Original Paper

Silencing of Long Non-Coding RNA NONHSAT009968 Ameliorates the Staphylococcal Protein A-Inhibited Osteogenic Differentiation in Human Bone Mesenchymal Stem Cells

Yi Cui^{a,b} Sheng Lu^b Hongbo Tan^b Jun Li^b Min Zhu^b Yongqing Xu^{a,b}

^aThe Third Military Medical University, Chongqing, ^bDepartment of Orthopaedic Surgery, Kunming General Hospital of Chengdu Military Command, Kunming, China

Key Words

Human bone mesenchymal stem cells • SpA • lncRNA microarray • Osteogenic differentiation • NONHSAT009968

Abstract

Background/Aims: Osteomyelitis is defined as an inflammation of the bones and bone marrow. The inflammatory microenvironment attenuates the osteogenic differentiation capacity of stem cells and inhibits osteoblast-mediated bone formation, leading to net bone loss. However, the whole expression profile, function and side effect of long non-coding RNAs (lncRNAs) on osteogenic differentiation of stem cells in an inflammatory microenvironment of osteomyelitis are not known. **Methods:** In the present study, human bone mesenchymal stem cells (hBMSCs) were treated with different concentrations of Staphylococcal protein A (SpA) to trigger an inflammatory microenvironment *in vitro* to partly duplicate the inflammatory microenvironment of osteomyelitis, which was confirmed using ELISA for detecting the inflammatory cytokines. The complete expression profiles of lncRNAs and mRNA during osteogenic differentiation of hBMSCs in an inflammatory microenvironment triggered by SpA were analyzed using a lncRNA microarray. lncRNA expression levels were verified by quantitative reverse transcription PCR analysis (qRT-PCR). The expression of NONHSAT009968 in hB-MSCs was silenced by infection with lentivirus expressing NONHSAT009968-shRNA. The expression of Runx2, OCN, OPN, COL1A1, and alkaline phosphatase (ALP) activity was detected by western blot. Alizarin red staining and ALP activity detection were carried out. **Results:** The results of ELISA showed that SpA treatment induced secretion of inflammatory cytokines IL-1A, IL-6, and TNFA. The results of alizarin red staining and ALP detection showed that SpA treatment suppressed the osteogenic differentiation of hBMSCs. A total of 2033 lncRNAs were found with aberrant expression in SpA-treated hBMSCs compared to controls. Among these lncRNAs, 641 were down-regulated and 1392 were up-regulated. Based on the results of qRT-PCR, lncRNA NONHSAT009968 was chosen for further investigation. The results of alizarin red staining, ALP activity detection, and western blot detection of Runx2, OCN,

Yongqing Xu

Department of Orthopaedic Surgery, Kunming General Hospital of Chengdu Military Command, NO.212 Duguan Road, Xi Shan District, Kunming city, Yunnan Province (China) E-Mail xuyongqing_kunming@163.com

OPN, COL1A1, and ALP indicated that NONHSAT009968 silencing ameliorates SpA-inhibited osteogenic differentiation in hBMSCs. **Conclusion:** Our present study provides a basis for future analyses of the role of lncRNAs in osteoblastic differentiation in an inflammatory environment triggered by SpA, and lncRNA NONHSAT009968 might be a new target for promoting osteoblast formation.

© 2016 The Author(s)
Published by S. Karger AG, Basel

Introduction

Osteomyelitis, an inflammation of the bones and bone marrow, develops as a result of contiguous spread, from adjacent soft tissues and joints, hematogenous seeding, or direct inoculation of microorganisms into the bone as a result of trauma or surgery [1]. Despite advances in surgical and medical management of osteomyelitis, it is still considered one of the most difficult to treat infectious diseases. Osteomyelitis is characterized by progressive destruction of the bone and the formation of sequestra. Osteoblasts function as the major directors of net bone formation or resorption during normal physiological turnover of bone and following infection [2]. Thus, promoting osteoblast formation is a key strategy for suppressing the progressive bone destruction and formation of sequestra induced by osteomyelitis.

Bone mesenchymal stem cells (BMSCs) are multi-potent stem cells with the ability to self-renew, proliferate, and differentiate. Thus, BMSCs play an important role in the bone formation process. However, the inflammation microenvironment of osteomyelitis is known to attenuates the osteogenic differentiation capacity of stem cells and inhibits osteoblast-mediated bone formation, leading to a net bone loss [3, 4]. *Staphylococcus aureus* is the most common microorganism recovered from osteomyelitis [5, 6]. Staphylococcal protein A (SpA) is an important virulence factor of *Staphylococcus aureus* [5, 6]. Previous studies show that SpA can induce an inflammatory response [7, 8]. However, whether SpA can induce an inflammatory response in BMSCs has not yet been investigated. In the present study, human BMSCs (hBMSCs) were treated with SpA to investigate whether SpA could induce an inflammatory response in BMSCs, and determine if SpA treatment suppresses osteogenic differentiation ability of BMSCs. Our results showed that treatment of hBMSCs with SpA resulted in marked increases in the concentrations of inflammatory cytokines IL-1A, IL-6, and TNFA in the supernatants of treated cells. In addition, SpA treatment suppressed calcium mineralization and ALP activity during the osteogenic differentiation process. Altogether, these results indicate that SpA treatment can successfully trigger an inflammatory environment and suppresses the osteogenic differentiation of hBMSCs. Thus, this cell model was used to simulate inflammatory environment of osteomyelitis in hBMSCs *in vitro*.

Long noncoding RNAs (lncRNAs), non-protein coding transcripts longer than 200 nucleotides, have been implicated in a range of developmental processes and diseases [9]. Previous studies indicate that some lncRNAs play a role in regulating osteogenic differentiation of stem cells, such as AK141205, ANCR, and MEG3 [10-12]. Certain lncRNAs, such as ANCR, and H19, also promote osteoblast differentiation [13, 14]. In addition, the lncRNAs, ANRIL, MALAT1, LOC100652951, and LOC100506036, play key roles in regulating the inflammatory response [15-17]. However, the complete expression profile, function and side effect of lncRNAs during the osteogenic differentiation of stem cells in an inflammatory microenvironment of osteomyelitis were not known. Thus, in the present study, we first investigated the whole expression profile of lncRNAs on osteogenic differentiation of hBMSCs in an inflammatory microenvironment triggered by SpA. We identified 2033 lncRNAs with aberrant expression during osteogenic differentiation in SpA-treated hBMSCs compared to controls. Then the expression of potential key lncRNAs related to osteogenic differentiation identified by cis-100k analysis was validated by quantitative reverse transcription PCR analysis (qRT-PCR). Finally, the function of lncRNA NONHSAT009968 in regulating osteogenic differentiation of hBMSCs was investigated, and the results showed that NONHSAT009968 silencing ameliorates SpA-inhibited osteogenic differentiation in hBMSCs.

Materials and Methods

Cell culture and osteogenic differentiation

hBMSCs were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The hBMSCs were cultured in MSC growth medium. The hBMSCs were seeded at a concentration of 2×10^4 cells/cm² in 6-well plates in MSC growth medium to 70% confluency. Then the medium was replaced with osteogenic differentiation medium (50 μ M ascorbate 2-phosphate, 10 mM β -glycerol phosphate, 0.1 μ M dexamethasone and 10% FBS in DMEM low glucose medium, all from Cyagen Corporation, Guangzhou, China). The media were completely replaced with fresh osteogenic differentiation media every 3 days for 2-3 weeks.

siRNA synthesis, siRNA transfection, and lentivirus packaging

The siRNA targeting NONHSAT009968 was obtained from GenePharma Co., Ltd. The sense siRNA-1 sequence is GGAGUAGUGACCAACUUCUdTdT; sense siRNA-2 sequence is GCAAUGACCUUAGAAAGCAdTdT; and sense siRNA-3 sequence is CUUUAAGCAAGCAUGACAdTdT. Approximately 2×10^4 hBMSCs were plated in a 6-well plate the day before transfection. Approximately 200 nM siRNA was transfected into hBMSCs using Lipofectamine 2000 reagent according to the manufacturer's instructions (Promega, Madison, WI, USA). A scramble sequence was used as a negative control [2]. The efficiency of siRNA was determined by quantitative reverse transcription PCR.

We selected siRNA-2 for lentivirus packaging. NONHSAT009968 shRNA was generated and inserted into the pLVX-IRES-ZsGreen1 plasmid. pLVX lentiviral particles containing NONHSAT009968 shRNA were generated by transiently transfecting 293T cells. Lentivirus production, concentration, and titration were performed according to standard procedures.

SpA treatment and cell sample preparation

For construction of the cell model, different concentrations of SpA (100 ng/mL, 500 ng/mL, 1000 ng/mL, 10 μ g/mL, 100 μ g/mL) were added to MSC growth medium; hBMSCs were seeded in 96-well plates or 24-well plates in MSC growth medium or SpA-treated MSC growth medium, and incubated for 72 h. After treatment, cell supernatants from the 24-well plates were harvested for ELISA and alkaline phosphatase (ALP) detection. For alizarin red staining, hBMSCs were seeded in 96-well plates in osteogenic differentiation medium or osteogenic differentiation medium treated with different concentrations of SpA (100 ng/mL, 500 ng/mL, 1000 ng/mL, 10 μ g/mL, 100 μ g/mL), and cultured for 3 weeks. For lncRNA microarray analysis, hBMSCs were seeded at a concentration of 2×10^4 cells/cm² in 6-well plates in osteogenic differentiation medium or osteogenic differentiation medium treated with 1000 ng/mL SpA, and cultured for 2 weeks. For qRT-PCR validation, hBMSCs were seeded at a concentration of 2×10^4 cells/cm² in 6-well plates in osteogenic differentiation medium or osteogenic differentiation medium treated with 1000 ng/mL SpA, and cultured for 2 or 3 weeks.

After infection with the lentivirus expressing NONHSAT009968-shRNA or empty lentivirus for 3 days, hBMSCs were harvested for qRT-PCR to confirm that the shRNA could successfully silence NONHSAT009968. After infection with lentivirus expressing NONHSAT009968-shRNA or empty lentivirus for 3 days, hBMSCs were seeded in 6-well plates or 96-well plates in osteogenic differentiation medium treated with 1000 ng/mL SpA, and cultured for 2 and 3 weeks. Cells from each group were prepared in 96-well plates for alizarin red staining. Cell supernatants from 6-well plates were harvested for ALP detection. Cells from 6-well plates were harvested for western blot and qRT-PCR assays.

ELISA

Human IL-1A, IL-6, and TNFA ELISA kits were purchased from MultiSciences Biotech Co., Ltd. (Hangzhou, China). Briefly, all reagents and working standards were prepared per the manufacturer's instructions. Microwell strips were washed twice with 300 μ L Wash Buffer per well followed by thorough aspiration of the microwell contents. Then, 300 μ L of Wash Buffer was added per well, and incubated for about 15 min before aspiration. After the last wash step, the wells were emptied and the microwell strips were tapped on an absorbent pad or paper towel to remove excess Wash Buffer. We then added 50 μ L of Assay Buffer to each well, followed by 50 μ L of the standard, sample, or control per well. Subsequently, we added 50 μ L of Detect Antibody to each well, covered the microplate with an adhesive strip, and incubated

at room temperature (18 to 25°C) for 2 hours on a microplate shaker set at 100 rpm. After the shaking incubation, the wells were washed with 300 µL of Wash Buffer each time. After the last wash, the plates were inverted and blotted on clean paper towels. Next, we added 100 µL of Streptavidin HRP to each well, incubated at room temperature for 45 min on a microplate shaker set at 100 rpm, and repeated the aspiration/wash step as above. After washing, 100 µL of Substrate Solution were added to each well, and the plates were incubated for 10 – 30 min at room temperature. Lastly, we added 100 µL of Stop Solution to each well, and determined the optical density within 30 minutes using a microplate reader (Multiscan MK3; Thermo Fisher Scientific, Waltham, MA, USA) set to 450 nm.

ALP activity detection

The ALP detection kits were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Briefly, 50 µL buffer solution, 50 µL substrate solution, and 5 µL ddH₂O were added to 96-well plates for blank wells; 50 µL buffer solution, 50 µL substrate solution, and 5 µL 0.1 mg/mL phenol solution were added to 96-well plates for standard wells; and 50 µL buffer solution, 50 µL substrate solution, and 5 µL cell supernatant were added to 96-well plates for experimental wells. After thorough mixing, the 96-well plates were incubated at 37°C for 15 min. Then, 150 µL of color-developing agent was added to each well, and the absorbance at 520 nm was measured using a microplate reader (Multiscan MK3; Thermo Fisher Scientific, Waltham, MA, USA) after mixing gently. All experiments were performed in triplicate and repeated three times. ALP activity could be expressed using King units/100 mL. The calculation formula for King units/100 mL:

$$\text{King unit}/100 \text{ ml} = \frac{\text{OD}_{\text{tested}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{blank}}} \times 0.1 \text{ mg/ml} \times 100 \text{ ml} \times \text{dilution ratio}$$

lncRNA microarray

The Agilent Human lncRNA (Design ID: 0629180) was used in this experiment. Total RNA was extracted from harvested cells by using Trizol reagent and as per the manufacturer's instructions (Invitrogen, CA, USA) and quantified using a NanoDrop ND-2000 (Thermo Scientific). RNA integrity was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies). The sample labeling, microarray hybridization, and washing were performed according to the manufacturer's standard protocols. Briefly, total RNA were transcribed to double-stranded cDNA, synthesized into cRNA, and labeled with Cyanine-3-CTP. The labeled cRNAs were hybridized onto the microarray. After washing, the arrays were scanned using an Agilent Scanner G2505C (Agilent Technologies).

Feature Extraction software (version 10.7.1.1, Agilent Technologies) was used to analyze array images to obtain raw data. Genespring (version 13.1, Agilent Technologies) was employed to finish the basic analysis of the raw data. The raw data were first normalized with the quantile algorithm. The probes with at least 1 out of 2 conditions with flags in 'P' were chosen for further data analysis. Differentially expressed genes or lncRNAs were then identified through fold change. A fold change of ≥ 2.0 was used as a threshold for identifying up- and down-regulated genes.

Functional group analysis

GO analysis was derived from Gene Ontology (www.geneontology.org), which provides three structured networks of defined terms that describe gene product attributes. The P value denotes the significance of GO term enrichment in the differentially expressed mRNA list. We also performed pathway analysis for the differentially expressed mRNAs based on the latest Kyoto Encyclopedia of Genes and Genomes (KEGG) database. This analysis allowed us to determine the biological pathways for which a significant enrichment of differentially expressed mRNAs existed (P<0.05 was considered statistically significant). Cis-100K analysis was carried out to define cis-regulated genes as protein-coding genes coexpressed with one dysregulated lncRNA and within 100 kbp upstream or downstream in the same allele.

RNA extraction and quantitative reverse transcription PCR analysis (qRT-PCR)

Total RNA was extracted from harvested cells using Trizol reagent (Invitrogen, CA, USA). To quantify RNA levels of NONHSAT125464, ENST00000504555, NONHSAT098635, NONHSAT054627, and NONHSAT009968, reverse transcription PCR was performed using a PrimeScript RT Reagent Kit with cDNA Eraser (Takara, Dalian, China), and qRT-PCR was performed using SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan). U6 was used as an internal control. The primer sequences used in qRT-PCR are shown

in Table 1. Gene expression was measured in triplicate, quantified using the $2^{-\Delta\Delta CT}$ method, and normalized to a control. Housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalize

Western blot

Each group of hBMSCs was lysed using RIPA buffer (Beyotime Biotechnology, Nantong, China). The total protein concentration was determined using a BCA Protein Assay kit (Beyotime Biotechnology, Nantong, China). Thirty micrograms of protein were loaded and separated on 10% SDS polyacrylamide gels, and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked for 1 h at room temperature with 5% milk in TBS containing 0.05% Tween-20 (TBST), incubated

for 1 h with rabbit polyclonal anti-COL1A1 antibody (1:800, BS60771, Bioworld Technology, Inc., St. Louis Park, MN, USA), rabbit polyclonal anti-OPN antibody (1:1000, BS1264, Bioworld Technology, Inc., St. Louis Park, MN, USA), rabbit polyclonal anti-OCN antibody (1:500, BS60987, Bioworld Technology, Inc., St. Louis Park, MN, USA), rabbit monoclonal anti-RUNX2 antibody (12556S, 1:1000, Cell Signaling Technologies, Beverly, MA, USA), rabbit monoclonal anti-ALP antibody (1:2500, ab186422, Abcam, Cambridge, MA, USA), or rabbit polyclonal anti-GAPDH antibody (1:3000, ab9485 Abcam, Cambridge, MA, USA), and washed three times with TBST. Membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG H&L secondary antibody (1:10000, ab97080, Abcam, Cambridge, MA, USA) for 40 min, and washed three times with TBST. Proteins were visualized using ECL (Thermo Scientific Pierce ECL Plus).

Statistical Analysis

All statistical analyses were performed using SPSS 19.0 software (IBM, Chicago, IL, USA). Results are represented as means $\pm$ standard deviation (SD). A Student t-test was used to compare means from different groups; P values < 0.05 were regarded as statistically significant.

Results

SpA treatment induced secretion of inflammatory cytokines

To investigate whether SpA could induce the inflammatory response, hBMSCs were treated with different concentrations (0, 0.1, 0.5, 1, 10, 100 μ g/mL) of SpA. After treating for 72 h, cell supernatants were harvested for ELISA analysis. The results showed that SpA treatment could induce secretion of inflammatory cytokines IL-1A, IL-6, and TNFA (Fig. 1). The secretion of inflammatory cytokines IL-1A, IL-6, and TNFA was time- and dose-dependent.

Fig. 1. The effects of different SpA concentrations on secretion of inflammatory cytokines IL-1A, IL-6, and TNFA.

Fig. 2. The effect of different concentrations (0, 0.1, 0.5, 1, 10, 100 $\mu\text{g/mL}$) of SpA on osteogenic differentiation of hBMSCs. A: The effect of different concentrations (0, 0.1, 0.5, 1, 10, 100 $\mu\text{g/mL}$) of SpA on calcium mineralization stained by alizarin red. B: The effect of different concentrations (0, 0.1, 0.5, 1, 10, 100 $\mu\text{g/mL}$) of SpA on ALP activity.

SpA treatment suppresses osteogenic differentiation of hBMSCs

To investigate whether SpA treatment suppress the osteogenic differentiation of hBMSCs, hBMSCs were cultured in osteogenic differentiation medium with different concentrations (0, 0.1, 0.5, 1, 10, 100 $\mu\text{g/mL}$) of SpA for 3 weeks. Then, cells were stained with alizarin red and cell supernatants were harvested for ALP detection. The alizarin red staining revealed that the five different concentrations of SpA could suppress calcium mineralization, with the degree of inhibition directly correlated to the SpA concentration (Fig. 2A). Thus, the results of ALP detection showed that SpA treatment could suppress ALP activity in a dose-dependent manner (Fig. 2B).

Aberrant expression profiles of lncRNA during osteogenic differentiation of hBMSCs treated by SpA

hBMSCs were cultured

in osteogenic differentiation medium with 1 $\mu\text{g/mL}$ SpA for 2 weeks and cells were harvested for lncRNA microarray assays. We identified 2033 lncRNAs with aberrant expression in SpA-treated hBMSCs compared to controls. Among these lncRNAs, 641 were down-regulated and 1392 were up-regulated (fold change > 2.0, P value < 0.05) compared to controls. The top 20 up-regulated and down-regulated lncRNAs are listed in Tables 2 and 3, respectively. From the mRNA expres-

Table 2. The top 20 up-regulated lncRNAs and mRNAs during osteogenic differentiation of hBMSCs treated with SpA, as identified by microarray test

lncRNA Accession	Source	Fold change	mRNA Gene Symbol	Fold change
NONHSAT055511	NONCODE v4	98.13	TECRL	11.49
NONHSAT101432	NONCODE v4	71.57	LOC100128651	9.42
NONHSAT032399	NONCODE v4	58.00	ADCY1	7.92
NONHSAT017755	NONCODE v4	54.90	PAX6	7.91
NONHSAT093218	NONCODE v4	54.21	ADAM9	7.40
NONHSAG044332	NONCODE v4	46.50	PDIK1L	7.31
NONHSAT071625	NONCODE v4	44.62	SLC10A6	7.18
NONHSAT081659	NONCODE v4	44.56	C2orf61	6.76
NONHSAG015211	NONCODE v4	44.35	ARMC3	6.30
NONHSAT070565	NONCODE v4	43.79	TGM5	6.16
TCONS_00023069	broadlincRNA	41.04	C10orf62	5.86
NONHSAT129104	NONCODE v4	40.61	KCNN1	5.80
TCONS_I2_00014301	broadlincRNA	38.06	SULT1B1	5.67
NONHSAG038331	NONCODE v4	34.86	SLC7A14	5.59
NONHSAT107532	NONCODE v4	34.49	MAP3K15	5.48
NR_038325.1	RefSeq	34.28	TDRD12	5.46
NONHSAT132944	NONCODE v4	34.24	PRAM1	5.45
ENST00000579138	Ensembl	33.97	OR5D18	5.41
NONHSAT004306	NONCODE v4	32.65	ZNF192P1	5.37
TCONS_00000693	broadlincRNA	32.56	Q442D3	5.33

sion profiling data, 449 mRNAs were identified as differentially expressed between the two groups. Among these mRNAs, 318 were obviously down-regulated and 131 were obviously up-regulated (fold change > 2.0, P value < 0.05). The top 20 up-regulated and down-regulated mRNAs are listed in Tables 2 and 3, respectively.

KEGG pathway analysis

KEGG assignments were used to classify functional annotations of the identified differentially expressed genes. Based on KEGG pathway analysis, the up-regulated coding mRNAs encompass 20 different pathways, and the most enriched pathways were salivary secretion, bile secretion, glutamatergic synapse, olfactory transduction, and insulin secretion (Fig. 3A). The down-regulated transcripts corresponded to 20 pathways, and the most enriched were hematopoietic cell lineage, retinol metabolism, drug metabolism, cell adhesion molecules, and metabolism of xenobiotics by cytochrome (Fig. 3B).

GO analysis

To understand the functions of differentially expressed genes, all the differentially expressed genes were mapped to terms in the GO database and compared with the background. Based on the GO analysis for biological processes, the up-regulated transcripts were highly enriched for plasminogen activation, and water transport (Fig. 4A), while the down-regulated transcripts were highly enriched for cellular responses to glucocorticoid

Table 3. The top 20 down-regulated lncRNAs and mRNAs during osteogenic differentiation process of hBMSCs treated with SpA, as identified by microarray analysis

lncRNA Accession	Source	Fold change	mRNA Gene Symbol	Fold change
TCONS_00008691	broadlincRNA	1464.84	LOC100132174	12.12
NONHSAG000201	NONCODE v4	52.26	CSF3	6.25
NONHSAT114067	NONCODE v4	27.47	KY	5.70
NR_033772.1	RefSeq	25.51	SFRP4	5.50
TCONS_00021560	broadlincRNA	24.80	ZFYVE26	5.34
FR281130	frnadb v3.4	24.40	C19orf81	5.25
ENST00000562349	Ensembl	23.00	MYOD1	4.05
NONHSAT120131	NONCODE v4	21.50	ABCB5	4.04
TCONS_00003998	broadlincRNA	20.78	CD177	4.00
NONHSAT079902	NONCODE v4	19.98	GHITM	3.98
TCONS_00002788	broadlincRNA	18.49	MED14	3.95
NONHSAG053014	NONCODE v4	16.19	RBM14	3.92
NONHSAG037523	NONCODE v4	15.24	UGT2A1	3.91
NONHSAT027305	NONCODE v4	14.83	FAM170B	3.89
TCONS_00019443	broadlincRNA	14.50	C19orf71	3.86
FR142221	frnadb v3.4	13.70	DDX26B	3.86
TCONS_I2_00020368	broadlincRNA	13.64	PPP1R1C	3.57
NONHSAG015130	NONCODE v4	13.46	LOC644662	3.56
NONHSAT137687	NONCODE v4	13.45	IFNAR2	3.47
TCONS_00008691	NONCODE v4	12.90	TXNDC8	3.47

Fig. 3. Pathways corresponding to up-regulated (A) and down-regulated mRNAs (B).

stimulus, and cellular glucuronidation (Fig. 5A). In the GO analysis for molecular function, we found that the up-regulated transcripts were highly enriched for neurotransmitter transporter activity, and calmodulin binding (Fig. 4B), whereas down-regulated transcripts were highly enriched for nuclear receptor transcription coactivator activity, and glucuronosyltransferase activity (Fig. 5B).

Cis-100k analysis

lncRNAs are uniquely adapted to regulate their genomic neighborhoods in cis [18]. Through cis-100k analysis, five potential key lncRNAs related to osteogenic differentiation were identified, including NONHSAT125464, ENST00000504555, NONHSAT098635, NONHSAT054627, and NONHSAT009968 (Table 4). Their potential cis-regulated mRNA targets included bone morphogenetic protein 1 (BMP1), SMAD family member 1 (SMAD1), collagen type I alpha 1 (COL1A1), and Wnt family member 3A (WNT3A), which have been demonstrated to function as regulators of osteoblast differentiation, or osteogenic differentiation markers [19-22].

Verification of differentially expressed lncRNAs

The expression of the 5 lncRNAs potentially related to osteogenic differentiation based on cis-100k analysis was verified by qRT-PCR. hBMSCs were cultured in osteogenic differentiation medium with or without 1 µg/mL of SpA for 2, 3 weeks. Cells were then harvested for qRT-PCR. The results of qRT-PCR (Fig. 6) demonstrated that the expression levels of NONHSAT125464, ENST00000504555, NONHSAT098635, NONHSAT054627, and NONHSAT009968 were obviously increased in the SpA treatment group compared with the normal culture group, which is

Fig. 4. GO analysis corresponding to up-regulated mRNAs.

Fig. 5. GO analysis corresponding to down-regulated mRNAs.

Table 4. Potential key lncRNAs related to osteogenic differentiation identified by cis-100k analysis

lncRNA Accession	Source	Fold change	Regulation	Cis-100K mRNA
NONHSAT125464	NONCODE v4	4.47	Up	BMP1[19]
ENST00000504555	Ensembl	21.10	Up	SMAD1[20]
NONHSAT098635	NONCODE v4	2.10	Up	SMAD1[20]
NONHSAT054627	NONCODE v4	2.13	Up	COL1A1[21]
NONHSAT009968	NONCODE v4	3.80	Up	WNT3A[22]

Fig. 6. Expression levels of NONHSAT009968, ENST00000504555, NONHSAT098635, NONHSAT054627, and NONHSAT125464 in hBMSCs cultured in osteogenic differentiation medium with or without 1 μ g/mL SpA for 2 or 3 weeks. The results of qRT-PCR were quantified using the $2^{-\Delta\Delta CT}$ method. Results are represented as means $\pm$ standard deviation (SD). *P values < 0.05, SpA vs. Normal.

Fig. 7. Expression of NONHSAT009968 in hBMSCs after transfection with siRNA-1, -2, or -3, (A) or after infection with lentivirus expressing NONHSAT009968-shRNA (B).

consistent with the microarray experiment data. The expression of NONHSAT009968 exhibited the greatest increase.

Silencing of NONHSAT009968 lncRNA by lentivirus expressing NONHSAT009968-shRNA

To decrease the expression of NONHSAT009968, three siRNAs targeting different NONHSAT009968 sequences or NC were synthesized and transfected into hBMSCs to

Fig. 8. The effect of NONHSAT009968 silencing on SpA-inhibited osteogenic differentiation in hBMSCs. A: The effect of NONHSAT009968 silencing on calcium mineralization, stained by alizarin red after SpA treatment. B: Western blot depicting the effect of NONHSAT009968 silencing on the protein expression of Runx2, OCN, OPN, COL1A1, and ALP. C: The effect of NONHSAT009968 silencing on ALP activity.

determine the best siRNA for silencing NONHSAT009968. We used qRT-PCR to determine the efficiency of siRNA-based silencing. Among the siRNAs tested, transfection with siRNA-2 resulted in the lowest expression of NONHSAT009968, and was thus the best for silencing this lncRNA target (Fig. 7A). Therefore, siRNA-2 was chosen for lentivirus packaging. After infecting cells with lentivirus expressing NONHSAT009968-shRNA or NC, the expression level of NONHSAT009968 was reassessed by qRT-PCR. The results showed that NONHSAT009968 was successfully silenced via infection with the lentivirus (Fig. 7B).

NONHSAT009968 silencing ameliorates SpA-inhibited osteogenic differentiation in hBMSCs

To investigate the role of lncRNA NONHSAT009968 in SpA-inhibited osteogenic differentiation in hBMSCs, hBMSCs were infected with lentivirus expressing NONHSAT009968 shRNA or NC and then cultured in osteogenic differentiation medium with or without 1 μ g/mL SpA for 14 days or 21 days. After treatment, cells were harvested for alizarin red staining and western blotting. Cell supernatants were harvested for ALP activity detection. The alizarin red staining showed that NONHSAT009968 silencing increased calcium mineralization by the cells compared to the NC group at both 14 days and 21 days (Fig. 8A). The results of ALP detection showed that NONHSAT009968-silencing also increased ALP activity compared to NC group at both 14 days and 21 days (Fig. 8B). In addition, NONHSAT009968-silencing increased the protein expression levels of Runx2, OCN, OPN, and COL1A1, which are involved in osteogenic differentiation (Fig. 8C). All of these results indicate that NONHSAT009968 silencing ameliorates SpA-inhibited osteogenic differentiation in hBMSCs.

Discussion

BMSCs are capable of osteogenic differentiation and play an important role in bone formation. The network regulating the osteogenic differentiation process of BMSCs has been

studied comprehensively. The osteogenic differentiation ability of BMSCs may be affected by many regulatory factors, such as proteins, microRNAs, and lncRNAs [23-27]. Osteomyelitis, which commonly occurs during bone healing, is known to inhibit osteogenic differentiation and bone formation [28]. However, the regulatory mechanism of osteomyelitis on the osteoblastic differentiation of BMSCs remains unclear. *In vivo*, the inflammatory environment of osteomyelitis is complex, so it is difficult to duplicate the inflammatory environment of osteomyelitis *in vitro*. SpA is an important virulence factor of *Staphylococcus aureus*, the most common microorganism recovered from osteomyelitis [5, 6]. And previous studies show that SpA can induce an inflammatory response *in vitro* [7, 8]. So SpA was chosen to trigger an inflammatory environment in hBMSCs to construct a cell model that could partly duplicate the inflammatory environment of osteomyelitis *in vitro*. We then investigated lncRNA and mRNA expression profiles under the inflammatory environment triggered by SpA during early osteogenic differentiation. We confirmed the role of lncRNA NONHSAT009968 on osteogenic differentiation in a SpA-triggered inflammatory environment triggered by SpA.

In previous studies, lncRNAs have been implicated in a range of developmental processes and diseases [9]. In a recent study, Song et al. identified lncRNAs associated with osteogenic differentiation from mesenchymal stem cells (MSCs) using high-throughput RNA sequencing (RNA-Seq) data [23]. Song et al. identified 574 lncRNAs that were differentially expressed during osteogenic differentiation [23], and the function of some of these lncRNAs on osteogenic differentiation has been confirmed. For example, down-regulation of lncRNA-ANCR promotes osteogenic differentiation of periodontal ligament stem cells [11] and up-regulation of lncRNA MEG3 promotes osteogenic differentiation of MSCs [23]. These studies indicate that lncRNAs play an important role in regulating osteogenic differentiation of MSCs. However, prior to this study, the complete expression profile and function of lncRNAs on the osteogenic differentiation of stem cells in an inflammatory microenvironment triggered by SpA were not yet known. Our firstly comprehensive study on the whole expression profile of lncRNAs will provide a theoretical basis for further studies on the function of lncRNAs in osteogenic differentiation in inflammatory environments triggered by SpA.

Through Cis-100k analysis, we identified five lncRNAs as potentially affecting osteogenic differentiation. We then verified the expression of these five lncRNAs by qRT-PCR, and confirmed that all five lncRNA expression levels were consistent with results from our microarray analysis. Among these five lncRNAs, lncRNA NONHSAT009968 was chosen as the object for further study. This decision was based on the following reasons: 1) expression of NONHSAT009968 exhibited the greatest increase in the SpA treatment group compared with the normal culture group; 2) its potential target, WNT3A has been identified as an important regulator of osteoblast differentiation [22]. NONHSAT009968 is a novel lncRNA that has not been previously studied. To investigate the function of NONHSAT009968, its expression was silenced using shRNA. We then assessed the role of NONHSAT009968 on osteogenic differentiation of hBMSCs in an inflammatory microenvironment triggered by SpA. Based on our results, NONHSAT009968 silencing ameliorates SpA-inhibited osteogenic differentiation in hBMSCs. Since this is the first study on NONHSAT009968, further investigations are required to confirm its function. In addition, we found that Wnt3A might be the cis-gene target of NONHSAT009968. In a previous study Wnt3A was shown to induce osteogenic differentiation of MSCs [29]. The relationship between NONHSAT009968 and Wnt3A also remains to be confirmed.

In conclusion, our present study is the first to characterize lncRNA and mRNA expression profiles in an inflammatory environment triggered by SpA during early osteogenic differentiation. This provides a foundation for future studies analyzing the involvement of lncRNAs in osteoblastic differentiation in an inflammatory microenvironment. In addition, we demonstrated that the novel lncRNA, NONHSAT009968, suppresses osteogenic differentiation in a SpA-triggered inflammatory microenvironment. Thus, our overall results indicate that lncRNA NONHSAT009968 might be a new target for promoting osteoblast formation.

Acknowledgement

This work was funded by the National Natural Sciences Foundation of China (Grant No.81472096) and the youth development project of military medicine and technology (Grant No.15QNP031)

Disclosure Statement

The authors declare they have no competing interests.

References

- 1 Spellberg B, Lipsky BA: Systemic antibiotic therapy for chronic osteomyelitis in adults. *Clin Infect Dis* 2012;54:393-407.
- 2 Sanchez CJ, Jr, Ward CL, Romano DR, Hurtgen BJ, Hardy SK, Woodbury RL, Trevino AV, Rathbone CR, Wenke JC: Staphylococcus aureus biofilms decrease osteoblast viability, inhibits osteogenic differentiation, and increases bone resorption in vitro. *BMC Musculoskelet Disord* 2013;14:1471-2474.
- 3 Tan J, Zhou L, Xue P, An Y, Luo L, Zhang R, Wu G, Wang Y, Zhu H, Wang Q: Tumor necrosis factor- α attenuates the osteogenic differentiation capacity of periodontal ligament stem cells by activating protein kinase like endoplasmic reticulum kinase signaling. *J Periodontol* 2016;18:1-22.
- 4 Matzelle MM, Shaw AT, Baum R, Maeda Y, Li J, Karmakar S, Manning CA, Walsh NC, Rosen V, Gravalles EM: Inflammation in arthritis induces expression of bmp3, an inhibitor of bone formation. *Scand J Rheumatol* 2016;16:1-5.
- 5 Votintseva AA, Fung R, Miller RR, Knox K, Godwin H, Wyllie DH, Bowden R, Crook DW, Walker AS: Prevalence of staphylococcus aureus protein a (spa) mutants in the community and hospitals in oxfordshire. *BMC Microbiol* 2014;14:1471-2180.
- 6 Dayan G, Naglaa M, Scully I, Cooper D, Begier E, Eiden J, Jansen K, Gurtman A, Anderson A: Staphylococcus aureus: The current state of disease, pathophysiology and strategies for prevention. *Expert Rev Vaccines* 2016;27:27.
- 7 Kumar A, Tassopoulos AM, Li Q, Yu FS: Staphylococcus aureus protein a induced inflammatory response in human corneal epithelial cells. *Biochem Biophys Res Commun* 2007;354:955-961.
- 8 Kozlowski LM, Li W, Goldschmidt M, Levinson AI: In vivo inflammatory response to a prototypic b cell superantigen: Elicitation of an arthus reaction by staphylococcal protein a. *J Immunol* 1998;160:5246-5252.
- 9 Kung JT, Colognori D, Lee JT: Long noncoding rnas: Past, present, and future. *Genetics* 2013;193:651-669.
- 10 Li H, Zhang Z, Chen Z, Zhang D: Osteogenic growth peptide promotes osteogenic differentiation of mesenchymal stem cells mediated by lncrna ak141205-induced upregulation of cxcl13. *Biochem Biophys Res Commun* 2015;466:82-88.
- 11 Jia Q, Jiang W, Ni L: Down-regulated non-coding rna (lncrna-ancr) promotes osteogenic differentiation of periodontal ligament stem cells. *Arch Oral Biol* 2015;60:234-241.
- 12 Zhuang W, Ge X, Yang S, Huang M, Chen P, Zhang X, Fu J, Qu J, Li B: Upregulation of lncrna meg3 promotes osteogenic differentiation of mesenchymal stem cells from multiple myeloma patients by targeting bmp4 transcription. *Stem Cells* 2015;33:1985-1997.
- 13 Zhu L, Xu PC: Downregulated lncrna-ancr promotes osteoblast differentiation by targeting ezh2 and regulating runx2 expression. *Biochem Biophys Res Commun* 2013;432:612-617.
- 14 Huang Y, Zheng Y, Jia L, Li W: Long noncoding rna h19 promotes osteoblast differentiation via tgfbeta1/smads3/hdac signaling pathway by deriving mir-675. *Stem Cells* 2015;33:3481-3492.
- 15 Puthanveetil P, Chen S, Feng B, Gautam A, Chakrabarti S: Long non-coding rna malat1 regulates hyperglycaemia induced inflammatory process in the endothelial cells. *J Cell Mol Med* 2015;19:1418-1425.

- 16 Lu MC, Yu HC, Yu CL, Huang HB, Koo M, Tung CH, Lai NS: Increased expression of long noncoding rnas loc100652951 and loc100506036 in t cells from patients with rheumatoid arthritis facilitates the inflammatory responses. *Immunol Res* 2016;64:576-583.
- 17 Zhou X, Han X, Wittfeldt A, Sun J, Liu C, Wang X, Gan LM, Cao H, Liang Z: Long non-coding rna anril regulates inflammatory responses as a novel component of nf-kappab pathway. *RNA Biol* 2016;13:98-108.
- 18 Quinn JJ, Chang HY: Unique features of long non-coding rna biogenesis and function. *Nat Rev Genet* 2016;17:47-62.
- 19 Li M, Li X, Meikle MC, Islam I, Cao T: Short periods of cyclic mechanical strain enhance triple-supplement directed osteogenesis and bone nodule formation by human embryonic stem cells in vitro. *Tissue Eng Part A* 2013;19:2130-2137.
- 20 Jang WG, Jeong BC, Kim EJ, Choi H, Oh SH, Kim DK, Koo SH, Choi HS, Koh JT: Cyclic amp response element-binding protein h (crebh) mediates the inhibitory actions of tumor necrosis factor alpha in osteoblast differentiation by stimulating smad1 degradation. *J Biol Chem* 2015;290:13556-13566.
- 21 Xu SJ, Qiu ZY, Wu JJ, Kong XD, Weng XS, Cui FZ, Wang XM: Osteogenic differentiation gene expression profiling of hmscs on hydroxyapatite and mineralized collagen. *Tissue Eng Part A* 2016;22:170-181.
- 22 He X, Wang H, Jin T, Xu Y, Mei L, Yang J: Tlr4 activation promotes bone marrow msc proliferation and osteogenic differentiation via wnt3a and wnt5a signaling. *PLoS One* 2016;11:e0149876
- 23 Song WQ, Gu WQ, Qian YB, Ma X, Mao YJ, Liu WJ: Identification of long non-coding rna involved in osteogenic differentiation from mesenchymal stem cells using rna-seq data. *Genet Mol Res* 2015;14:18268-18279.
- 24 Zheng L, Tu Q, Meng S, Zhang L, Yu L, Song J, Hu Y, Sui L, Zhang J, Dard M, Cheng J, Murray D, Lian J, Stein G, Chen J: Runx2/dicer/mirna pathway in regulating osteogenesis. *J Cell Physiol* 2016;11:25406.
- 25 Lee SH, Park Y, Song M, Srikanth S, Kim S, Kang MK, Gwack Y, Park NH, Kim RH, Shin KH: Orai1 mediates osteogenic differentiation via bmp signaling pathway in bone marrow mesenchymal stem cells. *Biochem Biophys Res Commun* 2016;473:1309-1314.
- 26 Li Y, Wagner ER, Yan Z, Wang Z, Luther G, Jiang W, Ye J, Wei Q, Wang J, Zhao L, Lu S, Wang X, Mohammed MK, Tang S, Liu H, Fan J, Zhang F, Zou Y, Song D, Liao J, Haydon RC, Luu HH, He TC: The calcium-binding protein s100a6 accelerates human osteosarcoma growth by promoting cell proliferation and inhibiting osteogenic differentiation. *Cell Physiol Biochem* 2015;37:2375-2392.
- 27 Zhang H, Li L, Dong Q, Wang Y, Feng Q, Ou X, Zhou P, He T, Luo J: Activation of pka/creb signaling is involved in bmp9-induced osteogenic differentiation of mesenchymal stem cells. *Cell Physiol Biochem* 2015;37:548-562.
- 28 Daigang L, Jining Q, Jinlai L, Pengfei W, Chuan S, Liangku H, Ding T, Zhe S, Wei W, Zhong L, Kun Z: Lps-stimulated inflammation inhibits bmp-9-induced osteoblastic differentiation through crosstalk between bmp/mapk and smad signaling. *Exp Cell Res* 2016;341:54-60.
- 29 Zhang S, Chen X, Hu Y, Wu J, Cao Q, Chen S, Gao Y: All-trans retinoic acid modulates wnt3a-induced osteogenic differentiation of mesenchymal stem cells via activating the pi3k/akt/gsk3beta signalling pathway. *Mol Cell Endocrinol* 2016;422:243-253.