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# MicroRNA-223 Is a Key Factor in Osteoclast Differentiation

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**Abstract** MicroRNAs (miRNAs) are a class of noncording RNAs that control gene expression by translational inhibition and messenger RNAs (mRNAs) degradation in plants and animals. Although miRNAs have been implicated in developmental and homeostatic events of vertebrates and invertebrates, the role of miRNAs in bone metabolism has not been explored. Here, we show that microRNA-223 (miR-223) is expressed in RAW264.7 cells, mouse osteoclast precursor cell lines, and plays a critical role in osteoclast differentiation. We constructed miR-223 short interfering RNA (siRNA) or precursor miR-223 (pre-miR-223) overexpression retroviral vectors, and established miR-223 knockdown by siRNA or pre-miR-223 overexpression in stably infected RAW264.7 cells. Tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells were observed in miR-223 knockdown cells as well as control cells. In contrast, pre-miR-223 overexpression completely blocked TRAP-positive multinucleated cell formation compared with control cells. Apoptotic cells were not observed in this study. Our results indicate that miR-223 plays an essential role during osteoclast differentiation, and miR-223 might be a viable therapeutic target for a range of bone metabolic disorders with excess osteoclast activity. J. Cell. Biochem. 101: 996–999, 2007. © 2007 Wiley-Liss, Inc.

**Key words:** microRNAs; microRNA-223; osteoclast; differentiation

MicroRNAs (miRNAs) are an abundant class of noncoding small (~22 nt) RNAs that regulate cell differentiation, cell proliferation, and apoptosis through post-transcriptional suppression of gene expression by binding to the complementary sequence in the 3′ untranslated region of target mRNAs [Ambros, 2004]. Hundreds of miRNAs have been identified in human and they are evolutionarily conserved [Cullen, 2004]. It is estimated that up to one-third of all human genes may be miRNA targets [Valencia-Sanchez et al., 2006]. The first miRNA, *lin*-4, was identified in 1993 in a genetic screen for mutants that disrupt the timing of post-embryonic development in *Caenorhabditis elegans* [Lee et al., 1993]. However, the precise molecular function of miRNAs in mammals is still largely unknown.

Most miRNA genes are located in the introns of host genes or out-side genes [Sarnow et al., 2006]. The miRNAs are synthesized through multiple steps [Kim, 2005]. A primary miRNA transcript (pri-miRNA), which is frequently synthesized from intronic regions of protein-coding RNA polymerase II transcripts, is first processed by a protein complex containing the double-strand (ds)-specific ribonuclease Drosha in the nucleus to produce a hairpin intermediate of ~70 nucleotide. This precursor miRNA (pre-miRNA) is subsequently transported by Exportin-5/Ran-GTP to the cytoplasm where it is cleaved by another dsRNA specific ribonuclease, Dicer, into miRNA duplexes. After strand separation of the duplexes, the mature single-stranded miRNA is incorporated into an RNA-induced silencing complex (RISC)-like ribonucleoprotein particle (miRNP). This complex inhibits translation or, depending on the degree of Watson–Crick complementarity, induces degradation of target mRNAs.

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Osteoclast differentiation is controlled by exogenous hormones and cytokines as well as many transcriptional factors which are involved in osteoclast differentiation and function such as *c-Src* [Soriano et al., 1991], *c-fos* [Grigoriadis et al., 1994], PU.1 [Tondravi et al., 1997], NFκB (p50 and p52) [Iotsova et al., 1997], and NFATc1 [Takayanagi et al., 2002]. In this study, we showed that pre-miR-223 overexpression in RAW264.7 osteoclast precursors completely blocked TRAP-positive multinucleated cells formation. Our results indicate that miR-223 is a key small RNA for gene regulation during osteoclastogenesis and a potential therapeutic target for bone metabolic disease.

## MATERIALS AND METHODS

### Construction of Retroviral Vector

Synthetic scrambled and miR-223 siRNA, pre-miR-223, and mutant pre-miR-223 were synthesized by Integrated DNA Technologies (Fig. 1). The synthetic oligonucleotides were annealed and the ds product was inserted into pSuppressor Retro (Imgenex) digested with *Xho*1 and *Xba*1 restriction enzymes. Clones containing the oligonucleotide insertion were verified by DNA sequencing.

### Establishment of Stably Infected Cells

Subconfluent BOSC 23 packaging cells were transfected with retroviral vectors (3 μg DNA/60 mm dish) using FuGene 6 (Roche). After 48 h, the supernatant was collected, filtered through a 0.45 μm syringe filter, and used to transduct target cells. Each supernatant (1 ml) was mixed with polybren (8 μg/ml). This infection cocktail (1 ml) was used to infect into RAW264.7 cells ($8.5 \times 10^4$ cells/35 mm dish). Infected cells were selected in the presence of 800 μg/ml G418 (Sigma) for a week. Colonies were isolated by digestion with trypsin/EDTA for 5 min at 37°C within stainless steel cloning rings. Four independent clones were established in each expression vector.

### Osteoclast Formation Assay

Stably infected RAW264.7 cells were cultured in DMEM containing 10% FBS ($2 \times 10^5$ cells in 0.5 mi/well for 24-well plates) for 6 days in the presence of soluble RANKL (100 ng/ml). The cells were stained for TRAP activity (Sigma) as a osteoclast marker [Sugatani et al., 2005]. TRAP-positive multinucleated cells containing more than three nuclei were counted as osteoclasts under microscopic examination [Sugatani and Hruska, 2005].

### Ribonuclease Protection Assay (RPA)

Small RNAs (<200 nucleotides) were isolated from the each stably infected RAW264.7 cells using the miRNA isolation kit (Ambion). For each reaction, 1 μg of small RNAs was mixed with biotin-labeled riboprobes, which were transcribed from restriction-digested plasmid DNA using the miRNA probe construction kit (Ambion). RPAs were performed using the RPA 3 kit from Pierce according to the manufacturer's instructions. Protected fragments were separated by electrophoresis through 8 M urea/15% acrylamide gel and were then transferred to a nylon membrane. The biotinylated probes are detected using a streptavidin-HRP conjugate and SuperSignal chemiluminescent substrate (Pierce). The X-ray film (Pierce) was exposed for 30 min at room temperature and scanned into Adobe Photoshop 7.0 for digital imaging.

**synthetic pre-miR-223**

5'-ucuggccaucugcagugucacgcuccguguauuugacaagcugaguuggacacucugugugguagagug
ucaguuugucaaauaccccaaguguggcucaugccuaucag-3'

**synthetic mutant pre-miR-223**

5'-ucuggccaucugcagugucacgcuccguguauuu**u**acaagcugaguuggacacucugugu**uu**uagagu
gucaguuu**u**ucaaauacccc**c**aguguggcucaugccuaucag-3'

**scrambled siRNA**

5'-gcgcgcuuuguaggauuc-3'

**miR-223 siRNA**

5'-gacacucugugugguagaguguc-3'

**Fig. 1.** Sequences of synthetic pre-miR-223, mutant pre-miR-223, scrambled, and miR-223 siRNA. Bold letters (mutant pre-miR-223) indicate nucleotides different from those in the sequence of pre-miR-223.

## RESULTS AND DISCUSSION

The pluripotent hematopoietic stem cell gives rise to a myeloid stem cell, which can further differentiate to megakaryocytes, granulocytes, and monocyte-macrophages [Tanaka et al., 2005]. The osteoclast is derived from the cells in the monocyte-macrophage lineage [Tanaka et al., 2005]. miR-223, a mouse bone marrow-specific miRNA, is specifically expressed in CD11b positive myeloid cell lineages [Chen et al., 2004]. CD11b is expressed along the osteoclast differentiation pathway from mononucleated early progenitor cells until mature multinucleated osteoclasts [Ferron and Vacher, 2005]. In the current study, we confirmed that miR-223 is expressed in RAW264.7 osteoclast precursors and primary mouse bone marrow macrophages (data not shown). We hypothesized that miR-223 plays a key role in differentiation into osteoclasts from bone marrow macrophages. To study the roles of miR-223 during osteoclastogenesis, we established stable miR-223 knockdown by siRNA or pre-miR-223 overexpression in RAW264.7 cells. The expression level of miR-223 was examined by RPA. miR-223 expression was markedly inhibited in stably infected miR-223 knockdown cells (Fig. 2, lane 1) compared with scrambled siRNA expressing cells. In contrast, miR-223 expression was enhanced in pre-miR-223 overexpressing cells (Fig. 2, lane 4) compared with mutant pre-miR-223 expressing cells (Fig. 2, lane 3). U6 small nuclear RNA was used as a loading control.

Next, we examined whether miR-223 regulates the differentiation of RAW264.7 osteoclast precursors. TRAP-positive multinucleated cells were observed in miR-223 knockdown cells as well as control cells in two independent clones (Fig. 3A–C). In contrast, pre-miR-223 overexpression in RAW264.7 cells completely blocked osteoclast differentiation compared with mutant pre-miR-223 expressing cells in two independent clones (Fig. 4A–D). Apoptotic cells were not observed in this study (data not shown). These data suggest that miR-223 plays a critical role in controlling the osteoclast differentiation. Moreover, the expression of miR-223 in osteoclasts is lower than bone marrow macrophages in primary culture (data not shown). On the basis of these results, we propose that miR-223 expression might suppress the differentiation into osteoclast in osteoclast precursors. However, we were not able to detect target mRNA of miR-223 during the differentiation of RAW264.7 osteoclast precursors in this study. Bioinformatic study shows no targets of miR-223 within the contexst of osteoclast differentiation as NFATc1 or *c-fos* so far. Identification of downstream targets of miR-223 will be an important issue to completely

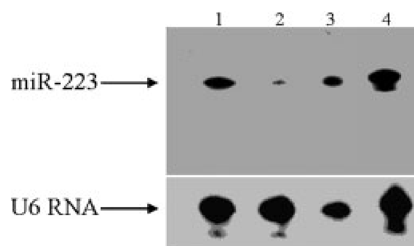


**Fig. 2.** Expression of miR-223 in stably infected RAW264.7 osteoclast precursor cells. We established stable miR-223 knockdown by siRNA or pre-miR-223 overexpression RAW264.7 cells as described in MATERIALS AND METHODS. The expression level of miR-223 was examined by RPA. Lane 1 and 2: miR-223 expression was markedly inhibited in stably infected miR-223 knockdown cells compared with scrambled siRNA expressing cells. Lane 3 and 4: miR-223 expression was enhanced two-fold in pre-miR-223 overexpressing cells compared with mutant pre-miR-223 expressing cells factored for U6 small nuclear RNA which was used as a loading control. Lane 1: scrambled siRNA, Lane 2: miR-223 siRNA, Lane 3: mutant pre-miR-223, Lane 4: pre-miR-223.

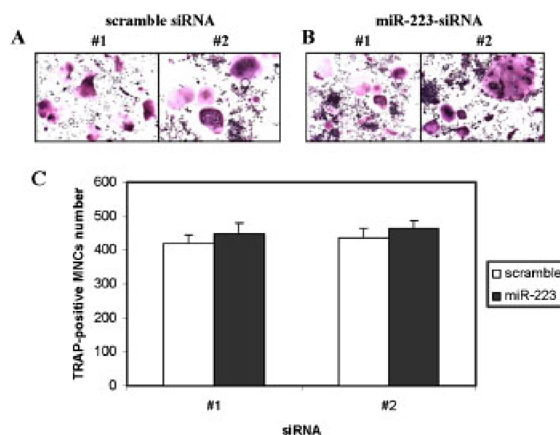


**Fig. 3.** The effect of miR-223 siRNA in osteoclast differentiation. The stably established RAW264.7 cells with scrambled or miR-223 siRNA retroviral vectors were cultured in DMEM containing 10% FBS for 6 days in the presence of soluble RANKL (100 ng/ml). Cells were then fixed and stained for TRAP as a marker of osteoclasts (**A** and **B**), and the number of TRAP-positive multinucleated cells (MNCs) was scored (**C**). TRAP activities in two independent clones (#1 and #2) are presented as mean ± SD of three experiments in duplicate. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

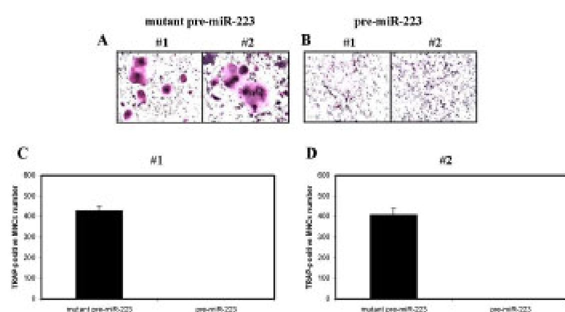


**Fig. 4.** The effect of pre-miR-223 overexpression in osteoclast differentiation. The stably established RAW264.7 cells with pre-miR-223 or mutant that retroviral vectors were cultured in DMEM containing 10% FBS for 6 days in the presence of soluble RANKL (100 ng/ml). Cells were then fixed and stained for TRAP as a marker of osteoclasts (**A** and **B**), and the number of TRAP-positive multinucleated cells was scored (**C** and **D**). TRAP activities in two independent clones (#1 and #2) are presented as mean $\pm$ SD of three experiments in duplicate. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

understand the role of miR-223 in osteoclastogenesis, which could be regulated by multiple miR-223 targets because of bioinformatic predictions indicate that each miRNA regulates on average ~200 target transcripts [Krutzfeldt et al., 2006].

There are a number of key regulators of osteoclast differentiation such as *c-fos* [Grigoriadis et al., 1994], PU.1 [Tondravi et al., 1997], NFκB (p50 and p52) [Iotsova et al., 1997], and NFATc1 [Takayanagi et al., 2002]. Besides these transcriptional factors, we report here for the first time that miRNAs expression and function are involved in osteoclast differentiation. Further investigation into the function of miR-223 in osteoclastogenesis will provide an insight on the role of small noncoding RNAs in bone metabolism. Understanding the role of miRNAs in bone metabolism opens the possibility that miRNAs represent novel therapeutic opportunities based on targeting of miRNAs in bone metabolic disorders.

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