ARTICLE
Journal of Cellular Biochemistry 116:2804–2813 (2015)

Journal of Cellular Biochemistry

The Role of MicroRNA, miR-24, and Its Target CHI3L1 in Osteomyelitis Caused by *Staphylococcus aureus*

Tao Jin,[1] Yong Lu,[2] Qing Xiao He,[1] Hai Wang,[1] Bing Fu Li,[1] Liang Yue Zhu,[1] and Qing Yong Xu[1]*

[1]*Department of Orthopedic Surgery, Kunming General Hospital Chengdu Military Command, Kunming, 650032, PR China*

[2]*Department of Radiology, School of Medicine, Ruijin Hospital, Shanghai Jiaotong University, Shanghai, 200025, PR China*

ABSTRACT

Osteomyelitis is a debilitating infectious disease of the bone which is predominantly caused by *Staphylococcus aureus* (*S. aureus*). MicroRNAs (miRNAs) have been shown to play a regulatory role in osteogenesis. In the present study, the expression levels of miRNAs proposed to potentially play a regulatory role in bone formation or differentiation (miR-24, miR-29b, miR-200a, miR-208, miR-322) were analyzed in the whole blood of patients with bacterial osteomyelitis or healthy controls, and in MC3T3-E1 cells infected with *S. aureus* by qRT-PCR. The expression of miR-24 was significantly down-regulated in osteomyelitis patients and *S. aureus*-infected MC3T3-E1 cells compared with the healthy controls or untreated control cells. Moreover, our results showed that *S. aureus* inhibited MC3T3-E1 cell proliferation, induced osteoblast apoptosis and prohibited bone formation and mineralization. We found that overexpression of miR-24 could reduce the effects of *S. aureus*, while inhibition of miR-24 intensified the effects. We also demonstrated that miR-24 suppressed the expression of chitinase 3-like 1 (CHI3L1) mRNA, thought to mediate multiple signaling pathways, by directly binding to the 3′-untranslated region. J. Cell. Biochem. 116: 2804–2813, 2015.

KEY WORDS: *Staphylococcus aureus*; OSTEOMYELITIS; miRNA; miR-24; CHI3L1

Osteomyelitis is an acute or chronic infection of the bone or bone marrow. Invasion of pathogenic bacteria into the usually sterile bone can lead to progressive bone destruction, severe inflammation and bone neoformation. In patients, osteomyelitis may lead to significant morbidity and mortality. There are several types of osteomyelitis based on the source of infection: a contiguous focus of infection (i.e., after trauma, surgery, or insertion of a joint prosthesis), vascular insufficiency (e.g., diabetic foot infections), hematogenous spread, or direct inoculation. *Staphylococcus aureus*, a member of the normal flora found on the skin and mucous membranes, is the causative agent in many cases of infection leading to osteomyelitis [Zuluaga et al., 2006]. In one study, *S. aureus* was reported in 38–67% of culture-positive cases of osteomyelitis [Wright and Nair, 2010].

MicroRNAs (miRNAs) are small endogenous non-coding RNAs (18–25 nucleotides in length) that negatively regulate gene expression through incomplete base-pairing to the 3′ untranslated region (3′-UTR) of target mRNAs [Ambros, 2004; Bartel, 2004, 2009; Rana, 2007]. More recently, it has been demonstrated that miRNAs can regulate target mRNAs by binding to the amino acid coding sequence [Forman et al., 2008; Tay et al., 2008; Ko et al., 2009]. Evidence suggests that miRNAs play a role in the regulation of diverse biological and pathological processes, such as developmental timing, organogenesis, apoptosis, cell proliferation, and differentiation [Filipowicz et al., 2008; Stefani and Slack, 2008]. Several miRNAs (miR-26a, miR-125b, miR-133, and miR-135) have been shown to play a role in osteogenesis by regulating osteoblast cell growth or differentiation in human or mouse cell lines

Tao Jin andYong Lu contribute equally to this article.

Grant sponsor: National Natural Science Foundation; Grant number: 81171734; Grant sponsor: Science and Technology Plan Projects; Grant number: 2012BC007.

*Correspondence to: Yong Qing Xu, Department of Orthopedic Surgery, Kunming General Hospital Chengdu Military Command, Kunming 650032, PR China.
E-mail: yongqingxu123@163.com

Manuscript Received: 22 January 2015; Manuscript Accepted: 8 May 2015
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 14 May 2015
DOI 10.1002/jcb.25225 • © 2015 Wiley Periodicals, Inc.

[Luzi et'al., 2008; Mizuno et al., 2008]. More recent studies have implicated a range of other miRNAs in mesenchymal stem cell differentiation toward osteoblasts (miR-24, miR-29b, miR-200a, miR-208, and miR-322) and some of these studies have started to elucidate the regulatory networks involved [Itoh et al., 2009, 2010; Eguchi et al., 2013; Suh et al., 2013; Vimalraj and Selvamurugan, 2014]. For example, miR-208 and miR-200a modulate bone morphogenetic protein-2-stimulated mouse preosteoblast differentiation by directly targeting V-ets erythroblastosis virus E26 oncogene homolog 1 and distal-less homeobox 5, respectively [Itoh et al., 2009, 2010]. However, much is still unknown about the role of miRNAs in bone formation.

The chitinase 3-like 1 (CHI3L1) gene, also known as YKL-40, a member of the "mammalian chitinase-like proteins," which encodes a secretory glycoprotein, is upregulated in a variety of inflammatory conditions and cancers [Hakala et al., 1993; Morrison and Leder, 1994; Shackelton et al., 1995]. CHI3L1 is thought to mediate multiple signaling pathways and is considered a biomarker for a variety of diseases. Overexpression of CHI3L1 is associated with increased patient mortality, although the mechanism for this has not been proven. It has been suggested that the specific receptors/binding partners of CHI3L1 may exist on the cell surface, due to its role in cell signaling, and are likely to be carbohydrate structures [Prakash et al., 2013].

In this study, we performed gene expression analysis of patients with osteomyelitis caused by *S. aureus*. Our findings indicate that the regulation of CHI3L1 mediated by miR-24 may play an important role in the process of osteogenesis.

MATERIALS AND METHODS

ETHICS STATEMENT

This study was approved by the Institutional Ethical Committee of Southwest Hospital and written informed consent was obtained from patients before blood samples were taken.

COLLECTION OF BLOOD SAMPLES

Blood samples were obtained from patients with bacterial osteomyelitis ($n = 27$, 11 females and 16 males, mean age 42.51 ± 11.08 years). All patients were confirmed to have *S. aureus*-induced osteomyelitis by a positive bacteremia result from one of the following sampling methods: percutaneous puncture aspiration, surgical sample, and/or blood culture. Blood samples were also obtained from 15 healthy volunteers comparable for age, hospitalized for minor surgical procedures. Blood samples were collected before they underwent surgery and they did not show any evidence of bacterial infection at the time of blood sampling. All blood samples were stored at −80°C.

BACTERIAL STRAIN AND CULTURE CONDITIONS

S. aureus strain 6,850 (ATCC 53,657; ATCC, Middlesex, UK) was used in this study. It was cultured in tryptic soy broth (TSB) at 37°C with shaking. *S. aureus* strain 6,850 suspensions were prepared. The multiplicity of infection (MOI), the average number of phage per bacterium, was determined by dividing the number of phage (PFU/ml) by the number of bacteria (cells/ml) added to the suspension.

CELL CULTURE AND INFECTION

The mouse clonal MC3T3-E1 pre-osteoblastic cell line (ATCC), commonly used to analyze osteoblast function [Murphy et al., 2010; Plunkett et al., 2010], was used in this study. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 2% penicillin-streptomycin solution and 1% L-glutamine (Sigma–Aldrich, St. Louis, MO) in 5% CO_2 at 37°C. The media was refreshed every 3–4 days and after confluency. Cells were harvested using trypsin-EDTA (Sigma–Aldrich) and resuspended in DMEM.

MC3T3-E1 cells were infected with *S. aureus* as previously described [Tuchscherr et al., 2010, 2011]. Briefly, MC3T3-E1 cells were infected with *S. aureus* at a MOI of 100. After incubation at 37°C (times varied depending on the experiment), cells were washed and treated with lysostaphin (20 mg/ml) for 30 min to lyse extracellular staphylococci. Then fresh medium was added to the cells. This process was repeated every 2–3 days to remove any extracellular staphylococci that may have been released from infected cells.

TRANSFECTION OF MC3T3-E1 CELLS WITH miR-24

MC3T3-E1 cells, at 30–50% confluence were transfected with miR-24 RNA, miR-NC, anti-miR-24, or anti-miR-NC at a concentration of 50 nM with oligofectamine (Invitrogen) following the manufacturer's instructions. After 24 h, the cells were infected with *S. aureus* (as described above) and were then harvested for mRNA and protein analysis.

CHI3L1 GENE KNOCKDOWN

An RNA interference (RNAi) target sequence within the mouse CHI3L1 gene was designed, short hairpin RNA (shRNA)-CHI3L1: 5′-CTGCGTTCTTATGGCTTTGAT-3′, along with a shRNA negative control, shRNA-NC: 5′-TCTCCGAACGACTCAGACCGTG-3′. Then pShuttle vectors containing the CHI3L1 RNAi sequences were constructed. A lentivirus was produced by co-transduction of the shRNA expression pShuttle vectors into 293T cells. MC3T3-E1 cells were then transfected with lentivirus expression shRNA-CHI3L1 or shRNA-NC for 24 h. Then, transfected and non-transfected MC3T3-E1 cells were infected with *S. aureus* at a MOI of 100 for 72 h and cells were then harvested for CHI3L1 mRNA and protein analysis.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was extracted from MC3T3-E1 cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was extracted from whole blood using the RNeasy Mini kit (Qiagen, Hilden, Germany). Purified RNA was quantified by measuring the absorbance at 260 nm using a spectrophotometer.

For miRNA analysis, quantitative real-time PCR (qRT-PCR) was performed using the TaqMan® MicroRNA Reverse Transcription Kit, TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and the corresponding primers. Expression levels were normalized against RNU6B levels (internal control). MicroRNA analysis was performed to analyze the expression levels of miR-24, miR-29b, miR-200a, miR-208, and miR-322 in whole blood and in MC3T3-E1 cells.

For mRNA analysis, qRT-PCR was performed using the TaqMan® High-Capacity cDNA Reverse Transcription Kit, TaqMan® Fast PCR Master Mix (Applied Biosystems) and the corresponding primers. Expression levels were normalized against GAPDH levels (internal control). Messenger RNA analysis was performed to analyze the expression levels of CHI3L1 in whole blood and in MC3T3-E1 cells. The sequences of primers used in PCR are shown in Table I.

Quantitative RT-PCR reactions were performed in triplicate on a RealPlex4 real-time PCR detection system (Eppendorf Co. Ltd., Germany). PCR parameters were as follows: 95°C for 10 min, then 40 amplification cycles of 95°C for 15 s and 60°C for 1 min (for miRNAs), and 95°C for 10 s and 72°C for 30 s (for mRNAs). The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passes the fixed threshold and the relative miRNA and mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [Livak and Schmittgen, 2001].

MTT ASSAY

Cell viability was quantitated by a colorimetric assay using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, MC3T3-E1 transfected cells (and non-transfected cells as a control) were infected with *S. aureus* at a MOI of 100 for 0, 1, 2, 3, or 4 days. Then, cells were seeded into 96-well plates (6×10^3 cells/ml) and maintained in growth media for 24 h at 37°C and 5% CO_2. At 60% confluence, the cells were treated with H_2O_2 (0–1 mmol/L) for 24, 48, and 72 h. Then, 10 μl of MTT solution (5 mg/ml) was added to each well, and the cells were incubated for 4 h at 37°C. Once the formation of formazan crystals was observed, the MTT medium was replaced with 150 μl of solubilization solution dimethyl sulfoxide (DMSO) to dissolve the crystals. The plates were shaken for 5 min. The absorbance of each well was recorded on a microplate spectrophotometer at 570 nm. Relative cell growth was calculated as a ratio of the average absorbance of treated cells versus the average absorbance of control cells. Cell viability was calculated as the ratio of optical densities.

ANALYSIS OF APOPTOSIS

Analysis of cellular apoptosis was performed using the Annexin V-PI Apoptosis Detection Kit (Clonetech Laboratories Inc.). Briefly, MC3T3-E1 transfected cells (and non-transfected cells as a control) were infected with *S. aureus* at a MOI of 100 for 24 h. The cells were then inoculated into six-well plates (4×10^6 cells/ml), harvested by trypsinization, then washed twice with cold PBS and centrifuged at $110 \times g$. Approximately 1×10^5–1×10^6 cells/ml were resuspended in 300 μl of 1 × binding buffer, centrifuged at $110 \times g$ for 5 min and the supernatant was removed. Cells were then resuspended again in 300 μl of 1 × binding buffer and transferred to a sterile flow cytometry glass tube. Then, 10 μl of Annexin V-PI was added and the cells were incubated in the dark for 30 min at room temperature. After further incubation in the dark with 5 μl of propidium iodide, cells were analyzed by flow cytometry using a FACSCalibur (Benton–Dickinson).

ALIZARIN RED STAINING

MC3T3-E1 transfected cells (and non-transfected cells as a control) were infected with *S. aureus* at a MOI of 100 for 21 days. The cells were then inoculated into 24-well plates (1×10^5 cells/ml), washed twice with PBS, then fixed with 500 μl of ice-cold 70% ethanol for 10 min. The fixed cells were stained with 500 μl of Alizarin red solution (Sigma–Aldrich) for the detection of calcification. Cells were analyzed using 200 × bright field microscopy. Staining was quantified by measuring the OD at an absorbance of 540 nm.

ALKALINE PHOSPHATASE (ALP) ACTIVITY

MC3T3-E1 transfected cells (and non-transfected cells as a control) were infected with *S. aureus* at a MOI of 100 for 7 days and then ALP activity was assayed. Briefly, treated cells, seeded into 24-well plates (1×10^5 cells/ml), were washed twice with PBS. Then, 200 μl of lysis buffer was added and the cells were incubated on ice for 5 min. The cell lysate was sonicated for 1 min, then centrifuged at $1{,}000 \times g$ for 10 min at 4°C. ALP activity was assayed using an ALP kit (Wako Pure Chemical Industries Ltd., Osaka, Japan). The absorbance of each well at 405 nm was measured with a microplate reader (Immuno-Mini NJ-2300; Nalge Nunc International K.K., Tokyo, Japan). ALP activity was then estimated using the TRAP and ALP double-staining kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions.

WESTERN BLOTTING

To prepare cell lysates, MC3T3-E1 cells were washed twice with PBS and harvested by centrifugation. The cell pellet was resuspended in RIPA buffer containing 25× Complete and Phosphatase Inhibitor Mixture (Roche Applied Science, IN). The protein content was measured with a DC protein assay kit (Bio-Rad, Richmond, CA). Each whole cell lysate was resuspended in SDS-PAGE buffer containing 2% 2-mercaptoethanol and boiled for 5 min. The proteins in the cell lysates were then separated by SDS-PAGE on 12% polyacrylamide gels. Beta-actin protein was included as a loading control. Proteins were then blotted onto PVDF membrane (PerkinElmer Life Sciences, Boston, MA). Non-specific binding sites were blocked by incubation of the membrane with 5% non-fat milk in TPBS (PBS and 0.1% Tween-20) for 1 h. The membrane was then incubated overnight with anti-CHI3L1 (1:1,000; Abcam, Cambridge, MA) or anti-β-actin (1:2,500; Abcam) primary antibody at 4°C. After three washes with

TABLE I. Sequences of Primers Used in qRT-PCR

		Forward	Reverse
miRNA	miR-24	5′-TTCTCCGGGCTGTCGATTGG-3′	5′-CAAGGGCTCGACTCCTGTTC-3′
	miR-29b	5′-CTTCTTCAGGAAGCTGGTTTCA-3′	5′-GGTCTCCCCCAAGAACACTG-3′
	miR-200a	5′-GCCCCTGTGAGCATCTTACC-3′	5′-GCGGGTCACCTTTGAACATC-3′
	miR-208	5′-TGACGGGCGAGCTTTTG-3′	5′-AACAAGCTTTTTGCTCGTCTTA-3′
	miR-322	5′-CTCGTTGACTCCGAAGGGC -3′	5′-TGTTGCAGCGCTTCATGTTT -3′
	RNU6B	5′-CTCGCTTCGGCAGCACA-3′	5′-AACGCTTCACGAATTTGCGT-3′
mRNA	CHI3L1	5′-CGGAGGATGGAACTTTGGGT-3′	5′-TTCGGCCTTCATTTCCTTGA-3′
	GAPDH	5′-GGGAGCCAAAAGGGTCATCA -3′	5′-TGGTCATGAGTCCTTCCACG -3′

TPBS, the membrane was incubated with goat polyclonal anti-rabbit IgG–H&L-pre-adsorbed (HRP) at 1:2,000 dilution (Abcam) at room temperature. After another three washes, proteins were detected using an enhanced ECL kit and a chemiluminescence detector (miR-24.AS-1000, Fuji, Japan). β-actin served as a loading control.

LUCIFERASE ASSAY

To confirm the interaction between the 3′-UTR of CHI3L1 mRNA and miR-24, MC3T3-E1 cells were inoculated into 12-well plates (1×10^5 cells/ml) and co-transfected with the wild-type or mutant pGL3-CHI3L1 3′-UTR and RNA oligonucleotides of miR-24 or miR-NC. Luciferase activity was calculated as a ratio of firefly to Renilla luciferase activity using a luciferase assay system (Promega, Madison, WI) according to the manufacturer's instructions. The relative luciferase activity was expressed as the ratio of the measured luciferase activity to the control.

STATISTICAL ANALYSIS

Statistical analysis was performed by one-way ANOVA using the SPSS 17.0 software (SPSS Inc., Chicago, IL). Data were interpreted using the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA). All results are presented as the mean ± standard deviation (SD). P values of <0.05 were considered statistically significant.

RESULTS

MIRNA LEVELS SIGNIFICANTLY DIFFER BETWEEN PATIENTS WITH *S. aureus*–INDUCED OSTEOMYELITIS AND HEALTHY CONTROLS AND BETWEEN MC3T3-E1 CELLS INFECTED WITH *S. aureus* AND UNTREATED CONTROL CELLS

To determine whether any differences exist in the miRNA expression levels in the whole blood of patients with osteomyelitis compared with healthy controls, qRT-PCR was performed on a range of miRNAs previously implicated in osteoblast cell growth and differentiation (miR-24, miR-29b, miR-200a, miR-208, and miR-322). A statistically significant difference was observed in the expression levels of miR-24 ($P < 0.01$), miR-29b ($P < 0.05$), and miR-200a ($P < 0.05$) in the whole blood of osteomyelitis patients compared with the healthy controls (Fig. 1A). To confirm this finding, expression levels were compared between MC3T3-E1 cells infected with *S. aureus* (MOI = 100) for 21 days, cells treated with the same volume of TSB for 21 days and untreated cells, by qRT-PCR. Again, a statistically significant difference was observed in the expression levels of miR-24 ($P < 0.01$), miR-29b ($P < 0.05$), miR-200a ($P < 0.05$), and this time, miR-208 ($P < 0.05$) in the *S. aureus*-infected MC3T3-E1 cells compared with the TSB-treated and the untreated cells (Fig. 1B).

MIR-24 EXPRESSION LEVELS ARE DECREASED IN *S. aureus*–INFECTED CELLS COMPARED WITH CONTROL CELLS, CORRELATING WITH AN INCREASE IN OSTEOGENIC MARKERS IN miR-24–TRANSFECTED CELLS COMPARED WITH INFECTED ONLY CELLS

To analyze the difference in miR-24 expression levels further, the relative miRNA levels in *S. aureus*-infected MC3T3-E1 cells compared with untreated cells were analyzed by qRT-PCR every three days over a time course of 21 days. A statistically significant difference was detected in the relative miR-24 levels between days 9 and 21 (days 9 and 12, $P < 0.05$; days 15, 18, and 21, $P < 0.01$) in *S. aureus*-infected MC3T3-E1 cells compared with untreated cells (Fig. 1C). This indicated that miR-24 levels were decreasing steadily in *S. aureus*-infected MC3T3-E1 cells over this time course.

Next, the expression of two specific biomarkers of osteogenesis, osteopontin (OPN), and Runx2, in MC3T3-E1 cells transfected with RNA oligonucleotides of miR-24 or miR-NC, then infected with *S. aureus* at a MOI of 100 for 7 days, was analyzed as an indicator of bone formation. The western blotting results indicated that both OPN and Runx2 protein expression levels were decreased in all of the treated cells compared with the untreated control cells, indicative of the inhibition of bone formation in patients with *S. aureus*-induced osteomyelitis. Furthermore, protein expression levels for both OPN and Runx2 showed a statistically significant increase in miR-24-transfected cells compared with the infected only (non-transfected) control cells ($P < 0.05$) (Fig. 1D), indicating partial restoration of osteogenesis with the overexpression of miR-24.

MIR-24 AFFECTS CELL VIABILITY, APOPTOSIS, AND MINERALIZATION IN MC3T3-E1 cELLS INFECTED WITH *S. aureus*

The effect of miR-24 expression on the growth, proliferation and differentiation of MC3T3-E1 cells was next examined. MC3T3-E1 cells were transfected with RNA oligonucleotides of miR-24 or miRNA negative control (miR-NC) (Fig. 2A), or anti-miR-24 or anti-miR-NC (Fig. 2B), for 24 h, and were then infected with *S. aureus* at a MOI of 100 for 0, 1, 2, 3, or 4 days. Firstly, cell viability was analyzed by an MTT assay. Untreated cells and infected only cells (not transfected) served as controls. A statistically significant decrease was observed in cell viability of *S. aureus*-infected cells compared with untreated cells ($P < 0.01$), and cell proliferation was partially restored in miR-24-transfected cells compared with untreated cells ($P < 0.05$), indicating that expression of miR-24 increases proliferation (Fig. 2A). When cell viability was analyzed in MC3T3-E1 cells transfected with anti-miR-24 or anti-miR-NC, again a statistically significant decrease was observed in cell viability of *S. aureus*-infected cells compared with untreated cells ($P < 0.01$). However, anti-miR-24-transfected cells showed a further decrease in cell proliferation compared with untreated cells ($P < 0.01$), indicating that silencing of miR-24 negatively affected cell viability (Fig. 2B). These findings confirmed the potential role for miR-24 in promoting proliferation of MC3T3-E1 cells.

Secondly, the four groups of transfected cells (miR-24, miR-NC, anti-miR-24, and anti-miR-NC) were infected with *S. aureus* at a MOI of 100 for 24 h, and apoptosis was determined by flow cytometry followed by Annexin V–PI double staining. As shown in Figure 2C, the percentage of apoptotic cells was increased in all of the *S. aureus*-infected cells compared with the untreated cells ($P < 0.01$). Furthermore, the number of apoptotic cells was decreased in miR-24-transfected cells and increased in anti-miR-24-transfected cells compared with the *S. aureus*-infected only cells ($P < 0.05$) (Fig. 2C), indicating a potential role for miR-24 in inhibiting apoptosis of MC3T3-E1 cells.

Thirdly, the four groups of transfected cells (miR-24, miR-NC, anti-miR-24, and anti-miR-NC) were infected with *S. aureus* at a MOI of 100 for 21 days and were then observed by Alizarin red staining (Fig. 2D). Mineralization was observed in all of the treated MC3T3-E1 cells compared with the untreated control cells. The highest degree of mineralization was observed with the cells transfected with anti-miR-24. Alizarin red staining was quantified by measuring the optical density of cells at an absorbance of 540 nm (Fig. 2E). Alizarin red staining was highest in untreated cells. In cells treated with *S. aureus*, a significant increase in staining was seen in miR-24-transfected cells ($P < 0.05$) compared with the negative control, and a significant decrease was seen in anti-miR-24-transfected cells ($P < 0.01$) compared with the negative control. These findings suggest that miR-24 plays a role in differentiation and bone formation in MC3T3-E1 cells.

Finally, the four groups of transfected cells (miR-24, miR-NC, anti-miR-24, and anti-miR-NC) were infected with *S. aureus* at a MOI of 100 for 7 days, and human bone alkaline phosphatase (ALP) activity was assayed, as another measure of bone mineralization, using an ALP kit. As shown in Figure 2F, the trends were the same as those observed for quantification of Alizarin red staining, with increased activity in miR-24-transfected cells compared with *S. aureus*-infected only cells ($P < 0.05$) and decreased activity in anti-miR-24-transfected cells compared with *S. aureus*-infected

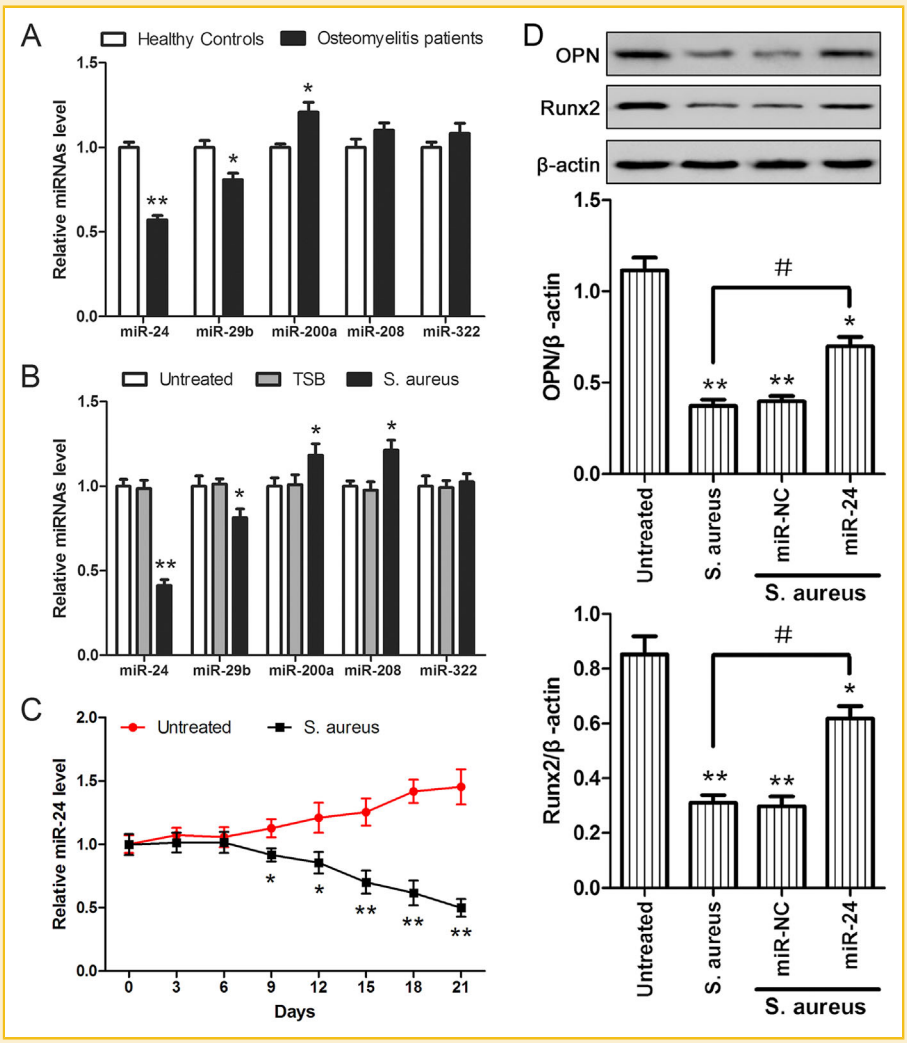

Fig. 1. MiR-24 expression levels significantly differ between MC3T3-E1 cells infected with *S. aureus* and untreated control cells. MiRNA expression levels were analyzed in MC3T3-E1 cells infected with *S. aureus* and in the whole blood of patients with osteomyelitis compared with healthy controls, by qRT-PCR. Expression levels of miR-24, miR-29b, miR-200a, miR-208, and miR-322 were analyzed. A: Differential miRNA levels in the whole blood of patients with bacterial osteomyelitis compared with healthy controls were analyzed by qRT-PCR. Expression levels were normalized against RNU6B. *$P < 0.05$, **$P < 0.01$ versus healthy controls. B and C: MC3T3-E1 cells were infected with *S. aureus* at a MOI of 100 or treated with the same volume of tryptic soy broth (TSB), as a control, for 21 days. B: Differential miRNA levels in the cells were analyzed by qRT-PCR. Expression levels were normalized against RNU6B. *$P < 0.05$, **$P < 0.01$ versus untreated group. C: Changes in miR-24 expression levels in MC3T3-E1 cells were analyzed further by qRT-PCR every three days. *$P < 0.05$, **$P < 0.01$ versus untreated group. D: MC3T3-E1 cells were transfected with 50 nM RNA oligonucleotides of miR-24 or miR-NC for 24 h, and transfected or non-transfected MC3T3-E1 cells were infected with *S. aureus* at a MOI of 100 for 7 days. Untreated cells served as a control. Protein levels of OPN and Runx2 were assessed by western blotting. β-actin was used as a loading control. The western blotting results were normalized to β-actin. *$P < 0.05$, **$P < 0.01$ versus untreated cells. #$P < <0.05$.

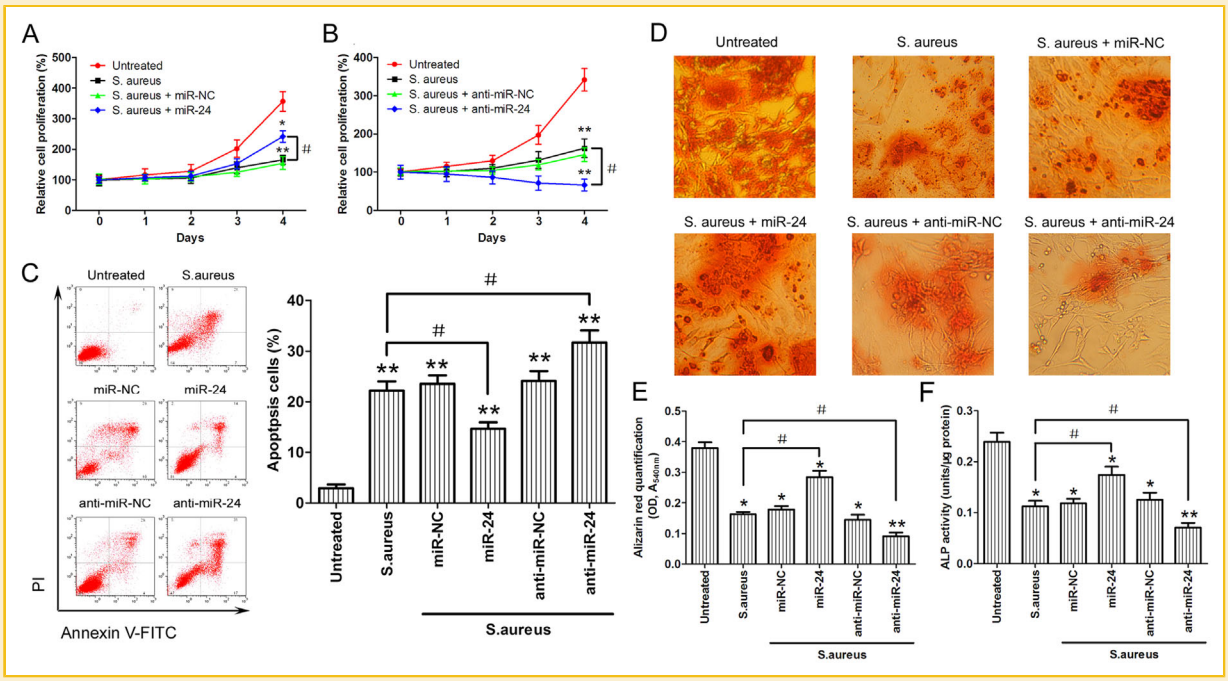

Fig. 2. MiR-24 affects cell viability, apoptosis, and mineralization of MC3T3-E1 cells infected with *S. aureus*. MC3T3-E1 cells transfected with oligonucleotides miR-24 or anti-miR-24 and infected with *S. aureus* were analyzed by MTT assay, apoptosis assay, ALP assay, and Alizarin red staining. MC3T3-E1 cells were transfected with: (A) 50 nM RNA oligonucleotides of miR-24 or miRNA negative control (miR-NC), (B) anti-miR-24 or anti-miR-NC, for 24 h, and then infected with *S. aureus* at a MOI of 100 for 0, 1, 2, 3, or 4 days. Cell viability was then analyzed by an MTT assay. Untreated cells and infected only cells (not transfected) served as controls. $^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated group. $\#P < 0.05$. C: The four groups of transfected cells (miR-24, miR-NC, anti-miR-24, anti-miR-NC) were infected with *S. aureus* at a MOI of 100 for 24 h, and apoptosis was determined by flow cytometry followed by Annexin V–PI double staining. $^{**}P < 0.01$ versus untreated group. $\#P < 0.05$. D: The four groups of transfected cells were infected with *S. aureus* at a MOI of 100 for 21 days and were then observed by Alizarin red staining, magnification 200×. E: Alizarin red staining was quantified by measuring the OD at an absorbance of 540 nm. $^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated group. $\#P < 0.05$. F: The four groups of transfected cells were infected with *S. aureus* at a MOI of 100 for 7 days, ALP activity was assayed using an ALP kit. $^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated group. $\#P < 0.05$.

only cells ($P < 0.05$). These findings confirmed the potential role of miR-24 in differentiation and bone formation in MC3T3-E1 cells.

MIR-24 BINDS TO THE 3′-UTR OF CHI3L1 mRNA AND AFFECTS CHI3L1 EXPRESSION DURING *S. aureus*-INFECTION OF MC3T3-E1 CELLS

To investigate the miR-24 binding site in the 3′-UTR of CHI3L1 mRNA, the miR-24 sequence and the 3′-UTR of CHI3L1 mRNA were aligned (Fig. 3A). Within these sequences, a conserved 9-bp "seed" sequence predicted as the miR-24 binding site was clearly observed. The sequence of an RNA mutant of the 3′-UTR of CHI3L1 was also included in the alignment. The mutant sequence contains four single base substitutions, disrupting the putative miR-24 binding site. To confirm the interaction between the 3′-UTR of CHI3L1 mRNA and miR-24, MC3T3-E1 cells were co-transfected with the wild-type or mutant pGL3-CHI3L1 3′-UTR and RNA oligonucleotides of miR-24 or miR-NC. Firefly and Renilla luciferases were used as bioreporters and the ratio of firefly to Renilla luciferase activity was calculated. The results indicated a significant decrease in luciferase activity of cells co-transfected with wild-type pGL3-CHI3L1 3′-UTR and miR-24 compared with those co-transfected with wild-type pGL3-CHI3L1 3′-UTR and miR-NC ($P < 0.05$) (Fig. 3B). There was no statistically significant difference between the cells co-transfected with mutant pGL3-CHI3L1 3′-UTR and miR-24 or miR-NC (Fig. 3B). These findings confirmed the interaction between the 3′-UTR of CHI3L1 mRNA and miR-24.

Next, the relative mRNA (Fig. 3C) and protein (Fig. 3D) expression levels of CHI3L1 were examined in MC3T3-E1 cells transfected with miR-24, miR-NC, anti-miR-24, or anti-miR-NC for 24 h, then infected with *S. aureus* at a MOI of 100 for 72 h. The results of qRT-PCR indicated that all of the transfected cells and the infected only cells (not transfected) showed increased expression of CHI3L1 mRNA compared with the untreated control. Furthermore, a significant decrease in CHI3L1 mRNA levels of miR-24-transfected cells and a significant increase in CHI3L1 mRNA levels of anti-miR-24-transfected cells was observed compared with the *S. aureus*-infected only cells ($P < 0.05$) (Fig. 3C). The same trends were observed for the relative protein expression levels of CHI3L1, as analyzed by western blotting, in this set of cells (Fig. 3D). These findings further confirm the interaction between CHI3L1 mRNA and miR-24, and suggest that miR-24 negatively affects CHI3L1 expression.

CHI3L1 EXPRESSION LEVELS INCREASE DURING *S. aureus*-INFECTION OF MC3T3-E1 CELLS OR THE WHOLE BLOOD OF OSTEOMYELITIS PATIENTS

Next, CHI3L1 mRNA levels were analyzed in the whole blood of patients with bacterial osteomyelitis by qRT-PCR and were found to be significantly increased compared with healthy controls ($P < 0.05$)

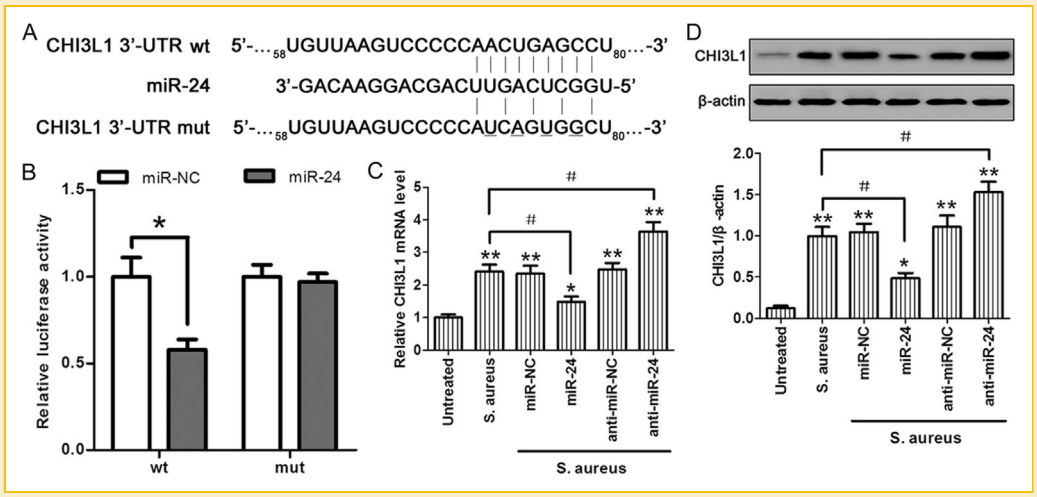

Fig. 3. MiR-24 binds to the 3′-UTR of CHI3L1 mRNA and affects CHI3L1 expression. A: Sequence alignment of the miR-24 RNA sequence and the 3′-UTR of CHI3L1 mRNA containing the conserved 9-bp "seed" sequence predicted as the miR-24 binding site. The paired miR-24 seed sequence and the seed-recognizing site in the wild-type (wt) and mutant (mut) 3′-UTR of CHI3L1 are indicated. B: MC3T3-E1 cells were co-transfected with the wt or mut pGL3-CHI3L1 3′-UTR and RNA oligonucleotides of miR-24 or miR-NC. The activities were calculated as a ratio of firefly to Renilla luciferase activity. $^{*}P < 0.05$. C and D: MC3T3-E1 cells were transfected with 50 nM RNA oligonucleotides of miR-24, miR-NC, anti-miR-24, or anti-miR-NC for 24 h, and the transfected and non-transfected MC3T3-E1 cells were infected with *S. aureus* at a MOI of 100 for 72 h. Untreated and infected only cells (not transfected) served as controls. C: CHI3L1 mRNA levels were analyzed by qRT-PCR. Expression levels were normalized to GAPDH. $^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated cells. $\#P < 0.05$. D: CHI3L1 protein levels were assessed by western blotting. β-actin was used as a loading control. The western blotting results were normalized to β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated cells. $\#P < 0.05$.

(Fig. 4A). Then, CHI3L1 mRNA (Fig. 4B) and protein (Fig. 4C) expression levels were analyzed in *S. aureus*-infected MC3T3-E1 cells by qRT-PCR and western blotting, respectively, over a time course of 72 h. CHI3L1 mRNA and protein expression levels increased steadily in MC3T3-E1 cells over the 72 h time course of infection. All time points showed a statistically significant difference in CHI3L1 mRNA levels compared with the untreated cells.

CHI3L1 EXPRESSION LEVELS AFFECT MINERALIZATION OF MC3T3-E1 CELLS INFECTED WITH *S. aureus*

To analyze the effects of CHI3L1 expression levels on *S. aureus* infection of MC3T3-E1 cells, cells were transfected with lentivirus expression CHI3L1 short hairpin RNA (shRNA) (shRNA-CHI3L1) or negative control shRNA (shRNA-NC) for 24 h. Then, transfected and non-transfected MC3T3-E1 cells were infected with *S. aureus* at a MOI of 100 for 72 h and CHI3L1 mRNA (Fig. 5A) and protein (Fig.5B) levels were analyzed by qRT-PCR and western blotting, respectively. CHI3L1 mRNA expression was significantly increased in *S. aureus*-infected cells compared with uninfected cells ($P < 0.01$). However, transfection with shRNA-CHI3L1 dramatically reduced CHI3L1 mRNA expression in both infected and uninfected cells compared with the non-transfected control ($P < 0.01$). The results of western blotting showed the same trends as for mRNA expression. These findings confirmed that transfection of cells with shRNA-CHI3L1 effectively silenced CHI3L1 mRNA and protein expression.

Next we used the shRNA-CHI3L1 construct to analyze the effects of CHI3L1 silencing in MC3T3-E1 cells. Relative miR-24 levels were investigated and transfection with shRNA-CHI3L1 resulted in a significant increase in miR-24 levels compared with the negative control in untreated cells ($P < 0.01$) and in *S. aureus*-infected cells ($P < 0.01$) (Fig. 5C), confirming the interaction between miR-24 and CHI3L1.

Then, cells transfected with shRNA-CHI3L1 or shRNA-NC were infected with *S. aureus* at a MOI of 100 for 21 days and observed by Alizarin red staining (Fig. 5D). Mineralization was observed in all of the treated MC3T3-E1 cells compared with the untreated control cells. However, low levels of mineralization were observed in the shRNA-CHI3L1-transfected cells compared with the control cells. Alizarin red staining was quantified by measuring the optical density of cells at an absorbance of 540 nm (Fig. 5E). Alizarin red staining was highest in untreated cells. In cells treated with *S. aureus*, a significant increase in staining was seen in shRNA-CHI3L1-transfected cells ($P < 0.05$) compared with the negative control. To confirm the effect of CHI3L1 on mineralization of MC3T3-E1 cells, cells transfected with shRNA-CHI3L1 or shRNA-NC for 24 h were infected with *S. aureus* at a MOI of 100 for 7 days and ALP activity was assayed using an ALP kit. As shown in Figure 5F, a statistically significant increase was detected between the infection only (non-transfected) cells and shRNA-CHI3L1-transfected cells ($P < 0.05$), confirming the potential involvement of CHI3L1 in differentiation and osteogenesis in this pre-osteoblastic cell line.

DISCUSSION

Osteomyelitis is a debilitating infectious disease of the bone, predominantly caused by *S. aureus*. In previous studies, several miRNAs have been shown to play a role in osteogenesis by regulating osteoblast cell growth or differentiation in human or mouse cell lines [Luzi et al., 2008; Mizuno et al., 2008]. However, the specific role of

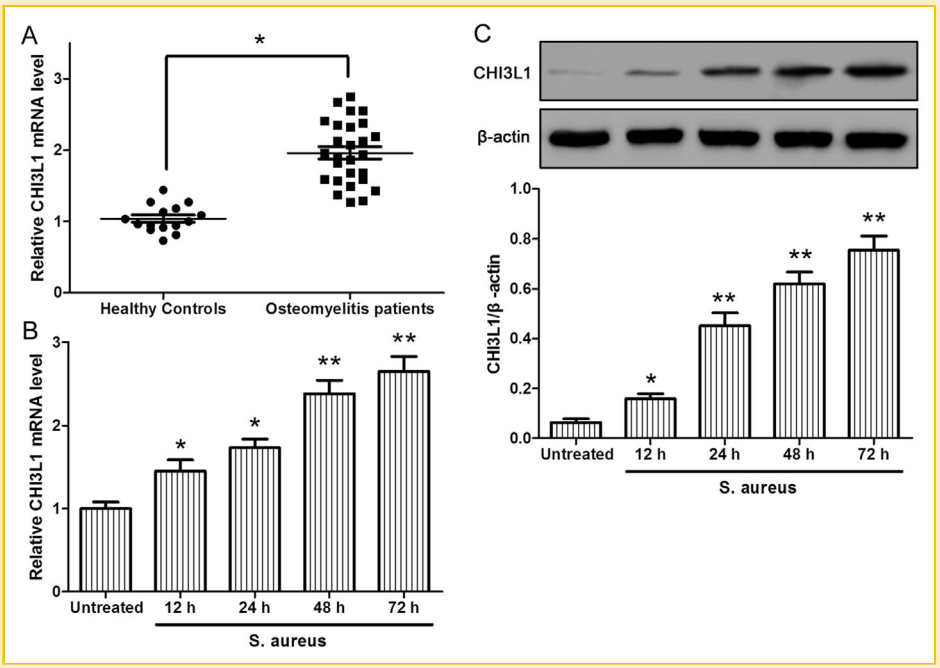

Fig. 4. CHI3L1 expression levels increase during *S. aureus*-infection of MC3T3-E1 cells or the whole blood of osteomyelitis patients. CHI3L1 expression was analyzed at the RNA and protein level in *S. aureus*-infected MC3T3-E1 cells, and at the mRNA level in the whole blood of patients with bacterial osteomyelitis. A: CHI3L1 mRNA levels in the whole blood of patients with bacterial osteomyelitis or healthy controls were analyzed by qRT-PCR. Expression levels were normalized with GAPDH. $^{*}P < 0.05$ versus healthy controls. B and C: MC3T3-E1 cells were infected with *S. aureus* at a MOI of 100 for 12, 24, 48, or 72 h. B: CHI3L1 mRNA levels were analyzed in *S. aureus*-infected MC3T3-E1 cells by qRT-PCR. Expression levels were normalized with GAPDH. $^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated. C: CHI3L1 protein levels were assessed in *S. aureus*-infected MC3T3-E1 cells by western blotting. β-actin was used as a loading control. The western blotting results were normalized to β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$ versus untreated.

miRNAs in bone formation remains to be determined. In the present study, the expression levels of miRNAs proposed to potentially play a regulatory role in osteogenesis (miR-24, miR-29b, miR-200a, miR-208, miR-322) [Itoh et al., 2009 2010; Eguchi et al., 2013; Suh et al., 2013; Vimalraj and Selvamurugan, 2014] were analyzed in the whole blood of patients with bacterial osteomyelitis or healthy controls, and in MC3T3-E1 cells infected with *S. aureus*, by qRT-PCR. The expression of miR-24 was significantly down-regulated in osteomyelitis patients and *S. aureus*-infected MC3T3-E1 cells compared with the healthy controls or untreated control cells.

Our findings suggest that miR-24 plays a regulatory role in the process of osteogenesis. We demonstrated that CHI3L1 is a target gene of miR-24 using a luciferase reporter assay. MicroRNA miR-24 suppressed the expression of CHI3L1 mRNA, thought to mediate multiple signaling pathways, by directly binding to the 3′-UTR region. Therefore, downregulation of miR-24 expression during osteomyelitis would remove the repression of CHI3L1 expression, as confirmed by our findings. CHI3L1, a member of the 'mammalian chitinase-like proteins', encodes a 40 kD secretory glycoprotein that is not expressed under physiological conditions but induced in a variety of inflammatory conditions and cancers, and during tissue remodeling [Hakala et al., 1993; Morrison and Leder, 1994; Shackelton et al., 1995]. The role of CHI3L1 in osteogenesis is indicated by its increased expression during osteomyelitis but the mechanism involved remains to be elucidated. Chitin is the most abundant polysaccharide in microorganisms. CHI3L1 does not possess any catalytic activity but interacts with chitin potentially affecting bacterial adherence and invasion. It is therefore proposed that CHI3L1 also plays a role in antibacterial responses. In a previous report, CHI3L1 was shown to play a central role in promoting bacterial clearance and mediating host tolerance during *Streptococcus pneumoniae* infection (Dela Cruz et al., 2012). Our findings indicate that the regulation of CHI3L1 mediated by miR-24 may play an important role in the process of osteogenesis and/or in antibacterial responses. Previous studies have reported other targets of miR-24, including FAF1 involved in cell survival [Qin et al., 2010], H2AX involved in DNA repair [Lal et al., 2009], and ALK4 involved in erythroid differentiation [Wang et al., 2008], demonstrating the wide range of regulatory roles played by miRNAs.

MC3T3-E1 cells were used in this study to investigate osteoblast function. Osteoblasts are the main functional cells in bone formation and are responsible for the synthesis, secretion and mineralization of the bone matrix. Our findings showed that *S. aureus* inhibited MC3T3-E1 cell proliferation, induced osteoblast apoptosis and prohibited bone formation and mineralization. CHI3L1 expression levels were found to be increased during *S. aureus* infection of MC3T3-E1 cells, negatively affecting ALP activity and bone mineralization. OPN and Runx2 protein levels were found to be decreased during *S. aureus* infection of MC3T3-E1 cells. OPN and Runx2 are specific biomarkers of osteogenesis and their decreased expression in *S. aureus*-infected MC3T3-E1 cells indicated inhibition of bone formation. CHI3L1 therefore plays a role in osteogenesis but further investigations are required to fully elucidate its role in *S. aureus*-induced osteomyelitis.

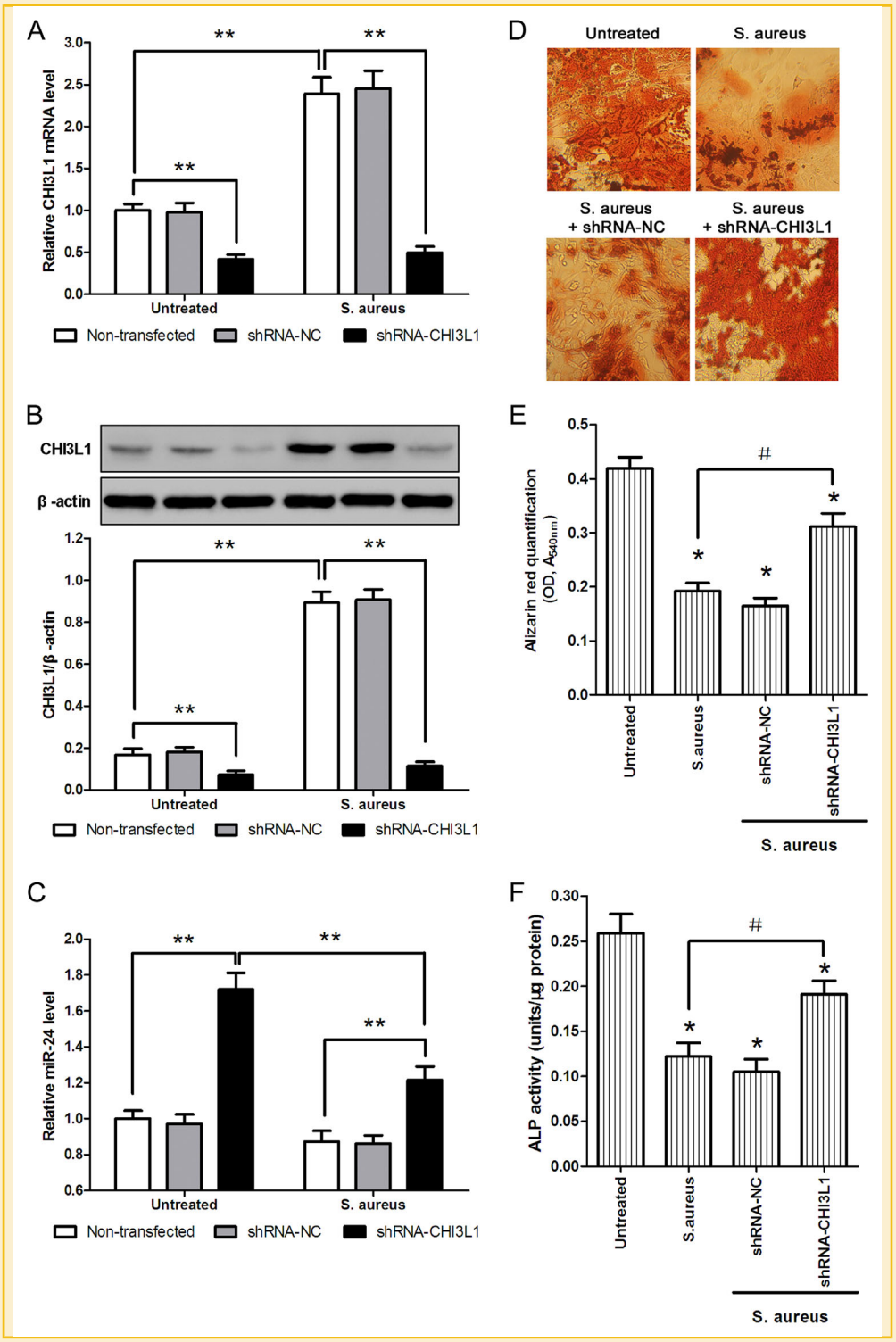

Fig. 5. CHI3L1 expression levels affect mineralization in MC3T3-E1 cells infected with *S. aureus*. Interference RNAs were used to analyze the effect of CHI3L1 expression levels on *S. aureus* infection of MC3T3-E1 cells. A–C: MC3T3-E1 cells were transfected with lentivirus expression CHI3L1 short hairpin RNA (shRNA) (shRNA-CHI3L1) or negative control shRNA (shRNA-NC) for 24 h. Then, transfected and non-transfected MC3T3-E1 cells were infected with *S. aureus* at a MOI of 100 for 72 h. A: CHI3L1 mRNA levels were analyzed by qRT-PCR. Expression levels were normalized to GAPDH. $^{**}P < 0.01$. B: CHI3L1 protein levels were assessed by western blotting. β-actin was used as a loading control. The western blotting results were normalized to β-actin. $^{**}P < 0.01$. C: Relative miR-24 levels were assessed by qRT-PCR. $^{**}P < 0.01$. D–F: MC3T3-E1 cells were transfected with 50 nM lentivirus expression shRNA-CHI3L1 or shRNA-NC for 24 h. D: Following 21 days of infection with *S. aureus* at a MOI of 100, mineralization in transfected and non-transfected MC3T3-E1 cells was observed by Alizarin red staining, magnification 200×. E: Alizarin red staining was quantified by measuring the OD at an absorbance of 540 nm. $^{*}P < 0.05$ versus untreated group. $\#P < 0.05$. F: Following 7 days of infection with *S. aureus* at a MOI of 100, ALP activity was assayed using an ALP kit. $^{*}P < 0.05$ vs untreated group. $\#P < 0.05$.

We also found that overexpression of miR-24 could reduce the effects of *S. aureus* on MC3T3-E1 cells, while inhibition of miR-24 intensified the effects. It is therefore possible that new therapies for osteomyelitis could exploit miR-24 as a drug target.

In summary, our findings provide new insight into the pathogenic mechanism behind *S. aureus*-induced osteomyelitis and the role of miRNAs in osteogenesis. An increased understanding of this disease is vital for the development of new treatments.

ACKNOWLEDGMENTS

The study was supported by National Natural Science Foundation of China (No: 81171734) and Science and technology plan projects of Yunnan province (No: 2012BC007).

REFERENCES

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

Bartel DP. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297.

Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. Cell 136(2):215–233.

Dela Cruz CS, Liu W, He CH, Jacoby A, Gornitzky A, Ma B, Flavell R, Lee CG, Elias JA. 2012. Chitinase 3-like-1 promotes *Streptococcus pneumoniae* killing and augments host tolerance to lung antibacterial responses. Cell Host Microbe 12(1):34–46.

Eguchi T, Watanabe K, Hara ES, Ono M, Kuboki T, Calderwood SK. 2013. OstemiR: A novel panel of microRNA biomarkers in osteoblastic and osteocytic differentiation from mesenchymal stem cells. PLoS One 8:e 58796.

Filipowicz W, Bhattacharyya SN, Sonenberg N. 2008. Mechanisms of post-transcriptional regulation by microRNAs: Are the answers in sight? Nat Rev Genet 9:102–114.

Forman JJ, Legesse-Miller A, Coller HA. 2008. A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence. Proc Natl Acad Sci U S A 105(39):14879–14884.

Hakala BE, White C, Recklies AD. 1993. Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. J Biol Chem 268(34):25803–25810.

Itoh T, Nozawa Y, Akao Y. 2009. MicroRNA-141 and -200a are involved in bone morphogenetic protein-2-induced mouse pre-osteoblast differentiation by targeting distal-less homeobox 5. J Biol Chem 284:19272–19279.

Itoh T, Takeda S, Akao Y. 2010. MicroRNA-208 modulates BMP-2-stimulated mouse preosteoblast differentiation by directly targeting V-ets erythroblastosis virus E26 oncogene homolog 1. J Biol Chem 285:27745–27752.

Ko HY, Lee DS, Kim S. 2009. Noninvasive imaging of microRNA 124a-mediated repression of the chromosome 14 ORF 24 gene during neurogenesis. FEBS J 276(17):4854–4865.

Lal A, Pan Y, Navarro F, Dykxhoorn DM, Moreau L, Meire E, Bentwich Z, Lieberman J, Chowdhury D. 2009. MiR-24-mediated downregulation of H2AX suppresses DNA repair in terminally differentiated blood cells. Nat Struct Mol Biol 16(5):492–498.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25(4):402–408.

Luzi E, Marini F, Sala SC, Tognarini I, Galli G, Brandi ML. 2008. Osteogenic differentiation of human adipose tissue-derived stem cells is modulated by the miR-26a targeting of the SMAD1 transcription factor. J Bone Miner Res 23:287–295.

Mizuno Y, Yagi K, Tokuzawa Y, Kanesaki-Yatsuka Y, Suda T, Katagiri T, Fukuda T, Maruyama M, Okuda A, Amemiya T, Kondoh Y, Tashiro H, Okazaki Y. 2008. MiR-125b inhibits osteoblastic differentiation by down-regulation of cell proliferation. Biochem Biophys Res Commun 368:267–272.

Morrison BW, Leder P. 1994. Neu and ras initiate murine mammary tumors that share genetic markers generally absent in c-myc and int-2-initiated tumors. Oncogene 9(12):3417–3426.

Murphy CM, Haugh MG, O′Brien FJ. 2010. The effect of mean pore size on cell attachment, proliferation, and migration in collagen-glycosaminoglycan scaffolds for bone tissue engineering. Biomaterials 31(3):461–466.

Prakash M, Bodas M, Prakash D, Nawani N, Khetmalas M, Mandal A, Eriksson C. 2013. Diverse pathological implications of YKL-40: Answers may lie in ′outside in′ signaling. Cell Signal 25(7):1567–1573.

Plunkett NA, Partap S, O′Brien FJ. 2010. Osteoblast response to rest periods during bioreactor culture of collagen-glycosaminoglycan scaffolds. Tissue Eng Part A 16(3):943–951.

Qin W, Shi Y, Zhao B, Yao C, Jin L, Ma J, Jin Y. 2010. MiR-24 regulates apoptosis by targeting the open reading frame (ORF) region of FAF1 in cancer cells. PLoS One 5(2):e9429.

Rana TM 2007. Illuminating the silence: Understanding the structure and function of small RNAs. Nat Rev Mol Cell Biol 8:23–36.

Shackelton LM, Mann DM, Millis AJ. 1995. Identification of a 38-kDa heparin-binding glycoprotein (gp38k) in differentiating vascular smooth muscle cells as a member of a group of proteins associated with tissue remodeling. J Biol Chem 270(22):13076–13083.

Stefani G, Slack FJ. 2008. Small non-coding RNAs in animal development. Nat Rev Mol Cell Biol 9:219–230.

Suh JS, Lee JY, Choi YS, Chung CP, Park YJ. 2013. Peptide-mediated intracellular delivery of miRNA-29b for osteogenic stem cell differentiation. Biomaterials 34:4347–4359.

Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I. 2008. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature 455(7216):1124–1128.

Tuchscherr L, Medina E, Hussain M, Volker W, Heitmann V, Niemann S, Holzinger D, Roth J, Proctor RA, Becker K, Peters G, Loffler B. 2011. *Staphylococcus aureus* phenotype switching: An effective bacterial strategy to escape host immune response and establish a chronic infection. EMBO Mol Med 3(3):129–141.

Tuchscherr L, Heitmann V, Hussain M, Viemann D, Roth J, von Eiff C, Peters G, Becker K, Loffler B. 2010. *Staphylococcus aureus* small-colony variants are adapted phenotypes for intracellular persistence. J Infect Dis 202(7):1031–1040.

Vimalraj S, Selvamurugan N. 2014. MicroRNAs expression and their regulatory networks during mesenchymal stem cells differentiation toward osteoblasts. Int J Biol Macromol 66:194–202.

Wang Q, Huang Z, Xue H, Jin C, Ju XL, Han JD, Chen YG. 2008. MicroRNA miR-24 inhibits erythropoiesis by targeting activin type I receptor ALK4. Blood 111(2):588–595.

Wright JA, Nair SP. 2010. Interaction of staphylococci with bone. Int J Med Microbiol 300:193–204.

Zuluaga AF, Galvis W, Saldarriaga JG, Agudelo M, Salazar BE, Vesga O. 2006. Etiologic diagnosis of chronic osteomyelitis: A prospective study. Arch Intern Med 166:95–100.