both in vivo and in vitro studies that TNF-alpha regulates osteoclasts and osteoblasts differentiation [Abu-Amer et al., 2000; Zhang et al., 2001]. However, the exact mechanism of TNF-induced osteoblasts apoptosis is still unclear.

Apoptosis is a programmed cell death pathway characterized by plasma-membrane blebbing, nuclear condensation, and nucleosomal DNA fragmentation [Elmore, 2007]. Apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway. The extrinsic pathway is initiated through the stimulation of the transmembrane death receptors, such as Fas/CD95/Apo-1 receptor and TNFR1. In contrast, the intrinsic pathway is initiated through the release of signal factors by mitochondria within the cell, which is triggered by many cellular abnormal events [Fulda and Debatin, 2006]. Previous studies showed that TNF-alpha can bind to two different receptors TNFR1 or p55 and TNFR2 or p75 [Kruglov et al., 2008; Neumann et al., 2012]. It has been reported that TNF-alpha binding to TNFR1 is a known activator of pro-apoptotic signals in osteoblasts [Bu et al., 2003]. Furthermore, TNF-alpha induces Fas protein in the human osteosarcoma cell line MG-63 and in mouse MC3T3-E1 cells [Kawakami et al., 1997; Ozeki et al., 2002]. It has been confirmed that Fas receptor plays an important role in TNF alpha-induced MC3T3-E1 cell apoptosis [Ozeki et al., 2002]. However, the

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precise molecular events underlying the effect of TNF-alpha on apoptotic pathways in osteoblasts need to be further explored.

MicroRNAs (miRNAs) have emerged as key negative regulators of diverse biological and pathological processes, including developmental timing, organogenesis, apoptosis, cell proliferation, and differentiation and in the control of tumorigenesis [Jaillon et al., 2007; Croce, 2008]. The miRNAs bind to the 3′-untranslational region (3′-UTR) of target mRNAs and either block the translation or initiate the transcript degradation. The miRNAs may also increase translation of selected mRNAs in a cell cycle-dependent manner. Although 1,048 miRNAs have been identified in the human genome, the biological functions of relatively few miRNAs have been characterized in detail [Ambros, 2004]. Recently, the impact of miRNAs on differentiation of osteoblasts and osteoclasts regulated by TNF-alpha have been investigated [Croce, 2008; Kagiya and Nakamura, 2012]. However, few are known about the complex regulation of gene expression in osteoblasts apoptosis induced by TNF-alpha. Notably, no previous studies have reported the expression profiling of miRNAs during TNF-alpha-regulated osteoblast apoptosis.

In this study, we screened for differentially expressed miRNAs during TNF-alpha induced MC3T3-E1 cell apoptosis and identified miRNA-23a (miR-23a) as a potential inhibitor of apoptosis. By modulating miR-23a activity, we show that inhibition of miR-23a by an 2′-O-methyl antisense inhibitory oligonucleotides (AMO-23a) markedly increased TNF-alpha induced MC3T3-E1 cell apoptosis, whereas miR-23a overexpression reversed these effects. Fas was identified as a direct target of miR-23a. Our findings that inhibition of miR-23a by AMO-23a leads to stimulation of Fas that coincides with increased osteoblasts apoptosis suggest that miR-23a modulates TNF-alpha-induced osteoblasts apoptosis by directly targeting Fas.

MATERIALS AND METHODS

CELL CULTURE

The clonal murine osteoblastic cell line, MC3T3-E1, was cultured in a-modified essential medium (a-MEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Human embryonic kidney cell line (HEK-293) was cultured in Dulbecco′s modified Eagle′s medium (DMEM). The cultures were supplemented with 10% FBS and 100 μg/ml penicillin/streptomycin.

FLOW CYTOMETRIC STAINING

The apoptosis and necrosis on the MC3T3-E1 cells were analyzed by flow cytometry as Jurisic et al. [2006] reports. Briefly, cells were washed in ice-cold PBS, pelleted, and resuspended in 0.3 ml of hypotonic fluorochrome solution containing 50 μg/ml propidium iodide (PI) (Sigma, St. Louis, MO), 0.1% sodium citrate, and 0.1% Triton X-100 (Sigma) to quantitate the cellular DNA content under the permeabilized condition. Phosphatidyl-serine (PS) exposure due to flipping of the plasma membrane, a concomitant feature during apoptosis, was evaluated by annexin V-FITC staining. Cells were then analyzed by standard procedure with FACS Calibur 440 E flow cytometer (Becton Dickinson). A total of 10,000 gated events were analyzed, using Cell QUEST software. Results were expressed as percentage of necrotic and apoptotic cells, calculated as mean values from three to four independent experiments.

HOECHST 33258 STAINING

MC3T3-E1 cells were fixed, stained with Hoechst 33258 and observed using fluorescence microscopy. Cells were designated as apoptosis with highly condensed, brightly staining nuclei and non-apoptosis with light green staining. The apoptotic index was defined as the ratio of the apoptotic cell number to total cell number.

TUNEL ASSAY

TdT-UTP nick end labeling (TUNEL) assays were performed with a commercially available in situ cell death detection kit (Roche) according to the manufacturer′s instruction. Briefly, the cells were fixed with 4% paraformaldehyde solution for 30 min at room temperature and then washed with PBS and incubated with permeabilization solution (0.1% Triton X-100, 0.01% sodium citrate) for 2 min on ice followed by TUNEL for 1 h at 37°C. The FITC-labeled TUNEL-positive cells were imaged under a fluorescent microscope by using 488 nm excitation and 530 nm emission. The cells with green fluorescence were defined as apoptotic cells.

MEASUREMENT OF CASPASE-3 ACTIVITY

Caspase-3 activity was measured by cleavage of chromogenic caspase substrates, Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Aspp-nitroanilide), which is a caspase-3 substrate. The optical density value at 405 nm was thus used as indication for the amount of caspase-3. The specific caspase-3 activity, normalized for total proteins of cell, was then expressed as fold of the baseline caspase activity of control cells.

SYNTHESIS OF miRNAs AND SEQUENCES OF miR-23a INHIBITORS

MiR-23a was synthesized by Integrated DNA Technologies (IDT). The sequences of miR-23a inhibitors (AMOs; anti-miRNA oligonucleotides) used are as follows: AMO-23a: 5′-GGAAAUCCCUGGCAAUGUGAU-3′. NC: 5′-CAGUACUUUUGUGUAGUACAA-3′. DNA fragments of the 3′-UTRs of Fas mRNA containing the putative miR-23a binding sequence were synthesized by Invitrogen. These fragments were then respectively cloned into the multiple cloning sites downstream the luciferase gene (HindIII and SpeI sites) in the pMIR-REPORT™ luciferase miRNA expression reporter vector (Ambion, Inc.), as described elsewhere [Yang et al., 2007].

MICRORNA MICROARRAY ANALYSIS

Quiescent (growth-arrested) MC3T3-E1 cells cultured in 0.3% FBS-aMEM were divided into two groups: control group, TNF-alpha group. Control group cells were cultured in normal culture medium. TNF-alpha group cells were treated with 1 nM TNF-alpha for 24 h, as Weinstein et al. [1998] report. Following treatment, total RNA was isolated from untreated samples, TNF-alpha-treated samples. RNAs were purified according to the manufacturer′s protocol before being subjected to Affymetrix® GeneChip® miRNA arrays. Five micrograms of total RNA from each sample was labeled and hybridized on miRNA microarray chips. Data were extracted from the images and analyzed using the miRNA QC tool from Affymetrix. Both an expression value of more than 50 and a *P* value of less than 0.05 were used as a cutoff for expressed miRNAs.

TRANSFECTION OF miRNAs AND LUCIFERASE ASSAY

DNA fragments of the 3′-UTRs of Fas mRNA containing the putative miR-23a binding sequence were synthesized by Invitrogen. This fragment was cloned into the multiple cloning sites downstream the luciferase gene (HindIII and SpeI sites) in the pMIR-REPORTTM luciferase miRNA expression reporter vector (Ambion, Inc.). HEK293 cells (1×10^5 per well) were transfected with 1 μg miR-23a or 1 μg PGL3-target DNA (firefly luciferase vector) and 0.1 μg PRL-TK (TK-driven Renilla luciferase expression vector), with Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Luciferase activities were measured 48 h after transfection with a dual luciferase reporter assay kit (Promega).

RNA INTERFERENCE

Fas siRNA (m) and control siRNAs containing a scrambled sequence (Santa Cruz Biotechnology) were dissolved in RNase-free PBS. Cells were plated in 6-well plates (2×10^5 cells/well) for 18–24 h before transfection. On the day of transfection, RNA-lipid complexes were introduced into each well of cells (4 μL/well siRNA duplex for MC3T3-E1). 24–72 h post-transfection, the level of target knockdown was assessed using Western blot.

QUANTIFICATION OF miRNA LEVELS

Expression levels of miRNAs were determined by quantitative RT-PCR (qRT-PCR). Total RNA was extracted using Trizol reagent (Invitrogen), treated with DNaseI (Ambion, Inc.). The qRT-PCR for miRNAs was performed using mirVanaTM qRT-PCR miRNA Detection Kit and SYBR Advantage qPCR Premix (Clontech) on an Applied Biosystems 7500 Fast system according to the manufacturer's instructions. Relative expression of each miRNA was normalized to the U6 snRNA (ΔCT) in each sample. Quantitative expression of a given miRNA between retinoic acid (RA) treatment and vehicle was determined according to the CT method ($2^{-\Delta\Delta CT}$).

WESTERN BLOT ANALYSIS

The protein samples were extracted from MC3T3-E1 cells, with the procedures essentially the same as described in detail elsewhere [Lin et al., 2013]. Protein samples were fractionated by SDS–PAGE. The primary antibodies against Fas and FasL with β-actin as an internal control were used.

RESULTS

EFFECTS OF TNF-ALPHA ON MC3T3-E1 CELL APOPTOSIS

To test the role of TNF-alpha on MC3T3-E1 cell apoptosis, cultures of MC3T3-E1 cells were treated with TNF-alpha and apoptosis was assessed through Hoechst 33258 dye and TUNEL. When MC3T3-E1 cells were treated with 1 nM TNF-alpha for 24 h, significant apoptosis was observed by fluorescence photomicrographs (Fig. 1A–D). Furthermore, apoptotic progression was also monitored in MC3T3-E1 cells via flow cytometric analysis of phosphatidylserine exposure and plasma membrane integrity. The percent of apoptotic cells in response to TNF-alpha was significantly increased. These cells exhibited phosphatidylserine externalization as determined by a significant increase in annexin-FITC fluorescence and decreased plasma membrane integrity demonstrated by a modest increase in propidium iodide fluorescence (Fig. 1E,F).

Due to the fact that caspase-3 plays an important role in the process of apoptosis, caspase-3 activities were measured to reflect apoptosis (Fig. 1G). Following 24 h treatment with 1 nM TNF-alpha, an increased caspase-3 activity was observed in MC3T3-E1 cells. These results indicated that TNF-alpha promotes MC3T3-E1 cell apoptosis.

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED miRNAs DURING TNF-ALPHA-INDUCED MC3T3-E1 CELL APOPTOSIS

To identify a miRNAs expression signature in TNF-alpha-induced MC3T3-E1 cell apoptosis, MC3T3-E1 cells were subjected to TNF-alpha assault and analyzed with a RNA/cDNA-based microarray screening. Among the 617 miRNAs represented on our chip, 31 were differentially expressed in response to TNF-alpha-induced apoptosis (Fig. 2A). According to the results from miRNAs chip, seven miRNAs which expression was decreased more than twofold were selected for validation by qRT-PCR (Fig. 2B). Microarray analysis and qRT-PCR data showed that the expression of miR-23a, -17, -20a, -92a, and -21, let-7c, and -7d are significantly down-regulated during TNF-alpha-induced MC3T3-E1 cell apoptosis (Fig. 2C).

To evaluate the biological effect of the differentially expressed miRNAs, we searched for their potential regulatory targets using algorithms based on miRNA-mRNA complementarity and its evolutionary conservation [TargetScan]. The screening identified miR-23a as a potential negative regulator of osteoblastic apoptosis. Furthermore, we firstly did the research about miR-23a because its expression was the most significantly downregulated during TNF-alpha-induced MC3T3-E1 cell apoptosis.

THE EFFECT OF miR-23a ON MC3T3-E1 CELL APOPTOSIS

For functional evaluation of miR-23a, we overexpressed or inhibited miR-23a levels using synthetic miR-23a and AMO-23a modified antimiR-oligonucleotides complementary to mature miR-23a. We found that inhibition of miR-23a significantly enhances TNF-alpha-induced MC3T3-E1 cell apoptosis, which is indicated by higher quantity of apoptotic cells monitored via flow cytometric analysis, visualized highly condensed, brightly staining nuclei by Hoechst 33258 dye and a significantly increased in the number of TUNEL-positive cells and caspase-3 activity (Fig. 3A–G). Similar results were obtained when AMO-23a-transfected MC3T3-E1 cells were not treated with TNF-alpha. In contrast, after treatment with miR-23a, quantities of apoptotic cells induced by TNF-alpha were reduced (Fig. 3A–G). Taken together, our results suggest that miR-23a is a negative regulator of TNF-alpha-induced osteoblasts apoptosis.

The degree of miRNA inhibition and overexpression was monitored by qRT-PCR after transfection of miR-23a or AMO-23a, respectively, to MC3T3-E1 cells. Mature miR-23a levels were elevated appropriately sevenfold relative to control-treated cells. By comparison, treatment of MC3T3-E1 cells with AMO-23a lead to inhibition of endogenous miR-23a by threefold after transfection (Fig. 3H). These results proved the feasibility of all experiments based on overexpressed or inhibited miR-23a levels.

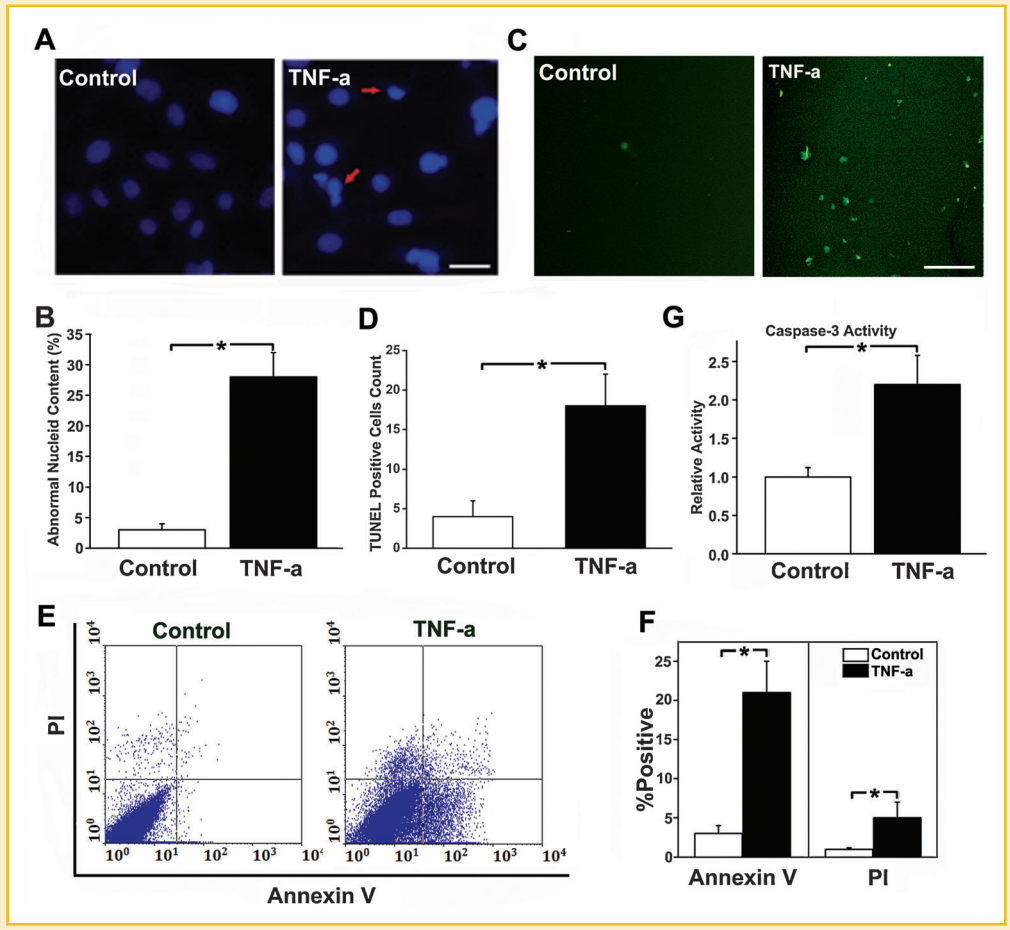

Figure 1. Regulation of MC3T3-E1 cell apoptosis by TNF-alpha. A,B: MC3T3-E1 cells nuclei were stained by DNA binding florescence dye Hoechst 33258 and examined by fluorescence microscopy. Scale bars are 10 μm. The results are expressed as the ratio of abnormal nuclei (crenation, condensation, and fractionation) to the total number of nuclei stained by Hoechst 33258 from six independent photographs shot in each group; $^{*}P < 0.05$. C,D: After TNF-alpha treatment for 24 h, the cells were TdT-UTP nick end labeled and imaged by fluorescent microscope. Scale bar = 100 μm. The results are expressed as the number of green points in each photograph. All values are denoted as positive mean values ± s.e.m. from six independent photographs shot in each group; $^{*}P < 0.05$. E,F: Apoptotic progression was monitored via flow cytometric analysis of phosphatidylserine exposure and plasma membrane integrity. The results are expressed as percentages of positive mean values ± s.e.m. n = 4, $^{*}P < 0.05$. G: Measurement of Caspase-3 activity from MC3T3-E1 cells treated with 1 nM TNF-alpha for 24 h; n = 4, $^{*}P < 0.05$. TNF-a indicates TNF-alpha.

REPRESSION OF FAS EXPRESSION BY miR-23a TRANSFECTION

Based on our observations above, miR-23a were involved in MC3T3-E1 cell apoptosis. It is possible that this action results from the regulation of distinct apoptotic factors by miR-23a. To understand the molecular mechanisms that underlie miR-23a-mediated regulation, we searched for potential targets of miR-23a implicated in osteoblast apoptosis using the miRNA target prediction algorithms TargetScan and PicTar. These explorations lead to the identification of candidate targets of miR-23a: Fas (Fig. 4A). To confirm the involvement of Fas, we studied the expression of Fas in TNF-treated MC3T3-E1 cells. Western blot analysis revealed that expression of Fas is increased during TNF-alpha-induced MC3T3-E1 cell apoptosis (Fig. 4B), but application of TNF alpha can not increase the levels of FasL (Fig. 4C). To further explore the role of Fas in TNF alpha-induced MC3T3-E1 cell apoptosis, we silenced Fas expression in MC3T3-E1 cells. SiRNAs was showed to deplete 80% of the expression of Fas in MC3T3-E1 cells by Western blot (Fig. 4D). Fas depletion significantly attenuated TNF alpha-induced MC3T3-E1 cell apoptosis (Fig. 4E,F). These results showed that TNF alpha modulates MC3T3-E1 cell apoptosis through Fas. Furthermore, miR-23a lowered markedly the levels of Fas proteins in MC3T3-E1 cells. Co-application of miR-23a and AMO-23a abolished almost completely the effect of miR-23a. Application of the AMO-23a alone increased the levels of Fas in osteoblasts, indicating that there is a basal level of miR-23a activity in osteoblasts (Fig. 4G).

According to in silico analysis, Fas has a 7-nt seed match site for miR-23a within its 3′-UTR, and this putative target site is highly conserved among the vertebrates. To determine whether miR-23a inhibits Fas gene expression by binding to the predicted target site in the 3′-UTR, we used a dual luciferase reporter gene system. Cotransfection of the Fas 3′-UTR luciferase reporter with miR-23a resulted in down-regulation of luciferase activity compared with the mock or scrambled oligonucleotide controls (Fig. 4H). In comparison, miR-23a had no effect on the luciferase control reporter

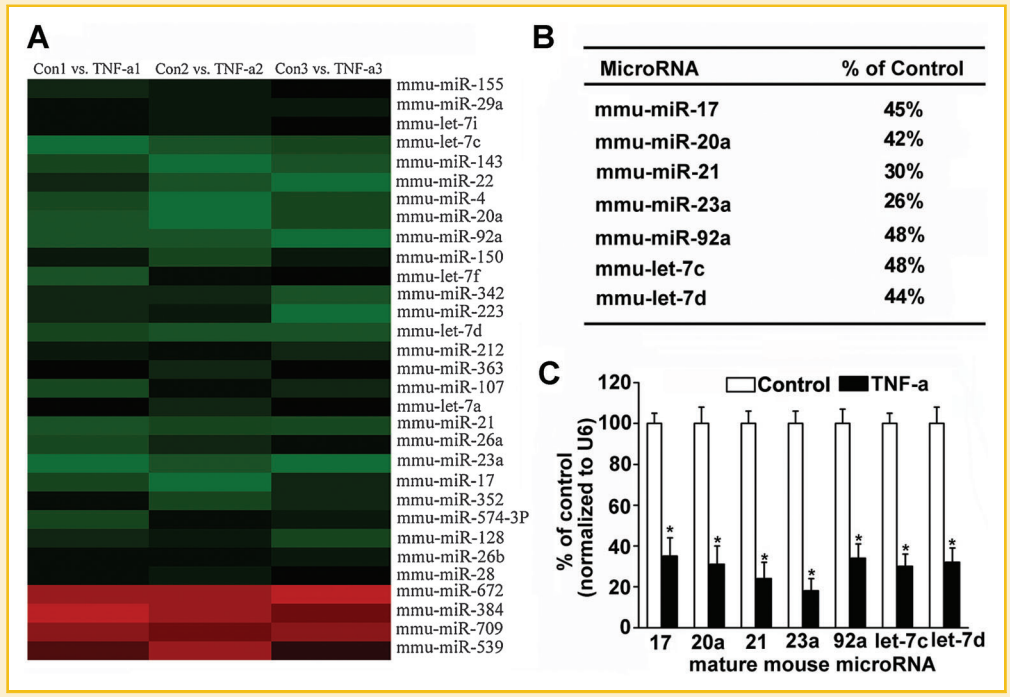

Figure 2. Altered miRNA expression during TNF-alpha-induced MC3T3-E1 cell apoptosis. A: Heat map representation of miRNAs differentially expressed in control and TNF-alpha-treated MC3T3-E1 cells. Red indicates miRNAs induced by TNF-alpha treatment, and green indicates miRNAs repressed. B: Percentage of repression of abundantly expressed miRNAs. The results are calculated as intensity TNF-alpha-treated/intensity untreated. C: Quantitative PCR analysis of miR-17, miR-20a, miR-21, miR-23a, miR-92a, let-7c, and let-7d expression in MC3T3-E1 cells treated with TNF-alpha. The results are expressed as percentages of control mean values ± s.e.m. n = 3, $^{*}P < 0.05$. TNF-a indicates TNF-alpha; Con indicates control.

without the Fas 3′-UTR, implying that Fas is a direct target of miR-23a.

DISCUSSION

The pro-apoptotic effect of TNF-alpha on osteoblasts is an important reason for bone loss caused by chronic inflammation and many other diseases, which mechanisms remain elusive. In the present study, we identified miR-23a as a negative regulator of TNF-alpha induced osteoblasts apoptosis. Data obtained from experiments revealed that inhibition of miR-23a function enhances MC3T3-E1 cell apoptosis caused by TNF-alpha, whereas miR-23a overexpression inhibited apoptotic potential of TNF-alpha. Moreover, miR-23a inhibited Fas expression through a miR-23a-binding site within the 3′-UTR of Fas. These findings suggest that miR-23a modulates TNF-alpha-induced MC3T3-E1 cell apoptosis by directly targeting Fas. This is the study linking a miRNA with TNF-alpha induced osteoblasts apoptosis, and provides key insights into the mechanisms underlying miR-23a-dependent apoptosis in MC3T3-E1 cells exposed to TNF-alpha.

Recently, studies showed an important role of miRNAs in TNF-alpha-mediated bone metabolism. TNF-alpha acts primarily through miR-21, miR-29b, miR-146a, miR-155 and miR-210 to regulate Raw264.7 cells differentiation in response to macrophage-colony-stimulating factor (M-CSF) and receptor activator of NF-kB ligand (RANKL) [Kagiya and Nakamura, 2012]. MiR-155 modulates TNF-alpha-regulated osteogenic differentiation by targeting SOCS1, at least partially through the SAPK/JNK pathway [Wu et al., 2012]. Furthermore, recent studies found that TNF-alpha caused by estrogen deficiency leads to the upregulation of miR-3077-5p and miR-705 expression, which synergistically mediate the shift of mesenchymal stem cells (MSCs) commitment to adipocyte in osteoporosis bone marrow [Liao et al., 2013]. However, only one report about miRNA involved in TNF-alpha-induced osteoblast apoptosis was found. They demonstrated miR-17–92a protected osteoblasts cells from apoptosis induced by TNF-alpha [Guo et al., 2012]. Aside from miR-17–92a, the identities of the miRNAs that play roles in TNF-alpha-induced osteoblasts apoptosis are unknown. Our study is the first effort to completely screen the miRNAs expression signature in MC3T3-E1 cell apoptosis regulated by TNF-alpha.

Our results identify the miR-23a is significantly reduced during TNF-alpha induced MC3T3-E1 cell apoptosis. MiR-23a depletion significantly enhanced TNF-alpha-induced apoptosis and over-expressing miR-23a remarkably attenuated apoptotic effects of TNF-alpha on osteoblasts. Previous studies reported that over-expression of miR-23a promotes the survival of MSCs exposed to hypoxia and serum deprivation. In contrast, down-regulation of miR-23a aggravates apoptosis of MSCs [Nie et al., 2011]. Furthermore, miR-23a could attenuate TNF-alpha-induced endothelial cell apoptosis through regulation of the caspase-7 and serine/threonine kinase 4-caspase-3 pathways [Ruan et al., 2012]. Chhabra′s study demonstrated that enhanced TNF-alpha induces apoptosis in

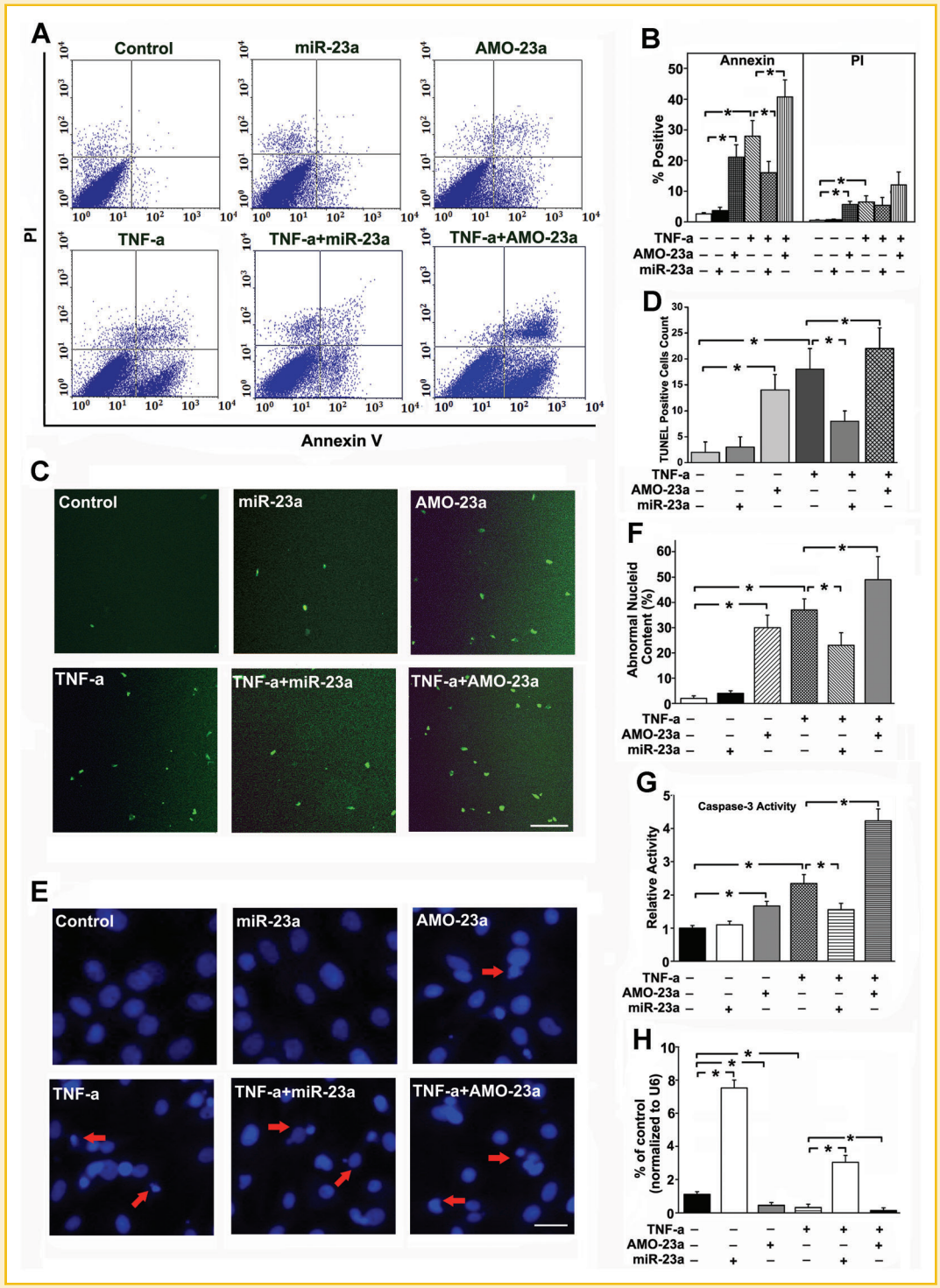

Figure 3. The effect of miR-23a on MC3T3-E1 cell apoptosis. A,B: Apoptotic progression was monitored in different group via flow cytometry. The results are expressed as percentages of positive mean values ± s.e.m. n = 3, $^{*}P < 0.05$. C,D: The cells were TdT-UTP nick end labeled and imaged by fluorescent microscope. Scale bar = 100 μm. The results are expressed as the number of green points in each photograph. All values are denoted as positive mean values ± s.e.m. from six independent photographs shot in each group; $^{*}P < 0.05$. E,F: MC3T3-E1 cells nuclei were stained by DNA binding florescence dye Hoechst 33258 and examined by fluorescence microscopy. Scale bars are 10 μm. The results are expressed as the ratio of abnormal nuclei (crenation, condensation, and fractionation) to the total number of nuclei stained by Hoechst 33258 from six independent photographs shot in each group; $^{*}P < 0.05$. G: Caspase-3 activity was measured to determine the effect of miR-23a during TNF-alpha-induced MC3T3-E1 cell apoptosis; n = 4, $^{*}P < 0.05$. H: MiR-23a levels in MC3T3-E1 cells with transfection of sequence determined by quantitative PCR; n = 4, $^{*}P < 0.05$. TNF-a indicates TNF-alpha.

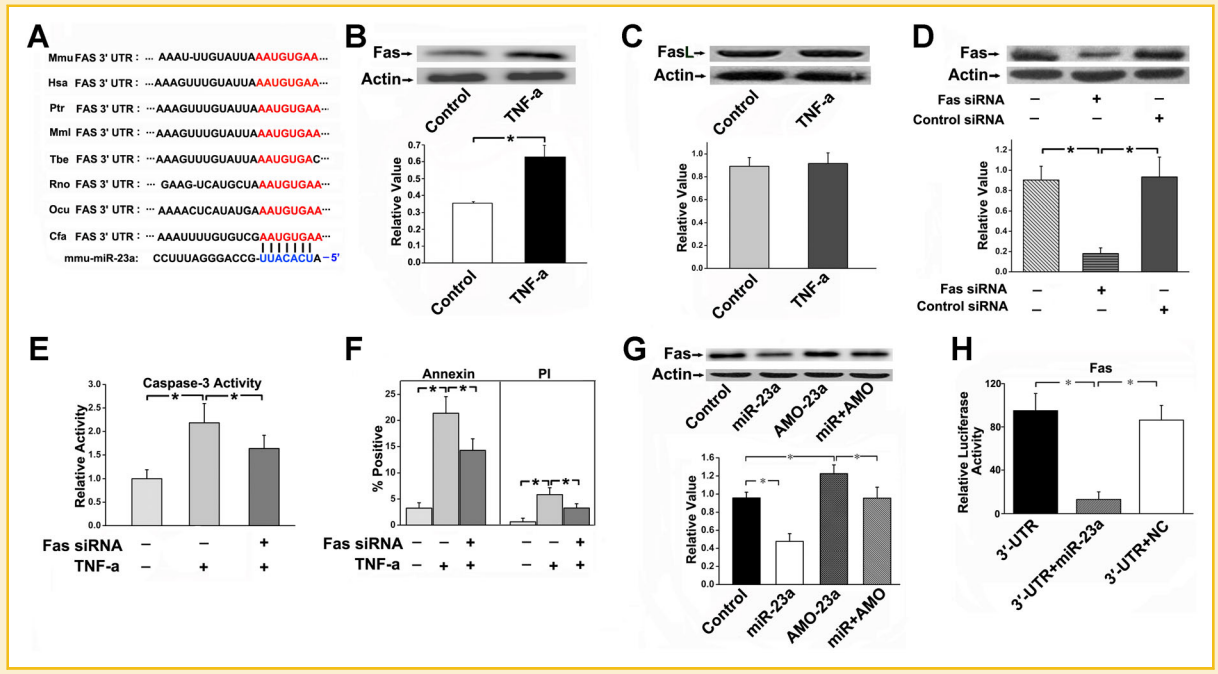

Figure 4. MiR-23a functional activity on target genes. A: Schematic of the miR-23a putative target site in the mouse Fas mRNA 3′-UTR. B: Western blot analysis of Fas expression in MC3T3-E1 cells exposed to 1 nM TNF-alpha for 24 h. Summarized data showed that Fas expression is significantly increased by TNF-alpha in MC3T3-E1 cells; n = 3, $^{*}P < 0.05$. C: Western blot analysis of FasL expression in MC3T3-E1 cells exposed to 1 nM TNF-alpha for 24 h. Summarized data showed that FasL expression is not significantly increased by TNF-alpha in MC3T3-E1 cells; n = 3, $^{*}P < 0.05$. D: Western blot analysis of Fas expression in MC3T3-E1 cells transfected with Fas siRNA. Summarized data showed that Fas siRNA significantly decrease Fas expression; n = 4, $^{*}P < 0.05$. E: After silencing Fas expression, Caspase-3 activity were measured from MC3T3-E1 cells treated with 1nM TNF-alpha for 24 h; n = 4, $^{*}P < 0.05$. F: Amplication of flow cytometry to monitor the effect of Fas depletion on TNF-alpha-induced MC3T3-E1 cell apoptosis. The results are expressed as percentages of positive mean values ± s.e.m. n = 3, $^{*}P < 0.05$. G: Western blot analysis of Fas expression in MC3T3-E1 cells transfected with miR-23a mimics and blocker; n = 4, $^{*}P < 0.05$. H: Data on luciferase reporter activities show the interaction between miR-23a and Fas 3′-UTRs; n = 4, $^{*}P < 0.05$. TNF-a indicates TNF-alpha.

HEK293T cells by over expression of miR-23a-27a-24-2 cluster [Chhabra et al., 2011]. These data, together with our findings, suggest a general role of miR-23a in cell apoptosis. Furthermore, based on the reports above, we found that the pro-apoptotic or anti-apoptosis effect of miR-23a is dependent of cell types. In our studies, we did not explore how TNF-alpha regulates miR-23a expression in osteoblasts. Further studies are required to investigate the regulation of TNF-alpha on miR-23a in osteoblasts.

To study the molecular mechanism by which miR-23a regulates osteoblast apoptosis, we searched for potential target genes that have an established function in promoting apoptosis. Interestingly, the 3′-UTR of Fas possesses a 7-nt perfect match site to the miR-23a seed region. Fas/APO-1 is a transmembrane protein of the TNF alpha receptor family, which triggers apoptosis when it interacts with Fas ligand. Furthermore, numerous studies also have showed that Fas can be activated in a ligand-independent, but FADD-dependent, manner [Kim et al., 2004; Li et al., 2007]. Previous studies found that TNF-alpha induces Fas protein in mouse MC3T3-E1 cells. Anti-Fas IgG (antagonistic antibody) significantly attenuated MC3T3-E1 cell apoptosis caused by TNF alpha in a dose-dependent manner [Ozeki et al., 2002]. Furthermore, Jilka et al. [1998] confirmed that Fas/FasL play an important role during TNF-alpha-induced MC3T3-E1 cell apoptosis. Coincidence with these reports, our results that Fas depletion significantly attenuated TNF alpha-induced MC3T3-E1 cell apoptosis suggests that TNF alpha modulates MC3T3-E1 cell apoptosis through Fas. However, the exact mechanisms of Fas involved in TNF alpha-mediated MC3T3-E1 cell apoptosis were not showed in the present study. Although our results demonstrated that application of TNF alpha can not increase the levels of FasL, Ozeki et al. confirmed that FasL protein is continuously present in MC3T3-E1 cells. Therefore, we speculated that the constitutive concentration of the FasL in MC3T3-E1 cells may be adequate for the activation of the Fas-FasL system. Moreover, it is possible that TNF alpha mediates FasL-independent Fas signaling to trigger apoptosis [Jayasooriya et al., 2013].

Based on these findings above, we speculated Fas may be a target gene of miR-23a for TNF-alpha-induced MC3T3-E1 cell apoptosis. To clarify this, we respectively silenced and overexpressed miR-23a in osteoblasts. We showed that miR-23a overexpression result in down-regulation of Fas at the protein level, whereas functional inhibition of miR-23a by AMO-23a leads to derepression of Fas, strongly suggesting that Fas is regulated by miR-23a during MC3T3-E1 cell apoptosis. Indeed, Fas 3′-UTR luciferase reporter assays confirmed that Fas is a direct target of miR-23a. Obviously, other undisclosed miR-23a targets may contribute to the apoptosis effects observed on inhibition or overexpression of miR-23a. There are more than 800 predicted targets for miR-23a in Targetscan, some of which are very relevant in the process of osteoblast apoptosis (e.g., caspase 7). The

involvement of other potential apoptotic targets should be elucidated in future studies.

In conclusion, our data provide new evidence that miR-23a plays a dominant role in the apoptotic effect of TNF-alpha on osteoblasts. The effect of miR-23a on apoptosis is attributable to the blocking of Fas expression. Functional increasing of miR-23a can attenuate TNF-alpha-induced osteoblasts apoptosis. This study is an effort to establish a mechanism of TNF-alpha-induced bone loss, and to provide insights into the potential contribution of miRNA in the regulation of osteoblasts apoptosis.

REFERENCES

Abu-Amer Y, Erdmann J, Alexopoulou L, Kollias G, Ross FP, Teitelbaum SL. 2000. Tumor necrosis factor receptors types 1 and 2 differentially regulate osteoclastogenesis. J Biol Chem 275:27307–27310.

Ambros V. 2004. The functions of animal miRNAs. Nature 431:350–355.

Bu R, Borysenko CW, Li Y, Cao L, Sabokbar A, Blair HC. 2003. Expression and function of TNF-family proteins and receptors in human osteoblasts. Bone 33:760–770.

Chhabra R, Dubey R, Saini N. 2011. Gene expression profiling indicate role of ER stress in miR-23a∼27a∼24-2 cluster induced apoptosis in HEK293T cells. RNA Biol 8:648–664.

Croce CM. 2008. Oncogenes and cancer. N Engl J Med 358:502–511.

David JP, Schett G. 2010. TNF and bone. Curr Dir Autoimmun 11:135–144.

Elmore S. 2007. Apoptosis: A review of programmed cell death. Toxicol Pathol 35:495–516.

Fulda S, Debatin KM. 2006. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 25:4798–4811.

Guo L, Xu J, Qi J, Zhang L, Wang J, Liang J, Qian N, Zhou H, Wei L, Deng L. 2012. MiRNA-17∼92a upregulation by estrogen leads to Bim targeting and inhibition of osteoblasts apoptosis. J Cell Sci 126:978–988.

Jaillon O, Aury JM, Noel B, Policriti A, Clepet C, Casagrande A, Choisne N, Aubourg S, Vitulo N, Jubin C, Vezzi A, Legeai F, Hugueney P, Dasilva C, Horner D, Mica E, Jublot D, Poulain J, Bruyere C, Billault A, Segurens B, Gouyvenoux M, Ugarte E, Cattonaro F, Anthouard V, Vico V, Del Fabbro C, Alaux M, Di Gaspero G, Dumas V, Felice N, Paillard S, Juman I, Moroldo M, Scalabrin S, Canaguier A, Le Clainche I, Malacrida G, Durand E, Pesole G, Laucou V, Chatelet P, Merdinoglu D, Delledonne M, Pezzotti M, Lecharny A, Scarpelli C, Artiguenave F, Pe ME, Valle G, Morgante M, Caboche M, Adam-Blondon AF, Weissenbach J, Quetier F, Wincker P. 2007. The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature 449:463–467.

Jayasooriya RG, Moon DO, Park SR, Choi YH, Asami Y, Kim MO, Jang JH, Kim BY, Ahn JS, Kim GY. 2013. Combined treatment with verrucarin A and tumor necrosis factor-alpha sensitizes apoptosis by overexpression of nuclear factor-kappaB-mediated Fas. Environ Toxicol Pharmacol 36:303–310.

Jilka RL, Weinstein RS, Bellido T, Parfitt AM, Manolagas SC. 1998. Osteoblast programmed cell death (apoptosis): Modulation by growth factors and cytokines. J Bone Miner Res 13:793–802.

Jurisic V, Bogdanovic G, Kojic V, Jakimov D, Srdic T. 2006. Effect of TNF-alpha on Raji cells at different cellular levels estimated by various methods. Ann Hematol 85:86–94.

Jurisic V, Terzic T, Colic S, Jurisic M. 2008a. The concentration of TNF-alpha correlate with number of inflammatory cells and degree of vascularization in radicular cysts. Oral Dis 14:600–605.

Jurisic V, Terzic T, Pavlovic S, Colovic N, Colovic M. 2008b. Elevated TNF-alpha and LDH without parathormone disturbance is associated with diffuse osteolytic lesions in leukemic transformation of myelofibrosis. Pathol Res Pract 204:129–132.

Kagiya T, Nakamura S. 2012. Expression profiling of miRNAs in RAW264.7 cells treated with a combination of tumor necrosis factor alpha and RANKL during osteoclast differentiation. J Periodontal Res 48:373–385.

Kawakami A, Eguchi K, Matsuoka N, Tsuboi M, Koji T, Urayama S, Fujiyama K, Kiriyama T, Nakashima T, Nakane PK, Nagataki S. 1997. Fas and Fas ligand interaction is necessary for human osteoblast apoptosis. J Bone Miner Res 12:1637–1646.

Kim SG, Jong HS, Kim TY, Lee JW, Kim NK, Hong SH, Bang YJ. 2004. Transforming growth factor-beta 1 induces apoptosis through Fas ligand-independent activation of the Fas death pathway in human gastric SNU-620 carcinoma cells. Mol Biol Cell 15:420–434.

Kruglov AA, Kuchmiy A, Grivennikov SI, Tumanov AV, Kuprash DV, Nedospasov SA. 2008. Physiological functions of tumor necrosis factor and the consequences of its pathologic overexpression or blockade: Mouse models. Cytokine Growth Factor Rev 19:231–244.

Li S, Zhou Y, Dong Y, Ip C. 2007. Doxorubicin and selenium cooperatively induce Fas signaling in the absence of Fas/Fas ligand interaction. Anticancer Res 27:3075–3082.

Liao L, Yang X, Su X, Hu C, Zhu X, Yang N, Chen X, Shi S, Shi S, Jin Y. 2013. Redundant miR-3077-5p and miR-705 mediate the shift of mesenchymal stem cell lineage commitment to adipocyte in osteoporosis bone marrow. Cell Death Dis 4:e600.

Lin Y, Liu L, Zhang T, Liu J. 2013. Functional investigation of Fas ligand expressions in human non-small cell lung cancer cells and its clinical implications. Ann Thorac Surg 95:412–418.

Mucci JM, Scian R, De Francesco PN, Garcia FS, Ceci R, Fossati CA, Delpino MV, Rozenfeld PA. 2012. Induction of osteoclastogenesis in an in vitro model of Gaucher disease is mediated by T cells via TNF-alpha. Gene 509:51–59.

Neumann S, Bidon T, Branschadel M, Krippner-Heidenreich A, Scheurich P, Doszczak M. 2012. The transmembrane domains of TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 co-regulate apoptotic signaling capacity. PLoS ONE 7:e42526.

Nie Y, Han BM, Liu XB, Yang JJ, Wang F, Cong XF, Chen X. 2011. Identification of MiRNAs involved in hypoxia- and serum deprivation-induced apoptosis in mesenchymal stem cells. Int J Biol Sci 7:762–768.

Ozeki N, Mogi M, Nakamura H, Togari A. 2002. Differential expression of the Fas–Fas ligand system on cytokine-induced apoptotic cell death in mouse osteoblastic cells. Arch Oral Biol 47:511–517.

Ruan W, Xu JM, Li SB, Yuan LQ, Dai RP. 2012. Effects of down-regulation of miRNA-23a on TNF-alpha-induced endothelial cell apoptosis through caspase-dependent pathways. Cardiovasc Res 93:623–632.

Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC. 1998. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Invest 102:274–282.

Wu T, Xie M, Wang X, Jiang X, Li J, Huang H. 2012. miR-155 modulates TNF-alpha-inhibited osteogenic differentiation by targeting SOCS1 expression. Bone 51:498–505.

Yang B, Lin H, Xiao J, Lu Y, Luo X, Li B, Zhang Y, Xu C, Bai Y, Wang H, Chen G, Wang Z. 2007. The muscle-specific miRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. Nat Med 13:486–491.

Zhang YH, Heulsmann A, Tondravi MM, Mukherjee A, Abu-Amer Y. 2001. Tumor necrosis factor-alpha (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways. J Biol Chem 276:563–568.