

Differential Expression Profiles of Long Noncoding RNA and mRNA of Osteogenically Differentiated Mesenchymal Stem Cells in Ankylosing Spondylitis

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ABSTRACT. Objective. We previously demonstrated that mesenchymal stem cells (MSC) from patients with ankylosing spondylitis (AS; ASMSC) have a greater osteogenic differentiation capacity than MSC from healthy donors (HDMSC) and that this difference underlies the pathogenesis of pathological osteogenesis in AS. Here we compared expression levels of long noncoding RNA (lncRNA) and mRNA between osteogenically differentiated ASMSC and HDMSC and explored the precise mechanism underlying abnormal osteogenic differentiation in ASMSC.

Methods. HDMSC and ASMSC were induced with osteogenic differentiation medium for 10 days. Microarray analyses were then performed to identify lncRNA and mRNA differentially expressed between HDMSC and ASMSC, which were then subjected to bioinformatics analysis and confirmed by quantitative real-time PCR (qRT-PCR) assays. In addition, coding-non-coding gene co-expression (CNC) networks were constructed to examine the relationships between the lncRNA and mRNA expression patterns.

Results. A total of 520 lncRNA and 665 mRNA were differentially expressed in osteogenically differentiated ASMSC compared with HDMSC. Bioinformatics analysis revealed 64 signaling pathways with significant differences, including transforming growth factor- β signaling. qRT-PCR assays confirmed the reliability of the microarray data. The CNC network indicated that 4 differentially expressed lncRNA, including lnc-ZNF354A-1, lnc-LIN54-1, lnc-FRG2C-3, and lnc-USP50-2 may be involved in the abnormal osteogenic differentiation of ASMSC.

Conclusion. Our study characterized the differential lncRNA and mRNA expression profiles of osteogenically differentiated ASMSC and identified 4 lncRNA that may participate in the abnormal osteogenic differentiation of ASMSC. These results provide insight into the pathogenesis of pathological osteogenesis in AS. (First Release May 15 2016; J Rheumatol 2016;43:1523–31; doi:10.3899/jrheum.151181)

Key Indexing Terms:

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MESENCHYMAL STEM CELLS
LONG NONCODING RNA

Ankylosing spondylitis (AS) is a type of rheumatic disease characterized by chronic inflammation and pathological osteogenesis¹. Several studies have examined the precise

pathogenesis of chronic inflammation in AS, which has been demonstrated to involve HLA-B27 misfolding², bacterial infection³, macrophage activation⁴, and certain cytokines^{5,6}.

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However, the mechanism of pathological osteogenesis remains poorly understood. Thus, in-depth studies of the precise mechanisms of pathological osteogenesis in AS should provide valuable information.

Mesenchymal stem cells (MSC) are a type of multipotent stromal cells that can be isolated from bone marrow, adipose tissue, and the umbilical cord. MSC are capable of immunoregulation and trilineage differentiation into osteoblasts, chondroblasts, and adipoblasts, which play important roles in the maintenance of homeostasis *in vivo*⁷. Additionally, MSC dysfunction has been demonstrated to be involved in the pathogenesis of several diseases^{8,9,10}. Recently, we demonstrated that an imbalance between bone morphogenetic protein 2 (BMP2) and Noggin (NOG) induces abnormal osteogenic differentiation of MSC in AS, representing a pivotal mechanism of pathological osteogenesis in AS¹¹. However, the specific mechanism of abnormal osteogenic differentiation and the reason for the imbalance between BMP2 and NOG in MSC from patients with AS remain to be elucidated.

Long noncoding RNA (lncRNA) are non-protein-coding transcripts that are more than 200 nucleotides in length. They have comprehensive functions in regulating gene expression and play roles in cell differentiation and development¹². Recent studies have demonstrated that lncRNA participate in the osteogenic differentiation of MSC¹³. Further, the abnormal expression of lncRNA alters the osteogenic differentiation capacity of MSC, leading to the development of disease¹⁴. The aim of our study is therefore to examine the role of lncRNA in the abnormal osteogenic differentiation of MSC in AS.

We investigated the differential expression profiles of lncRNA and mRNA in osteogenically differentiated MSC from healthy donors (HDMSC) and patients with AS (ASMSC). Bioinformatics analyses were performed to predict the roles of the differentially expressed lncRNA and mRNA in the abnormal osteogenic differentiation of ASMSC, and quantitative real-time PCR (qRT-PCR) assays were performed to confirm the results. We found that 4 differentially expressed lncRNA may participate in BMP2 and NOG dysregulation, an action that may be the underlying mechanism of abnormal osteogenic differentiation in ASMSC. These results provide greater insight into the precise mechanisms of pathological osteogenesis in AS.

MATERIALS AND METHODS

Patients and controls. Our study was approved by the ethics committee of Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, China. Twelve healthy donors and 12 patients with AS (diagnosed according to the New York modified criteria¹⁵) were enrolled, and all gave informed consent. The characteristics of the healthy donors and the patients are shown in Supplemental Table 1, available from the authors on request.

Cell isolation and culture. To minimize the effect of therapy, all patients with AS discontinued their treatment at 14 days before bone marrow puncture. Bone marrow punctures were performed by skilled allied health professionals. MSC were immediately isolated from the bone marrow samples by density gradient centrifugation, as described¹⁶. MSC were then cultured in

Dulbecco modified Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco) at 37°C in 5% CO₂. The medium was replaced every 3 days and MSC were passaged at 90% confluence. MSC at passage 3-4 were used for the experiments.

Flow cytometry. MSC were digested with 0.25% trypsin supplemented with 0.53 mM EDTA (Gibco). After centrifugation, MSC were resuspended in phosphate-buffered saline (PBS) and incubated for 30 min with antibodies against human CD14- phycoerythrin (PE), CD29-PE, CD44-FITC, CD45-FITC, CD105-FITC, or HLA DR-PE. Flow cytometry was performed to identify the MSC phenotypes.

Osteogenic differentiation. MSC were seeded in 12-well plates in osteogenic differentiation medium for 0 to 21 days. This medium was composed of DMEM containing 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerol phosphate, 50 μM ascorbic acid, 100 IU/ml penicillin, and 100 IU/ml streptomycin (Sigma). The medium was replaced every 3 days.

Alizarin red S assay. For alizarin red S staining, MSC were washed with PBS and fixed with 4% paraformaldehyde for 30 min. MSC were then stained with 1% alizarin red S for 15 min. The cells were washed to remove nonspecific staining, and images of the stained MSC were captured. For alizarin red S quantification, cells were destained with 10% cetylpyridinium chloride monohydrate (Sigma). After 1 h, absorbance was measured at 562 nm.

Microarray detection and analysis. Three HDMSC and 3 ASMSC were randomly selected on Day 10 of osteogenic differentiation. The characteristics of the study subjects used for the microarray analysis are shown in Supplemental Table 2, available from the authors on request. Total RNA was extracted using TRIzol reagent (Invitrogen) and purified using NucleoSpin RNA cleanup kits (Macherey-Nagel) according to the manufacturer's instructions. RNA integrity was determined by formaldehyde denaturing gel electrophoresis. Total RNA was reverse-transcribed into cDNA, which was successively labeled with fluorescent dye and hybridized with lncRNA + mRNA Human Gene Expression Microarrays v4.0 (4 × 180K; CapitalBio Co.). The microarrays were washed and scanned using a G2565CA Microarray Scanner (Agilent). Data were normalized and analyzed using GeneSpring software (Agilent). Differentially expressed genes with a fold change of > 2.0 or ≤ 2.0 and p values < 0.05 were selected for further analyses.

Bioinformatics analysis was performed using Molecule Annotation System 3.0 (MAS 3.0, CapitalBio Co.). Gene ontology (GO) analysis was performed to determine the primary functions of the differentially expressed genes. Pathway analysis of the differentially expressed genes was also carried out based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Further, coding-non-coding gene co-expression (CNC) networks were constructed for the HDMSC or ASMSC data based on the results of correlation analyses between differentially expressed mRNA and lncRNA (Pearson correlation coefficients > 0.99 or ≤ 0.99). P values < 0.05 were considered statistically significant.

Total RNA was extracted and reverse-transcribed into cDNA as described above. A LightCycler 480 PCR System (Roche) was used to perform qRT-PCR, using SYBR Premix Ex Taq™ (TAKARA). Data were normalized by GAPDH, and the relative expression levels of each gene were analyzed using the 2^{-ΔΔCt} method. The forward and reverse primers for each gene are presented in Supplemental Table 3, available from the authors on request.

Statistical analysis. Statistical analysis was performed with SPSS software. All data are expressed as means ± SD. P values < 0.05 were considered to indicate a statistically significant difference. All lncRNA were named according to the LNCipedia Database.

RESULTS

Phenotypes and osteogenic differentiation capacities of HDMSC and ASMSC. Both HDMSC and ASMSC expressed typical MSC surface markers with positivity for CD29, CD44, and CD105 and negativity for CD14, CD45, and

Table 1. Pathways with largest significant difference in KEGG analysis.

Pathway	Count	p	q	Gene Symbol
DNA polymerase	8	3.22e ⁻¹⁰	2.98e ⁻⁸	PRIM1, DNA2, RFC3, MCM7, MCM3, POLA2, PRIM2, PCNA
TGF-β signaling pathway	9	2.95e ⁻⁸	1.01e ⁻⁶	BMP2, SMAD1, ID1, THBS3, BMPR1B, NOG, SMURF1, DCN, BMP8A
Pathogenic <i>Escherichia coli</i> infection	7	2.47e ⁻⁷	3.82e ⁻⁶	TUBA1A, ARPC5, TUBB2A, TUBB, NCL, TUBA1B, CDC42
Focal adhesion	10	5.64e ⁻⁶	4.04e ⁻⁵	LAMA4, IBSP, PRKCB, IGF1, TNC, THBS3, COL3A1, DIAPH1, CAV2, CDC42
Calcium signaling pathway	9	1.62e ⁻⁵	7.88e ⁻⁵	ATP2A1, PRKCB, TNNC1, TNNC2, PLCB1, VDAC1, ATP2B1, LTB4R2, BDKRB2

KEGG: Kyoto Encyclopedia of Genes and Genomes database; TGF: transforming growth factor-β; PRIM: primase DNA polypeptide; RFC3: replication factor C 3; MCM: minichromosome maintenance complex component; POLA2: polymerase α 2; PCNA: proliferating cell nuclear antigen; BMP2: bone morphogenetic protein 2; SMAD1: SMAD family member 1; ID1: inhibitor of DNA binding 1; THBS3: thrombospondin 3; BMPR1B: bone morphogenetic protein receptor type IB; NOG: noggin; SMURF1: SMAD specific E3 ubiquitin protein ligase 1; DCN: decorin; BMP8A: bone morphogenetic protein 8a; TUBA1A: tubulin α 1a; ARPC5: actin-related protein 2/3 complex subunit 5; TUBB2A: tubulin β 2A; TUBB: tubulin β; NCL: nucleolin; TUBA1B: tubulin α 1b; CDC42: cell division cycle 42; LAMA4: laminin α 4; IBSP: integrin-binding sialoprotein; PRKCB: protein kinase C β; IGF1: insulin-like growth factor 1; TNC: tenascin C; COL3A1: collagen type III α 1; DIAPH1: diaphanous-related formin 1; CAV2: caveolin 2; ATP2A1: ATPase: Ca⁺⁺ transporting, cardiac muscle, fast twitch 1; TNNC: troponin C type; PLCB1: phospholipase C β 1; VDAC1: voltage-dependent anion channel 1; ATP2B1: ATPase, Ca⁺⁺ transporting, plasma membrane 1; LTB4R2: leukotriene B4 receptor 2; BDKRB2: bradykinin receptor B2.

Table 2. The characteristics of lncRNA with largest fold change in ASMSC.

lncRNA	Fold Change	Regulation	Chromosome	Strand	Start	End	Class	Size (bp)
lnc-KCNA5-1	4.293	Down	12	+	5088059	5090604	Intergenic	817
lnc-COMMD6-1	3.749	Up	13	-	76203370	76206271	Intronic	2044
lnc-C3orf77-1	3.594	Up	3	+	44040886	44059829	Intergenic	1368
lnc-PIK3C2G-2	3.345	Up	12	+	16573586	16590381	Intergenic	223
lnc-SOD3-2	3.298	Down	4	+	23781076	23784409	Intergenic	913
lnc-MAT2B-3	3.278	Up	5	+	163723702	164192411	Intergenic	804
lnc-LYZL1-3	3.241	Up	10	+	29698468	29776674	Antisense	767
lnc-DERA-2	3.200	Up	12	+	16720345	16726874	Intronic	603
lnc-IL17RA-4	3.141	Up	22	+	17130820	17134695	Intergenic	1067
lnc-TPTE2-1	3.031	Up	13	-	19982182	19983669	Intergenic	1059

lncRNA: long noncoding RNA; ASMSC: mesenchymal stem cells from patients with ankylosing spondylitis; KCNA5: potassium channel, voltage-gated shaker-related subfamily A, member 5; COMMD6: COMM domain containing 6; C3orf77: chromosome 3 open reading frame 77; PIK3C2G: phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 γ; SOD3: superoxide dismutase 3; MAT2B: methionine adenosyltransferase II, β; LYZL1: lysozyme-like 1; DERA: deoxyribose-phosphate aldolase; IL17RA: interleukin 17 receptor A; TPTE2: transmembrane phosphoinositide 3-phosphatase and tensin homolog 2.

Table 3. Correlation analysis between key mRNA and lncRNA expression (n = 12).

	HDMSC				ASMSC			
	BMP2		Noggin		BMP2		Noggin	
	Pearson Value	p	Pearson Value	p	Pearson Value	p	Pearson Value	p
Noggin	0.916	0.039	—	—	0.055	0.839	—	—
lnc-ZNF354A-1	0.902	0.007	0.962	< 0.001	-0.008	0.977	0.518	0.48
lnc-LIN54-1	0.883	< 0.001	0.828	< 0.001	0.21	0.825	0.622	0.18
lnc-FRG2C-3	0.994	< 0.001	0.966	0.044	0.018	0.95	0.304	0.271
lnc-USP50-2	0.941	0.006	0.947	< 0.001	-0.25	0.926	0.014	0.96

ZNF354A: zinc finger protein 354A; LIN54: lin-54 DREAM MuvB core complex component; FRG2C: FSHD region gene 2 family member C; USP50: ubiquitin specific peptidase 50; BMP2: bone morphogenetic protein 2; HDMSC: healthy donor mesenchymal stem cells; lncRNA: long noncoding RNA; ASMSC: ankylosing spondylitis MSC.

HLA-DR (Figure 1A). HDMSC and ASMSC were cultured in osteogenic differentiation medium for 0 to 10 days and then subjected to alizarin red S (ARS) assays. ARS staining intensity was increased in both HDMSC and ASMSC after osteogenic induction, although ASMSC outperformed

HDMSC in ARS staining on Day 10 of osteogenic differentiation. The ARS quantification assay yielded consistent results (Figure 1B). These results were in agreement with our previous conclusions¹¹, confirming that ASMSC have a greater capacity for osteogenic differentiation than HDMSC.

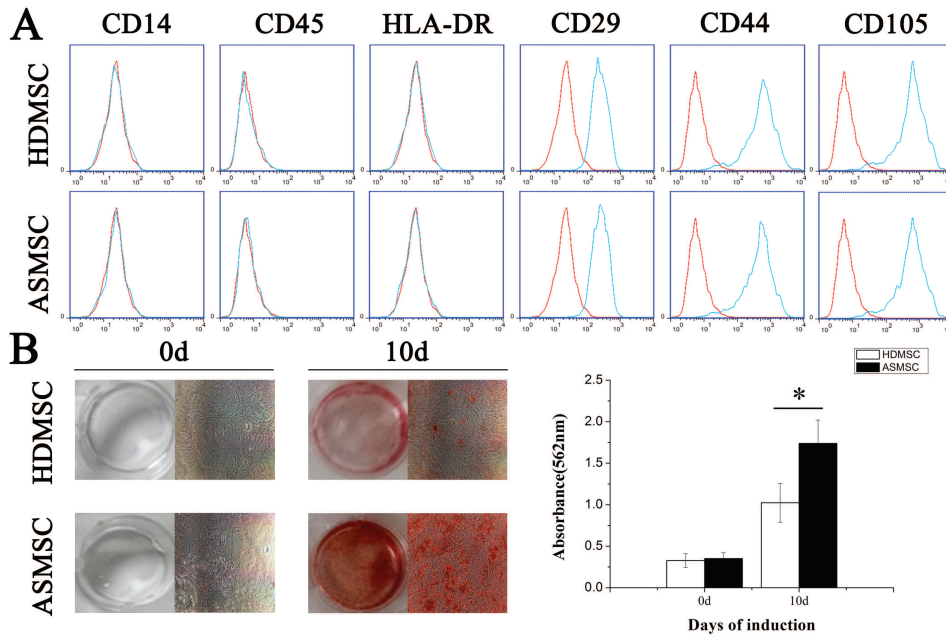


Figure 1. Phenotypes and osteogenic differentiation capacities of HDMSC and ASMSC. A. HDMSC (n = 12) and ASMSC (n = 12) were both positive for CD29, CD44, and CD105, and negative for CD14, CD45, and HLA-DR. B. Alizarin red S (ARS) assays were performed to assess the osteogenic differentiation capacity of MSC. ASMSC (n = 12) showed greater ARS staining than HDMSC (n = 12) after 10 days of induction. The absorbance of ARS quantification of ASMSC was also greater than that of HDMSC. *p < 0.05. HDMSC: healthy donor mesenchymal stem cells; ASMSC: ankylosing spondylitis MSC.

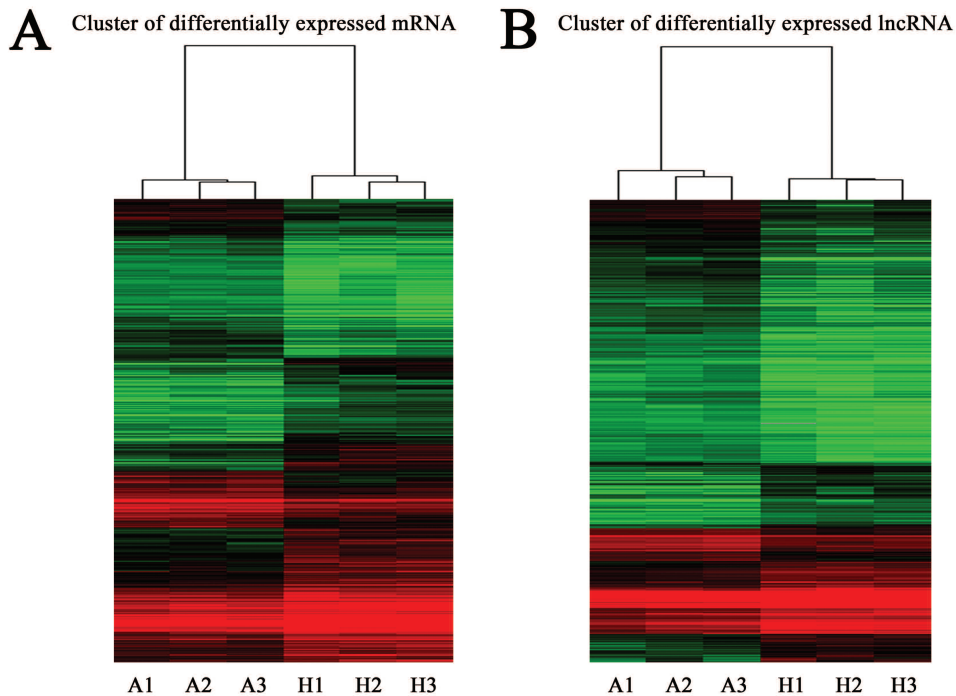


Figure 2. Clusters of differentially expressed mRNA and lncRNA. A. A total of 665 mRNA were differentially expressed in osteogenically differentiated ASMSC (n = 3) compared with HDMSC (n = 3), including 354 upregulated and 311 downregulated mRNA. Hierarchical clustering showed a distinguishable mRNA expression profile. B. A total of 520 lncRNA were differentially expressed in osteogenically differentiated ASMSC (n = 3) compared with HDMSC (n = 3), including 184 upregulated and 336 downregulated lncRNA. Hierarchical clustering showed a distinguishable lncRNA expression profile. HDMSC: healthy donor mesenchymal stem cells; ASMSC: ankylosing spondylitis MSC; lncRNA: long noncoding RNA.

Differential expression profile of mRNA in osteogenically differentiated ASMSC. A total of 665 mRNA were differentially expressed in osteogenically differentiated ASMSC compared with HDMSC. Among them, 354 mRNA were upregulated and 311 mRNA were downregulated (Figure 2A). GO analysis was performed to classify these differentially expressed mRNA into 3 domains, including biological process, molecular function, and cellular component (Supplemental Figure 1, available from the authors on request). In the biological process domain, the top 5 GO terms for the differentially expressed mRNA were regulation of transcription, signal transduction, visual perception, response to DNA damage stimulus, and cell cycle. In the molecular function domain, the top 5 GO terms were protein binding, nucleotide binding, ATP binding, zinc ion binding, and metal ion binding. In the cellular component domain, the top 5 GO terms were nucleus, cytoplasm, extracellular region, plasma membrane, and nucleolus. KEGG pathway analysis was conducted to identify the key signaling pathways and the relationships among the differentially expressed mRNA. We identified 64 signaling pathways that were enriched in ASMSC. The top 5 pathways were DNA polymerase, transforming growth factor- β (TGF- β) signaling, pathogenic *Escherichia coli* infection, focal adhesion, and calcium signaling pathway. The differentially expressed mRNA in these pathways are displayed in Table 1. In the TGF- β signaling pathway, we also observed that BMP2 was upregulated and NOG was downregulated in osteogenically differentiated ASMSC. These results suggested that these mRNA and pathways play important roles in the abnormal osteogenic differentiation of ASMSC. The characteristics of the differentially expressed mRNA in the TGF- β signaling pathway in ASMSC are shown in Supplemental Table 4, available from the authors on request.

Differential expression profile of lncRNA in osteogenically differentiated ASMSC. A total of 520 lncRNA were differentially expressed in ASMSC on Day 10 of osteogenic induction, including 184 upregulated and 336 downregulated lncRNA (Figure 2B). The 10 lncRNA with the largest fold changes are shown in Table 2. We categorized these differentially expressed lncRNA into 5 classes, including intergenic, intronic, bidirectional, sense, and antisense. These were identified: 219 intergenic, 57 intronic, 58 bidirectional, 100 sense, and 86 antisense lncRNA.

Confirmation of differentially expressed mRNA and lncRNA by qRT-PCR. To confirm the reliability of the microarray data, we randomly selected 12 differentially expressed mRNA (6 upregulated and 6 downregulated) and 12 differentially expressed lncRNA (6 upregulated and 6 downregulated) and measured their expression with qRT-PCR. Consistent with the microarray data, the following were upregulated: protocadherin 10, importin 5, paternally expressed 10, myopalladin, zinc finger protein 100, and TTK protein kinase. The following were downregulated: leukine

rich repeat interacting protein 2, myozenin 2, synaptotagmin XII, fibromodulin, acyl-CoA thioesterase 4, and tenascin C in osteogenically differentiated ASMSC compared with HDMSC. Moreover, in osteogenically differentiated ASMSC compared with HDMSC, these were upregulated: lnc-NOL6-4, lnc-PIK3C2G-2, lnc-AYM1A-3, lnc-BLID-2, lnc-KLF14-1, and lncMFN1-1, and these were downregulated: lnc-THBS2-3, lnc-DTHD1-8, lnc-GLRX5-2, lnc-NDUFS5, lnc-MPDZ, and lnc-FAM182B (Figure 3). These results demonstrate the reliability of the microarray data.

CNC network analysis of key differentially expressed mRNA and lncRNA. BMP2 and NOG are 2 key cytokines that induce abnormal osteogenic differentiation in ASMSC¹¹. We constructed CNC networks to evaluate the interactions among the key differentially expressed mRNA and lncRNA in HDMSC or ASMSC. The results showed that BMP2 expression was positively correlated with NOG expression in HDMSC. However, this relationship was not observed in ASMSC. Additionally, the expression levels of 4 differentially expressed lncRNA, including lnc-ZNF354A-1, lnc-LIN54-1, lnc-FRG2C-3, and lnc-USP50-2, were positively correlated with the expression of both BMP2 and NOG in HDMSC. However, these correlations were not detected in ASMSC. We confirmed these results by qRT-PCR. Correlation analysis of the expression of these mRNA and lncRNA showed consistent results with the CNC network (Table 3). These results suggested that differential relationships among these 4 lncRNA and BMP2 and NOG in ASMSC may be clues to studying the precise mechanisms of abnormal osteogenic differentiation of ASMSC. The characteristics of these key lncRNA are shown in Supplemental Table 5, and their expression profiles from Day 0 to 21 of osteogenic differentiation are shown in Supplemental Figure 2, both available from the authors on request.

DISCUSSION

MSC, defined as mesenchymal stem cells, are a major source of osteoblasts¹⁷. The normal osteogenic differentiation capacity of MSC is of great importance in regulating bone formation *in vivo*¹⁸. Previous studies have demonstrated that a deficiency in the osteogenic differentiation capacity of MSC contributes to osteoporosis in rheumatoid arthritis⁹, indicating that disruption of this process leads to disease symptoms. AS is a rheumatic disease characterized by pathological osteogenesis¹⁹. Similar to the above-mentioned studies, we previously found (and confirmed in this study) that the osteogenic differentiation capacity of ASMSC was intrinsically overactive, resulting from the secretion of more BMP2 and less NOG¹¹. These findings may be crucial to understanding the pathogenesis of new bone formation in AS. However, both the complicated mechanism of the BMP2-NOG imbalance in ASMSC and the precise mechanism of pathological osteogenesis in AS remain unclear. Day 10 of osteogenic differentiation was the point at which the

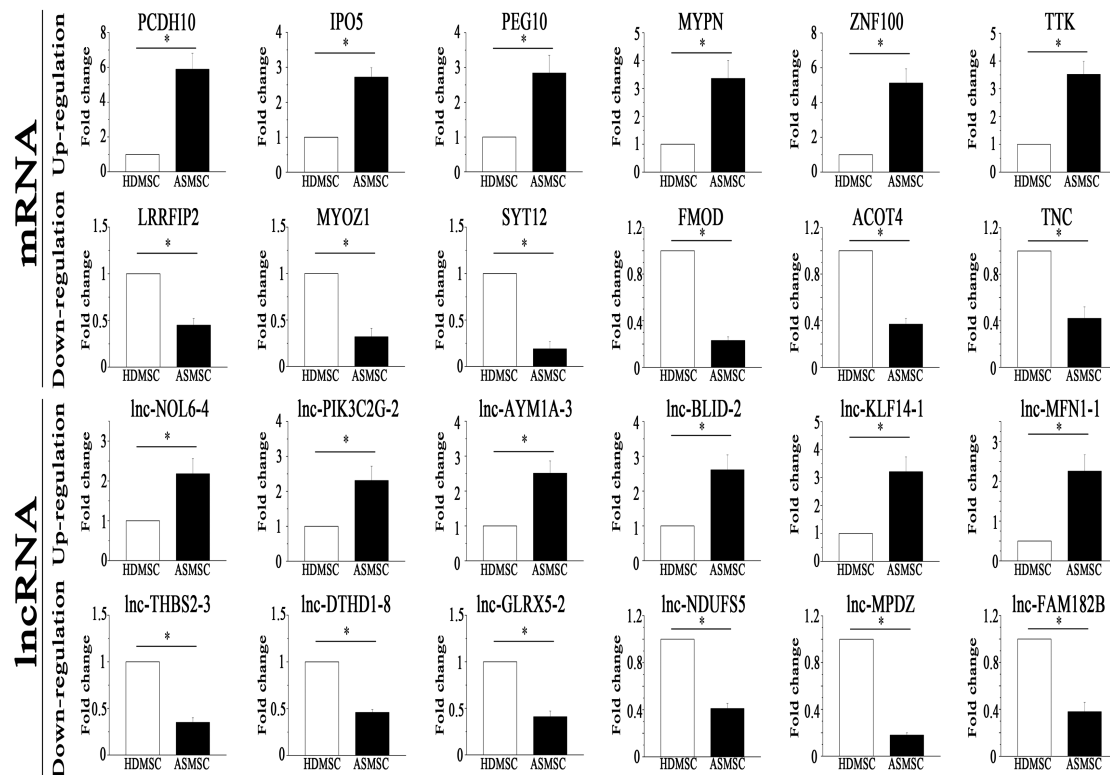


Figure 3. Quantitative real-time PCR (qRT-PCR) confirmation of differentially expressed mRNA and lncRNA. To confirm the reliability of the microarray data, 12 differentially expressed mRNA (6 upregulated and 6 downregulated) and 12 differentially expressed lncRNA (6 upregulated and 6 downregulated) were randomly chosen for qRT-PCR assays. Consistent with the microarray data, these were upregulated: protocadherin 10 (PCDH10), importin 5 (IPO5), paternally expressed 10 (PEG10), myopalladin (MYPN), zinc finger protein 100 (ZNF100), TTK protein kinase (TTK), lnc-nucleolar protein 6-4 (lnc-NOL6-4), lnc-phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 γ (lnc-PIK3C2G-2), lnc-amylase α 1A-3 (lnc-AYM1A-3), lnc-BH3-like motif containing cell death inducer (lnc-BLID-2), lnc-Kruppel-like factor-14 (lnc-KLF14-1), and lnc-mitofusin-1 (lnc-MFN1-1). These were downregulated: leukine rich repeat interacting protein 2 (LRRFIP2), myozenin 2 (MYOZ2), synaptotagmin XII (SYT12), fibromodulin (FMOD), acyl-CoA thioesterase 4 (ACOT4), tenascin C (TNC), lnc-thrombospondin 2-3 (lnc-THBS2-3), lnc-death domain containing 1-8 (lnc-DTHD1-8), lnc-glutaredoxin 5-2 (lnc-GLRX5-2), lnc-NADH dehydrogenase (ubiquinone) Fe-S protein 5 (lnc-NDUFS5), lnc-multiple PDZ domain protein (lnc-MPDZ), and lnc family with sequence similarity 182, member B (lnc-FAM182B). There were 12 in ASMSC and 12 in HDMSC, confirming the reliability of the microarray results. * $p < 0.05$. lncRNA: long noncoding RNA; HDMSC: healthy donor mesenchymal stem cells; ASMSC: ankylosing spondylitis MSC.

difference in osteogenic differentiation capacity between HDMSC and ASMSC began to manifest as well as when the most marked differences in BMP2 and NOG expression between HDMSC and ASMSC were observed. We therefore measured the differential expression profiles of lncRNA and mRNA in ASMSC on Day 10 of induction to provide insight into the precise mechanisms of both the abnormal osteogenic differentiation and the imbalance between BMP2 and NOG.

A cell's mRNA expression profile reflects its status and function. In this study, 665 differentially expressed mRNA were detected in osteogenically differentiated ASMSC compared with HDMSC. Among these differentially expressed mRNA, KEGG pathway analysis revealed that 64 signaling pathways exhibited significant differences in osteogenically differentiated ASMSC. Among these pathways, TGF- β signaling containing the BMP pathway was the second most prominent. Previous studies have found that the BMP

signaling pathway plays an important role in pathological osteogenesis in AS^{19,20}. Our previous study also demonstrated that overactivation of BMP signaling contributed to the enhanced osteogenic differentiation capacity of ASMSC because of an imbalance between BMP2 and NOG, 2 key molecules in this pathway¹¹. The new microarray data provide strong evidence at the whole-genome level that the BMP signaling pathway plays an important role in the abnormal osteogenic differentiation of ASMSC and in the subsequent pathological osteogenesis of AS. The WNT signaling pathway has also been suggested to play a role in new bone formation in AS²¹. However, no significant differences in this pathway were observed in either our previous or current study¹¹. Therefore, we hypothesize that this pathway may affect pathological osteogenesis in AS in a different manner.

BMP2 is a secreted cytokine belonging to the BMP

signaling pathway that promotes the osteogenic differentiation of MSC²². As an extracellular antagonist of BMP2, NOG inhibits the osteogenic differentiation of MSC²³. During the osteogenic differentiation of MSC, the levels of BMP2 and NOG remain balanced to maintain normal bone formation at different stages^{24,25,26,27}. However, the precise mechanism underlying the maintenance of this balance remains unclear. We demonstrated that ASMSC exhibit a greater osteogenic differentiation capacity than HDMSC owing to their secretion of more BMP2 but less NOG. The imbalance between BMP2 and NOG is an important mechanism contributing to abnormal osteogenic differentiation in ASMSC and pathological osteogenesis in AS¹¹. In our study, we observed that BMP2 was upregulated by 3.545-fold and that NOG was downregulated by 3.341-fold in ASMSC. Further, a positive correlation between BMP2 and NOG expression was observed in HDMSC but not in ASMSC. These results not only indicate that HDMSC maintain a balance between BMP2 and NOG, as reported by previous studies, but also reconfirm that BMP2 and NOG are imbalanced in osteogenically differentiated ASMSC.

The lncRNA are noncoding RNA that regulate the functions of genes and proteins through numerous mechanisms²⁸. Previous studies have demonstrated that lncRNA play important roles in maintaining homeostasis^{29,30}. Recently, differentially expressed lncRNA were found to be involved in rheumatic and autoimmune diseases³¹. LincRNA-p21 expression has been reported to be reduced in peripheral blood mononuclear cells from patients with rheumatoid arthritis³². Additionally, an lncRNA termed PRINS has been shown to be upregulated in the skin of patients with psoriasis³³. Moreover, lncRNA are also involved in other rheumatological and autoimmune diseases, including multiple sclerosis³⁴ and systemic lupus erythematosus³⁵. AS is an important type of rheumatic and autoimmune disease. To the best of our knowledge, our study is the first to use microarray analyses to examine the roles of lncRNA in AS.

The lncRNA have also been shown to be involved in regulating the differentiation of MSC, including osteogenic, chondrogenic, adipogenic, and myogenic differentiation. Moreover, abnormal expression of lncRNA can result in dysfunctional MSC differentiation, leading to abnormal tissue development and disease³⁶. Specifically, lncRNA-MEG3 expression was demonstrated to be downregulated in MSC from patients with multiple myeloma, resulting in inactivation of BMP4 and subsequent inhibition of osteogenic differentiation¹⁴. Compared with HDMSC, we found that osteogenically differentiated ASMSC contained 184 upregulated and 336 downregulated lncRNA. We found that 2 of the downregulated lncRNA, growth arrest-specific (GAS)5 and colorectal neoplasia differentially expressed (CRNDE), have been extensively studied. GAS5 is an lncRNA that acts as a tumor suppressor and is related to type 2 diabetes

mellitus, osteoarthritis, and certain tumors^{37,38,39}. The full name of CRNDE reflects its association with tumors^{40,41,42}. Some or all of these differentially expressed lncRNA, including GAS5 and CRNDE, may contribute to the abnormal osteogenic differentiation of ASMSC and the pathological osteogenesis of AS. Notably, lncRNA expression is both cell- and tissue-specific. Further study is needed to determine whether these lncRNA are differentially expressed in other cells and tissues of patients with AS and whether they can be used as biomarkers of AS.

To explore the precise mechanisms of both the abnormal osteogenic differentiation and the imbalance between BMP2 and NOG in ASMSC, we then constructed CNC networks to gain deeper insights into the relationships among BMP2 and NOG and the differentially expressed lncRNA. Four differentially expressed lncRNA, lnc-ZNF354A-1, lnc-LIN54-1, lnc-FRG2C-3, and lnc-USP50-2, were positively correlated with BMP2 and NOG expression in HDMSC. In ASMSC, however, these relationships were absent. We suggest that these lncRNA may have intimate connection with BMP2 and NOG expression in HDMSC. Moreover, we further speculate that the differential expression of these 4 lncRNA and the differential relationship among these 4 lncRNA and BMP2 and NOG may provide clues to study the mechanism of imbalance between BMP2 and NOG in ASMSC. These lncRNA may thus be involved in the precise mechanism underlying abnormal osteogenic differentiation in ASMSC and pathological osteogenesis in AS. Through diverse mechanisms, lncRNA regulate gene and protein expression. However, whether these differentially expressed lncRNA regulated BMP2 and NOG expression or how they exhibited their regulatory function is still unknown. Further studies still need to address this.

In our study we confirmed the abnormal osteogenic differentiation capacity of ASMSC and subsequently performed microarrays to identify lncRNA and mRNA differentially expressed in osteogenically differentiated ASMSC. After 10 days of osteogenic induction, 520 lncRNA and 665 mRNA were differentially expressed in ASMSC compared with HDMSC. Bioinformatics analysis revealed the main functions of the differentially expressed genes and 64 signaling pathways with significant differences, including the TGF- β signaling pathway. The reliability of the microarray data was confirmed by qRT-PCR. Additionally, CNC network and correlation analysis of gene expression data suggested that lnc-ZNF354A-1, lnc-LIN54-1, lnc-FRG2C-3, and lnc-USP50-2 may be involved in the mechanism leading to the imbalance between BMP2 and NOG that promotes the abnormal osteogenic differentiation of ASMSC. These results may help to provide clues for the BMP2-NOG imbalance and the abnormal osteogenic differentiation of ASMSC, thereby facilitating the development of new treatments for pathological osteogenesis in AS.

It should be noted, however, that our study has some

limitations: It remains unclear which of the 4 differentially expressed lncRNA is really responsible for the imbalance between BMP2 and NOG in osteogenically differentiated ASMSC. The mechanism by which the responsible lncRNA maintains the balance between BMP2 and NOG is likewise unknown. Further studies should address these questions.

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