Integrative analysis of long non-coding RNA (lncRNA) and mRNA expression in TLR4-primed MSCs of ankylosing spondylitis

Yuxi Li

Sun Yat-sen Memorial Hospital <https://orcid.org/0000-0002-4470-7679>

Ming Li

Sun Yat-sen Memorial Hospital

Ting Liu

Sun Yat-sen Memorial Hospital

Jiajun Huang

Sun Yat-sen Memorial Hospital

Yuwei Liang

Sun Yat-sen Memorial Hospital

Junshen Huang

Sun Yat-sen Memorial Hospital

Xiangge Liu

Sun Yat-sen Memorial Hospital

Ziying Cheng

Sun Yat-sen Memorial Hospital

Shixin Lu

Sun Yat-sen Memorial Hospital

Huiyong Shen

The Eighth Affiliated Hospital of Sun Yat-Sen University

Lin Huang (✉ huangl5@mail.sysu.edu.cn)

Sun Yat-sen Memorial Hospital

Research

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Abstract

Background: Ankylosing spondylitis (AS) is a chronic autoimmune disease, and the precise pathogenesis is largely unknown at present. Our previous study found that the expression of toll-like receptor 4 (TLR4) of mesenchymal stem cells from AS patients (AS-MSCs) was reduced and activation of TLR4 by lipopolysaccharide (LPS) could enhance the immunoregulatory ability of AS-MSCs. However, the potential mechanism by which TLR4 affect the immunoregulatory function of AS-MSCs remains unclear.

Objective: The goal of this study was to explore the expression profiles and functional networks of lncRNAs and mRNAs in TLR4-primed AS-MSCs and to clarify the potential mechanisms by which TLR4-primed AS-MSCs exert immunoregulatory effects.

Methods: Immunoregulatory effects of MSCs were determined after TLR4 activation. Then, the differentially expressed (DE) long non-coding RNAs (lncRNA) and messenger RNAs (mRNA) between AS-MSCs and TLR4-primed AS-MSCs (stimulated by LPS) were identified through high-throughput sequencing followed by qRT-PCR confirmation. Finally, bioinformatic analyses were performed to identify the critical biological functions, signalling pathways and associated functional networks involved in the TLR4-primed immunoregulatory function of AS-MSCs.

Results: A total of 147 DE lncRNAs and 698 DE mRNAs were identified between TLR4-primed AS-MSCs and unstimulated AS-MSCs. Of total, 107 lncRNAs were upregulated and 40 were downregulated (fold change ≥ 2 , $P < 0.05$), while 504 mRNAs upregulated and 194 downregulated (fold change ≥ 2 , $P < 0.05$). 5 lncRNAs and 5 mRNAs with largest fold changes were respectively verified by qRT-PCR. GO and KEGG analysis demonstrated that the DE mRNAs and lncRNAs were highly associated with the inflammatory response, such as NOD-like receptor (NLR) signalling pathway, the TNF signalling pathway and the NF-kappa B signalling pathway. Cis-regulation prediction revealed 8 novel lncRNAs while trans-regulation prediction revealed 15 lncRNAs, respectively. 8 core pairs of lncRNA and target mRNA in the lncRNA-TF-mRNA network were: PACERR-PTGS2, LOC105378085-SOD2, LOC107986655-HIVEP2, MICB-DT-MICB, LOC105373925-SP140L, LOC107984251-IFIT5, LOC112268267-GBP2 and LOC101926887-IFIT3, respectively.

Conclusion: In AS, TLR4 activation can enhance the immunoregulation ability of MSCs. Eight core pairs of lncRNA and target mRNA have been found in TLR4-primed AS-MSCs, which could contribute to elucidate the potential mechanism of immunoregulatory dysfunction of AS-MSCs.

Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory rheumatic disease characterized by inflammatory back pain and asymmetrical peripheral arthritis [1]. Previous studies have shown that AS is closely related to immune dysfunction. However, the pathogenesis of AS is largely unknown. Mesenchymal stem cells (MSCs) are a group of self-renewing cells that have a significant immunomodulatory ability that allows them to regulate T cell proliferation and differentiation and inhibit dendritic cell (DC) maturation [2, 3]. According to recent studies, abnormal immunoregulation by MSCs can lead to several autoimmune diseases, such as immune thrombocytopenia and systemic lupus erythematosus (SLE) [4–6]. In our previous study, we found the reduced immunoregulatory ability of MSCs from AS patients may participate a significant role in the pathogenesis of this disease [7]. Furthermore, our clinical trial demonstrated that intravenous infusion of MSCs from healthy donors (HDs) was a feasible, safe, and effective approach for the treatment of AS [8]. Our research results indicate that it is essential to elucidate the mechanism of abnormal immunoregulatory function of AS-MSCs. However, our understanding of the immunoregulatory function of AS-MSCs is still in its infancy, and further characterization and identification of key factors regulating these properties are still needed.

Toll-like receptors (TLRs), the most characterized pattern recognition receptors (PRRs), which are capable of potently activating different cell types, could be highly expressed on most immune cells, as well as other cell types, including chondrocytes, endothelial cells, and fibroblasts [9]. Their downstream signalling pathways lead to the production of a wide range of immune stimulatory cytokines and chemokines [10]. Aberrant activation of TLRs may result in unrestricted inflammatory responses, suggesting that the family of TLRs may play a pivotal role in the development of autoimmune diseases [11]. TLRs have also been demonstrated to play important roles in regulating the immunomodulatory properties of MSCs [12]. Our previous study found that the expression of TLR4 in MSCs was downregulated in AS patients compared to healthy donors. The inhibitory effects of MSCs on CD4⁺ T cell proliferation in AS were enhanced by stimulation by lipopolysaccharide (LPS), which is the specific ligand of TLR4. It has been suggested that TLR4 plays a significant role in regulating the immunomodulatory ability of MSCs in AS [13]. Liotta et al. found that ligation of TLR4 suppressed the inhibitory effects of human bone marrow (BM)-MSCs on T cell proliferation by downregulating Jagged-1 expression [14]. Waterman et al. demonstrated that MSCs could be primed towards a pro-inflammatory phenotype after TLR4 activation [15]. In contrast, Opitz et al. reported that TLR4 enhanced the immunosuppressive properties of human BM-MSCs by directly inducing indoleamine 2,3-dioxygenase 1 (IDO1) [16]. Inconsistent results have been reported in recent years, the specific immunoregulatory mechanisms by which TLR4 controls MSC immunoregulation remains unclear and must be addressed.

Noncoding RNAs (ncRNAs) are a class of non-protein-coding RNA and functional RNA molecules that include microRNA (miRNA), long noncoding RNAs (lncRNA) and circular RNAs (circRNA) [17]. Overwhelming evidence has indicated that various ncRNAs are implicated in human disease process. For example, miRNAs are the important molecules of various diseases [18]. In recent years, lncRNAs (> 200nt RNA molecules) have been found and studied to play a role in development, evolution and disease. lncRNAs are important epigenetic regulators and thus participate in

crucial roles in various cell biology behaviours [19]. For example, lncRNA-PCAT1 negatively regulated miR-145-5p, which promoted TLR4 expression to promote osteogenic differentiation by activating the TLR signalling pathway in human adipose-derived stem cells [20]. Specifically, lncRNAs are widely involved in the regulation of immune system homeostasis. lncRNA NEAT1 promotes inflammatory response in sepsis-induced liver injury via the Let-7a/TLR4 axis [21]. LPS-induced lncRNA Mirt2 can function as a checkpoint to prevent aberrant activation of inflammation, and is a potential regulator of macrophage polarization [22]. lncRNA MALAT1 regulates inflammatory cytokine production in lipopolysaccharide-stimulated human gingival fibroblasts through sponging miR-20a and activating TLR4 pathway [23]. However, the molecular mechanism by which lncRNAs participate in the immunoregulatory function of MSCs, particularly in AS, is still unclear.

The current study presents an integrative analysis of lncRNA-mRNA expression profiles and functional networks involved in the TLR4-primed immunoregulation of AS-MSCs. These results improve our understanding of the roles of lncRNAs in the immunoregulatory ability of AS-MSCs and could provide potential targets to improve the curative effect of MSCs on AS.

Results

The effect of TLR4 activation on MSCs is time and dose dependent

To investigate whether the activation of TLR4 in MSCs can affect the immunoregulatory ability of these cells, MSCs from AS patients were pre-stimulated with LPS before being co-cultured with PBMCs. To determine the best stimulation time and concentration for the TLR4 ligand used, the level of p38 phosphorylation was examined by western blotting. AS-MSCs were first exposed to LPS at a concentration of 1 µg/ml for the indicated times (0, 2, 4, 8, 12 and 24 hours) and then treated with three different concentrations (0, 0.1, 1 and 10 µg/ml) of stimuli for the previously selected time. The upregulation of the phospho-p38 level was highest at 4 hours with the LPS concentration of 1 µg/ml and declined thereafter (Fig. 1A).

TLR4-primed AS-MSCs demonstrate an enhanced inhibitory effect on CD4⁺ T cell proliferation

Previous reports have shown that co-culture of unprimed MSCs with PBMCs can inhibit PBMC proliferation and/or activation [24]. Thus, we sought to assess the potential influence of TLR4 activation on the immunoregulatory effect of MSCs derived from AS patients. To this end, MSC-PBMC co-culturing was conducted with CFSE-labelled PBMCs (responder cells) co-cultured with unprimed or TLR4-primed MSCs (effector cells) for 5 days, after which time the proliferating responders were sorted by flow cytometry for CD4 positivity and then gated on CFSE expression. As shown in Fig. 1C, 72.8% of the CD4⁺ T cells underwent proliferation when cultured without MSCs, but this proportion was reduced to 46.3% when cultured with AS-MSCs. Activation of TLR4 with 1 µg/ml LPS significantly enhanced the immunoregulatory effect of AS-MSCs, which reduced CD4⁺ T cell proliferation to 40.3% ($p < 0.05$) (Fig. 1B). qRT-PCR results suggested that the expression of several cytokines and chemokines (TNF-α, CXCL-9, PDL1, IL-1β, IL-6 and iNOS) was strengthened after stimulation with LPS (Fig. 1C).

Identification of DE mRNAs and lncRNAs

A total of 698 mRNAs were DE in TLR4-primed MSCs compared to unprimed MSCs from AS patients. Among these genes, 594 mRNAs were upregulated, and 104 mRNAs were downregulated. The DE mRNAs are depicted using a clustergram (Fig. 2A) and volcano plots (Fig. 2C). The 20 mRNAs with the largest fold changes are shown in Table 1. Several immunoregulatory cytokines and chemokines, such as CXCL10, CXCL11, IDO1, CXCL8, CXCL1, CCL20, IL6 and SOD2, which are secreted by MSCs and play important roles in regulating immunocytes, were included in this list. A total of 147 lncRNAs, including 107 upregulated and 40 downregulated lncRNAs, were differentially expressed in TLR4-primed MSCs compared to unprimed MSCs from AS patients. The DE lncRNAs are depicted in a clustergram (Fig. 2B) and volcano plots (Fig. 2D). The 10 lncRNAs with the largest fold changes are shown in Table 2.

Table 1
The characteristics of mRNAs with the largest fold change.

Gene name	Accession no.	Log2 Fold change	Regulation
CXCL10	NM_001565.3	12.58	up
CXCL11	NM_005409.4	10.14	up
RSAD2	NM_080657.4	8.27	up
IDO1	NM_002164.5	7.98	up
CXCL8	NM_000584.3	7.95	up
CXCL1	NR_046035.1	7.86	up
IFIT1	NM_001270930.1	7.30	up
OASL	NM_003733.3	6.95	up
CCL20	NM_004591.2	6.86	up
CXCL3	NM_002090.2	6.79	up
CCL5	NM_001278736.1	6.56	up
IFIT3	NM_001289758.1	6.54	up
CMPK2	NM_207315.3	6.39	up
CD274	NM_001267706.1	6.19	up
OASL	NM_198213.2	6.08	up
PTGS2	NM_000963.3	6.04	up
IL6	NM_001318095.1	5.81	up
SOD2	NM_001322816.1	5.79	up
CXCL8	NM_001354840.1	5.56	up
GBP5	NM_052942.3	5.47	up

Table 2
The characteristics of lncRNAs with the largest fold change.

Gene Symbol	Accession No.	Fold change	Regulation	Chromosome	Strand	Start	End	Feature	Size(bp)
GBP1P1	NR_003133.2	65.35	up	1	+	89407679	89424934	miscRNA	1234
LOC105370436	XR_943719.2	35.08	up	14	+	31271945	31276870	ncRNA	626
LOC105376014	XR_929550.3	18.12	up	9	-	32450235	32454362	ncRNA	2133
MIR3142HG	NR_132748.1	14.70	up	5	+	160468251	160487426	ncRNA	2318
LOC105371619	XR_922291.3	14.43	up	1	+	173637658	173681709	ncRNA	582
LOC105369543	XR_948124.3	14.25	up	11	+	123651728	123672257	ncRNA	3095
LOC105374444	XR_925284.2	11.54	up	4	+	47627252	47636569	ncRNA	1416
ANO7L1	XR_946991.2	11.17	up	1	-	16215438	16217420	miscRNA	1466
PACERR	NR_125801.1	9.48	up	1	+	186680654	186681446	ncRNA	793
LOC105375914	XR_001745967.1	9.23	up	8	+	78804578	78937632	ncRNA	3370

Validation of DE mRNA and lncRNA expression levels

To confirm the RNA-seq results, several important DE mRNAs and lncRNAs were assessed by qRT-PCR. We found that the expression of mRNAs (CXCL1, CXCL8, CXCL10, CXCL11 and CCL20) and lncRNAs (MIR3142HG, LOC105371619, LOC105374444, PACERR and LOC105375914) was

significantly upregulated in TLR4-primed AS-MSCs compared to unstimulated AS-MSCs ($P < 0.05$) (Fig. 3A and 3B). All qRT-PCR results were consistent with the RNA-seq results, confirming the reliability of the sequencing data.

GO and KEGG analyses

We performed GO analysis of the DE mRNAs and lncRNAs. The top 10 GO terms related to biological processes, cellular components and molecular functions are provided in Fig. 4A and supplementary table 1. In the biological process category, the top 5 GO terms associated with DE mRNAs were defense response to virus, type I interferon signalling pathway, interferon-gamma-mediated signalling pathway, inflammatory response and response to virus. In the molecular function category, the top 5 GO terms associated with the DE mRNAs were chemokine activity, CXCR chemokine receptor binding, ubiquitin-protein transferase activity, double-stranded RNA binding and TF activity, and sequence-specific DNA binding. In the cellular component category, the top 5 GO terms associated with the DE mRNAs were cytoplasm, cytosol, extracellular space, nucleus and extracellular region. KEGG analysis of the DE mRNAs determined that 75 pathways were significantly altered in TLR4-primed MSCs from AS patients. The top 30 affected pathways are shown in Fig. 4B. The top 10 pathways and DE mRNAs associated with these pathways are shown in supplementary table 2. The top pathways included the NOD-like receptor (NLR) signalling pathway, the TNF signalling pathway, the NF-kappa B signalling pathway, cytokine-cytokine receptor interaction, the IL-17 signalling pathway and the TLR signalling pathway, which contribute to the immunoregulatory function of AS-MSCs.

Interaction and co-expression network analyses

The interactions between proteins encoded by DE mRNAs are shown in Fig. 4C. OAS2, OAS3, OAS1, OASL, STAT1, IRF9, HLA-F, IRF1, IRF2 and HERC5 were identified as key genes that interact with many other DE mRNAs in this network. Based on the expression levels of DE lncRNAs and DE mRNAs, the PCC describing the co-expression association between 147 DE lncRNAs and 698 DE mRNAs was calculated. A total of 1,072 DE lncRNA-DE mRNA co-expression pairs were obtained with an absolute PCC value ≥ 0.85 and $P < 0.05$. Among these pairs, 706 lncRNA-mRNA pairs were identified as being positively co-expressed, whereas 366 lncRNA-mRNA pairs were found to be negatively co-expressed (Fig. 5B).

Cis-regulation prediction of DE lncRNAs

Cis-regulation, which regulates the transcription of nearby genes located on the same chromosome, is vital for gene expression. A total of 8 lncRNA transcripts and their predicted cis-regulated protein-coding genes were identified in the top 20 cis-regulated genes (Fig. 6A). LOC107986655 was predicted to cis-regulate HIVP2, PACERR was predicted to cis-regulate PTGS2, MICB-DT was predicted to cis-regulate MICB, LOC105373925 was predicted to cis-regulate SP140L, LOC107984251 was predicted to cis-regulate IFIT5, LOC112268267 was predicted to cis-regulate GBP2, LOC101926887 was predicted to cis-regulate IFIT3, and LOC105378085 was predicted to cis-regulate SOD2. These networks may provide valuable clues about these lncRNAs and their nearby coding genes in the development of AS.

Trans-regulation prediction of DE lncRNAs

One of the important mechanisms by which lncRNAs function is by participating in particular pathways regulated by TFs. A top 500 lncRNA-TF network, which showed that 15 lncRNAs participate in pathways regulated by TFs, was constructed to provide key data for subsequent research (Fig. 6B). Then, we selected the abovementioned relationships among the lncRNAs and TFs and further introduced the target mRNAs to build the lncRNA-TF-mRNA network. 8 core pairs of lncRNAs and target mRNAs in the lncRNA-TF-mRNA network were obtained: PACERR-PTGS2, LOC105378085-SOD2, LOC107986655-HIVP2, MICB-DT-MICB, LOC105373925-SP140L, LOC107984251-IFIT5, LOC112268267-GBP2 and LOC101926887-IFIT3 (Fig. 6C).

Discussion

In our present research, we utilized high-throughput sequencing followed by bioinformatic analysis to analyse the mRNA and lncRNA expression profiles and functional networks of TLR4-primed AS-MSCs. These findings were then confirmed by qRT-PCR. KEGG pathway analysis indicated that some key pathways, such as the NF-kappa B and TLR signalling pathways, might contribute to the immunoregulatory function of TLR4-primed AS-MSCs. In addition, we obtained novel findings by bioinformatic analyses of DE transcripts, including identification of the most significantly altered GO categories, construction of a co-expression network for lncRNA function prediction, and cis- and trans-regulation predictions of lncRNAs. Our results provide a model that can be used to explore the roles of lncRNAs and mRNAs in the immunoregulatory mechanisms of TLR4-primed AS-MSCs.

MSCs are one of the most important immunoregulatory cell types and regulate the functions of many immune cells, including T cells, B cells and DCs [25–27]. Abnormal immunoregulation by MSCs can lead to several autoimmune diseases [4]. Moreover, MSCs exert considerable therapeutic effects on several autoimmune diseases owing to their multilineage differentiation potential and highly immunoregulatory properties [28, 29]. In our previous study, we found that impairment in the immunoregulatory functions of MSCs played a key role in the pathogenesis of AS [7]. Additionally, our clinical trial study demonstrated that infusion of MSCs isolated from healthy individuals is a safe and efficient method for the treatment of AS [8].

Accumulating evidence suggests that TLR activation can modulate the immunoregulatory functions of MSCs [14–16]. In addition, emerging evidence further suggests a role for TLRs in the pathogenesis of spondyloarthropathies, including AS [30]. According to our previous study, the expression of TLR4 was downregulated in MSCs derived from AS patients, and compared with MSCs from healthy donors, TLR4-primed AS-MSCs possessed enhanced immunoregulatory effects limiting the proliferation of naive CD4⁺ T cells [31]. However, the precise mechanism underlying the enhanced immunoregulatory ability of TLR4-primed AS-MSCs remains unclear. Therefore, we measured the differential expression profiles of lncRNAs and mRNAs in AS-MSCs after TLR4 activation to identify the regulatory network of lncRNAs and mRNAs in these cells. The results showed that there were 698 DE mRNAs and 147 DE lncRNAs in TLR4-primed AS-MSCs compared with unstimulated cells. The top 5 mRNAs and lncRNAs, which may be involved in the regulatory dysfunction of AS-MSCs, were verified by qRT-PCR.

The mRNA expression profiles reflect the biological behaviours and functions of cells. In this study, KEGG pathway analysis revealed that 75 signalling pathways exhibited significant differences between TLR4-primed AS-MSCs and unstimulated AS-MSCs. Among these pathways, the NLR signalling pathway, the TNF signalling pathway, the NF- κ B signalling pathway, cytokine-cytokine receptor interaction and the TLR signalling pathway were prominent. Recent studies indicate that the activation of TLRs can activate NF- κ B and MAPK signalling pathways to promote the secretion of pro-inflammatory cytokines, such as IL-6, IL-12, TNF- α and type I IFNs, which drive inflammation in AS [32]. Consistent with these findings, we found that the expression of TNF- α , IL-6 and IL1 β in AS-MSCs was significantly increased after TLR4 activation, which supported the crucial roles for TLR4 in the NF- κ B and MAPK signalling pathways identified by microarray analysis. In addition, as pathogen recognition receptors, both TLR and NLR activate pathways mediated through different adaptor proteins that are commonly found to activate NF- κ B [33]. The NF- κ B-mediated activation of MSCs leads to the secretion of TNF- α and other cytokines. Elevated pro-inflammatory cytokine levels are one of the main manifestations of AS, as confirmed by previous research. Our results further confirmed the significant role of TLR4 in the pathology of AS. The selected top DE mRNAs and lncRNAs included in the NF- κ B pathway might be possible upstream targets of pathological inflammation in AS.

The mRNA expression profiles are under the control of a series of epigenetic regulators, of which lncRNAs are an important component [19, 34]. In recent years, an increasing number of lncRNAs have been reported to perform key roles in the pathogenesis and development of AS. For example, lncRNA-AK001085 expression was found to be downregulated in AS patients, which served as a potential diagnostic indicator; thus, this lncRNA is considered a potential suppressor of AS [35]. Our previous microarray study identified four lncRNAs (lnc-ZNF354A-1, lnc-LIN54-1, lnc-FRG2C-3 and lnc-USP50-2) that are involved in the abnormal osteogenic differentiation of AS-MSCs [36]. However, the immunoregulatory function of AS-MSCs regulated by lncRNAs has not been explored. Our research identifies the lncRNA expression profile in an inflammatory environment based on previous studies, which provides a possible way to further explore the regulatory function of lncRNAs in AS. To the best of our knowledge, this study is the first to use microarray analyses to examine the roles of lncRNAs in TLR4-primed AS-MSCs.

In this study, several lncRNAs with the largest fold changes among DE lncRNAs were studied. For example, GBP1P1 is a lncRNA that acts as a prognostic biomarker for hepatocellular carcinoma [37]. MIR3142HG can regulate the inflammatory response following IL-1 β -mediated activation of human lung fibroblasts, which is a positive regulator of IL-8 and CCL2 release [38]. The inflammatory response regulation by MIR3142HG indicates that it may contribute to the enhanced immunoregulatory ability of TLR4-primed AS-MSCs and the immunoregulatory dysfunction seen in AS. Unfortunately, most of the DE lncRNAs, such as LOC101926887, LOC105378410, and LOC107984251 have not been studied yet. Further exploration is needed in the future.

Regulatory lncRNAs act in a cis and/or trans manner to influence or interact with nearby or distant genes [39]. In our study, 8 lncRNA transcripts were predicted to cis-regulate nearby protein-coding genes. In addition, we predicted the functions of trans-regulatory lncRNAs using TFs that regulate protein-coding gene expression. 15 lncRNAs were identified to function in a trans-regulation manner. Then, 8 core pairs of lncRNAs and target mRNAs in the lncRNA-TF-mRNA network were obtained, which are PACERR-PTGS2, LOC105378085-SOD2, LOC107986655-HIVEP2, MICB-DT-MICB, LOC105373925-SP140L, LOC107984251-IFIT5, LOC112268267-GBP2 and LOC101926887-IFIT3. Among them, PACERR (PTGS2 Antisense NFKB1 Complex-Mediated Expression Regulator RNA, also known as PACER) is a novel long noncoding RNA that has been found to interact with NF- κ B transcriptional regulators to promote expression of prostaglandin-endoperoxide synthase 2 (PTGS2, cyclooxygenase-2, also known as COX2) [40]. COX2 could produce prostaglandin E2 (PGE2) and amplify the Th17 mediated autoimmune process through COX2–PGE2–EP2/EP4–NF- κ B loop [41]. Targeting COX2 activity with non-steroidal anti-inflammatory drugs (NSAIDs) or agents designed to specifically block COX-2 activity have been approved for therapeutic use [42]. Of note, NSAIDs are recommended as first-line treatment for AS patients for their high efficiency in reducing back pain and stiffness in patients with axial spondyloarthritis [43]. The pair of PACERR and PTGS2 may participate a key role in the immunoregulation in TLR4-primed AS-MSCs, which maybe an important potential biomark for diagnosis and target for drug therapy. SOD2 is a component of antioxidant defence systems, which are crucial in defending cells against oxidative stress. SOD2-overexpressing BM-MSCs have an enhanced therapeutic effect on brain injury treatment in traumatic brain injury mice [44]. Moreover, MSCs could enhance the expression of the SOD2 antioxidant gene to adapt to the oxidative environment and exert their therapeutic effect [45]. Exposure to LPS induces oxidative stress in AS-MSCs, LOC105378085 may regulate the expression of SOD2 in AS-MSCs to defend cells against oxidative stress and to exert the immunoregulatory effects of the AS-MSCs. Finally, the above mentioned 23 DE lncRNA and 8 lncRNA-target mRNA pairs identified in the present study provide novel information for understanding the biological functions of lncRNAs in AS-MSCs, but the underlying mechanisms of how “lncRNA-TF-target gene” networks affect TLR4-induced immunoregulation remain to be clarified.

Our study has several limitations. First, RNA-seq is an important method to screen possible lncRNAs and mRNAs associated with specific diseases, but the results of big-data analyses may include false positives. Therefore, we performed qRT-PCR to further verify differential expression. Second, we predicted lncRNA functions only indirectly using bioinformatic analysis and validated several DE lncRNAs. Further functional studies on the mechanism are warranted to clarify the roles of lncRNAs.

Conclusion

In AS, TLR4 activation can enhance the immunoregulatory ability of MSCs in AS. This study describes the lncRNA and mRNA expression profiles and functional networks in TLR4-primed AS-MSCs. Eight core pairs of lncRNA and target mRNA in the lncRNA-TF-mRNA network have been found in TLR4-primed AS-MSCs. These results provide insight into the pathogenesis of immunoregulatory dysfunction in AS-MSCs, which may help to elucidate possible molecular mechanisms and therapeutic targets in AS.

Methods

AS-MSC isolation and cell culture

MSCs were isolated from BM aspirates taken from AS patients who provided informed consent through density gradient centrifugation, as described in our previous study [46]. After density gradient centrifugation, MSCs were isolated through plastic adherence and grown at 37 °C in an atmosphere of 5% CO₂ for one week. The MSCs were trypsinized when the cultures reached 80–90% confluence. MSCs in passages 3–5 were used in subsequent experiments. MSCs were identified on the basis of immunological phenotypes and the triple-lineage differentiation capability, as previously described [46]. After identifying MSC immunophenotypic markers by flow cytometry, cells in passages three to five were used for subsequent experiments.

Pre-stimulation of TLR4 on AS-MSCs

To stimulate TLR4 on AS-MSCs effectively, determining the proper stimulating concentration and treatment time of the LPS (TLR4 ligand) was necessary. For the time-based stimulation test, 1.0 µg/ml LPS (Sigma-Aldrich, USA) was added to the culture medium for different times (0, 2, 4, 8, 12 and 24 hours) before a mixed lymphocyte reaction (MLR) was performed. For the concentration-based stimulation test, different concentrations (0, 0.1, 1 and 10 µg/ml) of LPS were added to cultured cells for the previously selected time. The best conditions for TLR4 activation in AS-MSCs were evaluated by assessing the level of p38 phosphorylation by western blotting. After determining the best stimulation time and concentration of LPS, MSCs were pre-primed before co-culture with peripheral blood mononuclear cells (PBMCs) and gene analysis.

Co-culture of AS-MSCs and PBMCs for PBMC proliferation analysis

AS-MSCs were primed with 1 µg/ml LPS for 4 hours prior to co-culture with PBMCs to activate TLR4. PBMCs were harvested from blood samples taken from healthy donors using the Ficoll-Paque density gradient centrifugation method and then labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE; BD Bioscience, USA). PBMCs were incubated with 5 µM CFSE in phosphate-buffered saline (PBS) at 37 °C for 5 min. After the incubation, the staining reaction was terminated by washing the PBMCs with complete medium twice. For co-culture with AS-MSCs (effector cells), AS-MSCs at a density of 1×10^5 cells/well seeded in 6-well plates were stimulated with or without 1 µg/ml LPS for 4 hours and then subjected to Co-60 irradiation (30 Gy). CFSE-labelled PBMCs (responder cells) at a cell density of 1×10^6 were then added to the MSC cultures. The co-cultures were stimulated with human anti-CD3/CD28 monoclonal antibodies (mAbs; CD3: 200 ng/ml; CD28: 1 µg/ml, BD Bioscience, USA) for 5 days, after which the PBMCs were harvested and stained with an anti-CD4-FITC antibody (BD Bioscience, USA), and cell proliferation was evaluated using flow cytometry. A minimum of 10,000 live cell events gated in scatter plots were analysed for each sample.

Library construction and high-throughput sequencing

Three MSC samples from five AS patients were selected randomly and separated into two subgroups: MSCs cultured without stimulation (control group, samples C1-C3) and MSCs stimulated with 1 µg/ml LPS for four hours to activate TLR4 specifically (experimental group, samples T1-T3). The total RNA concentration was quantified with a NanoDrop ND-2000 (Thermo Scientific), and RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies). Sample labelling, microarray hybridization and washing were performed based on the manufacturer's standard protocols. Briefly, total RNA was transcribed into double-stranded complementary DNA (cDNA), which was then synthesized into complementary RNA (cRNA) and labelled with Cyanine-3-CTP. The labelled cRNAs were hybridized onto the microarray. After washing, the arrays were scanned by an Agilent Scanner G2505C (Agilent Technologies). The Agilent Human lncRNA Microarray 2018 (4*180 k, Design ID: 085630) was used in this experiment, and data analysis of the 6 samples was conducted by OE Biotechnology Co., Ltd. (Shanghai, China).

Expression Analysis

Feature Extraction software (version 10.7.1.1, Agilent Technologies) was used to analyse array images to obtain raw data. GeneSpring (version 14.8, Agilent Technologies) was employed to complete the basic analysis with the raw data. First, the raw data were normalized with the quantile algorithm. The probes with at least 1 condition out of 2 conditions having flags in "Detected" were chosen for further data analysis. DE genes were

then identified through fold change data as well as the P value calculated with a t-test. The threshold set for up- and downregulated genes was a fold change ≥ 2.0 and a P value ≤ 0.05 . Afterwards, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were applied to determine the roles of these differentially expressed (DE) mRNAs. Finally, hierarchical clustering was performed to display the distinguishable gene expression patterns among samples.

qRT-PCR validation

To validate the reliability of high-throughput RNA-seq and explore the expression trends of mRNAs and lncRNAs, we performed quantitative real-time PCR (qRT-PCR) for biological validation. Total RNA was isolated from AS-MSCs with or without LPS stimulation using TRIzol according to the manufacturer's protocol. cDNA was transcribed using a PrimeScript RT reagent kit (Takara, Otsu). qRT-PCR was then performed, and the data were analysed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in the qRT-PCR assay are provided in Supplementary Table S1.

Co-expression network of lncRNAs with mRNAs

To further examine the potential roles of DE lncRNAs and mRNAs in TLR4-primed AS-MSCs, a DE lncRNA-mRNA co-expression network was constructed. First, the Pearson's correlation coefficient (PCC) between the expression levels of each DE lncRNA-DE mRNA pair in the TLR4-primed AS-MSCs and unstimulated AS-MSCs were calculated. Second, the DE lncRNA-DE mRNA pairs with an absolute PCC value ≥ 0.85 and $P < 0.05$ were defined as co-expressed DE lncRNA-DE mRNA pairs. The co-expressed DE lncRNA-DE mRNA pairs in which the expression level of the DE mRNA was positively correlated with the expression level of the DE lncRNA in TLR4-primed AS-MSCs were defined as positively co-expressed DE lncRNA-DE mRNA pairs. In contrast, negatively co-expressed DE lncRNA-DE mRNA pairs were defined as the expression level of the DE mRNA being negatively correlated with that of the DE lncRNA in TLR4-primed AS-MSCs. The co-expressed DE lncRNA-DE mRNA networks were visualized using Cytoscape 3.0 software.

Cis- and trans-regulation predictions of DE lncRNAs

It has been suggested that lncRNAs regulate gene expression through both cis- and trans-regulation. For cis-regulation prediction, we identified each paired lncRNA and mRNA by the following procedures: (1) the mRNA loci were within 100-kb windows upstream or downstream of the given lncRNA, and (2) the Pearson correlation of lncRNA-mRNA expression was significant ($P \leq 0.05$). For trans-regulation prediction, we enriched the co-expressed mRNAs with DE lncRNAs that significantly overlapped with the host genes of transcription factors (TFs). Using the threshold $P < 0.05$, each lncRNA could be connected with one to more than a dozen TFs, and each pair of lncRNA-TF was the result of several gene enrichments based on the hypergeometric cumulative distribution function. Then, we constructed the lncRNA-TF-mRNA network using Cytoscape software.

Statistical analysis

Data are expressed as the mean $\pm$ standard deviation (SD) and were analysed using the statistical software package SPSS16.0. Pearson correlation was used in lncRNA-mRNA co-expression analyses. A p value < 0.05 , fold enrichment > 2 , and $\log_2 FC > 1$ were considered statistically significant.

Abbreviations

lncRNA: long non-coding RNA;

AS: ankylosing spondylitis;

TLR4: toll-like receptor 4;

MSCs: mesenchymal stem cells;

AS-MSCs: mesenchymal stem cells from AS patients;

LPS: lipopolysaccharide;

DE: Differentially expressed;

mRNA: messenger RNAs;

DC: dendritic cell;

SLE: systemic lupus erythematosus;

HD: healthy donors;

TLRs: toll-like receptors;

PRRs: pattern recognition receptors;

PBMCs: peripheral blood mononuclear cells;

IDO1: indoleamine 2,3-dioxygenase 1;

ncRNAs: non-coding RNAs;

BM: bone marrow;

PTGS2: prostaglandin-endoperoxide synthase 2;

PACERR: PTGS2 Antisense NFKB1 complex-mediated expression regulator RNA;

COX2: cyclooxygenase-2

PGE2: prostaglandin E2;

NSAIDs: non-steroidal anti-inflammatory drugs;

MLR: mixed lymphocyte reaction;

CFSE: carboxyfluorescein diacetate succinimidyl ester;

PBS: phosphate-buffered saline;

GO: gene ontology;

KEGG: kyoto encyclopedia of genes and genomes;

qRT-PCR: quantitative real-time PCR;

PCC: Pearson's correlation coefficient;

TFs: transcription factors.

Declarations

Ethics approval and consent to participate

Sample collection was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital, Sun Yat-Sen University. The AS-MSCs used in this study were obtained from the Center for Biotherapy, Sun Yat-sen Memorial Hospital, Sun Yat-Sen University.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its Additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Yuxi Li: Conception and design; Ming Li: Manuscript writing; Ting Liu: Data analysis and interpretation; Jiajun Huang, Yuwei Liang and Junshen Huang: Provision of study material or patients and cell culture; Xiangge Liu, Ziyang Cheng and Shixin Lu: Collection and assembly of data; Huiyong Shen and Lin Huang: Conception and design, fund raising, management and adjustment.

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Authors' information

Yu-xi Li, Ming Li and Ting Liu contributed equally to this work.

Affiliations

Department of Orthopaedics, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, No.107, Yanjiang west Rd., Yuexiu Dist., Guangzhou, 510120, P.R. China

Yu-xi Li, Ming Li, Jia-jun Huang, Yu-wei Liang, Jun-shen Huang, Xiang-ge Liu, Zi-ying Cheng, Shi-xin Lu, Hui-yong Shen, Lin Huang

Department of Anaesthesia, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, No.107, Yanjiang west Rd., Yuexiu Dist., Guangzhou, 510120, P.R. China

Ting Liu

Department of Orthopaedics, Sun Yat-sen University Eighth Affiliated Hospital, Sun Yat-sen University, No.92, Fuhua Rd., Futian Dist., Shenzhen, 518033, P.R. China

Hui-yong Shen

Corresponding authors

Correspondence to Hui-yong Shen or Lin Huang.

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Supplementary Tables

Table S1. GO analysis of DE mRNA.

ID	Term	Category	Count	Fold Enrichment	P value
GO:0051607	defense response to virus	Biological process	47	10.03	4.03E-34
GO:0060337	type I interferon signaling pathway	Biological process	26	13.95	1.30E-23
GO:0060333	interferon-gamma-mediated signaling pathway	Biological process	26	12.58	3.15E-22
GO:0006954	inflammatory response	Biological process	52	4.81	2.45E-21
GO:0009615	response to virus	Biological process	26	8.35	3.55E-17
GO:0045071	negative regulation of viral genome replication	Biological process	16	14.09	4.25E-15
GO:0051092	positive regulation of NF-kappaB transcription factor activity	Biological process	26	6.77	8.34E-15
GO:0006955	immune response	Biological process	37	3.73	5.49E-12
GO:0032496	response to lipopolysaccharide	Biological process	24	5.12	4.75E-11
GO:0070098	chemokine-mediated signaling pathway	Biological process	16	7.97	9.63E-11
GO:0008009	chemokine activity	Molecular function	16	11.24	2.64E-13
GO:0045236	CXCR chemokine receptor binding	Molecular function	5	18.73	2.57E-06
GO:0004842	ubiquitin-protein transferase activity	Molecular function	26	2.74	3.57E-06
GO:0003725	double-stranded RNA binding	Molecular function	10	5.72	8.18E-06
GO:0003700	transcription factor activity, sequence-specific DNA binding	Molecular function	47	1.85	3.69E-05
GO:0016874	ligase activity	Molecular function	19	2.81	5.17E-05
GO:0005125	cytokine activity	Molecular function	16	3.10	6.26E-05
GO:0004871	signal transducer activity	Molecular function	17	2.95	6.83E-05
GO:0008270	zinc ion binding	Molecular function	57	1.69	7.09E-05
GO:0003950	NAD+ ADP-ribosyltransferase activity	Molecular function	6	7.49	1.15E-04
GO:0005737	cytoplasm	Cellular component	189	1.37	2.51E-07
GO:0005829	cytosol	Cellular component	136	1.50	2.92E-07
GO:0005615	extracellular space	Cellular component	65	1.79	2.98E-06
GO:0005634	nucleus	Cellular component	186	1.30	1.38E-05
GO:0005576	extracellular region	Cellular component	66	1.61	7.97E-05
GO:0043657	host cell	Cellular component	3	21.58	2.05E-04
GO:0033256	I-kappaB/NF-kappaB complex	Cellular component	3	21.58	2.05E-04
GO:0097342	riposome	Cellular component	3	15.41	6.88E-04
GO:0031264	death-inducing signaling complex	Cellular component	3	15.41	6.88E-04
GO:0000932	cytoplasmic mRNA processing body	Cellular	8	3.74	1.32E-

Table S2. Pathways with the largest significant difference of mRNA in KEGG analysis.

ID	Pathway	Count	Fold Enrichment	P value	Gene
hsa04621	NOD-like receptor signaling pathway	38	6.67	1.39E- 21	CXCL8; GBP3; NAMPT; RBCK1; CXCL2; GBP4; OAS1; MAPK8; GBP2; GBP1; TICAM1; AIM2; GBP5; IFNAR2; TNFAIP3; BIRC3; IL1B; CXCL1; RIPK2; BIRC2; IRF9; MYD88; NLRP3; OAS2; IL6; RIPK1; NFKB1; PANX1; CCL2; CCL5; IFNB1; CXCL3; ANTXR2; STAT2; STAT1; CASP1; NOD1; OAS3
hsa04668	TNF signaling pathway	31	8.47	4.75E- 21	CXCL2; FAS; ICAM1; CSF1; VCAM1; CASP7; PTGS2; MAP3K5; MAPK8; TNFAIP3; BIRC3; IL1B; CXCL1; BIRC2; IL18R1; MLKL; CCL20; LIF; MAP3K8; CX3CL1; IL6; RIPK1; MMP3; NFKB1; CCL2; CCL5; CXCL5; CXCL3; CXCL10; CFLAR; TRAF1
hsa05164	Influenza A	35	5.97	2.95E- 18	CXCL8; FAS; ICAM1; TLR3; RSAD2; OAS1; TNFSF10; CIITA; MAPK8; HLA-DOB; TRIM25; MX1; TICAM1; IFNAR2; PML; DDX58; IL1B; EIF2AK2; IRF9; MYD88; NLRP3; OAS2; IL6; NFKB1; CCL2; CCL5; JAK2; IFNB1; CXCL10; IFNGR2; STAT2; CASP1; STAT1; OAS3; IFIH1
hsa05168	Herpes simplex infection	34	5.42	2.12E- 16	HLA-F; FAS; SP100; TLR3; OAS1; MAPK8; HLA-DOB; CD74; TICAM1; IFNAR2; PML; TNFSF14; DDX58; IL1B; EIF2AK2; IRF9; MYD88; TRAF1; IFIT1; OAS2; NFKB1; IL6; CCL2; CCL5; JAK2; IFNB1; TAP1; TAP2; IFNGR2; STAT2; C3; STAT1; OAS3; IFIH1
hsa04064	NF-kappa B signaling pathway	22	6.83	4.16E- 13	CXCL8; CXCL2; ICAM1; VCAM1; PTGS2; TRIM25; TICAM1; TNFAIP3; TNFSF14; BIRC3; DDX58; IL1B; BIRC2; RELB; MYD88; RIPK1; NFKB1; BCL2A1; CCL4; NFKB2; CFLAR; TRAF1
hsa04060	Cytokine- cytokine receptor interaction	36	3.93	8.02E- 13	CXCL8; IL6R; FLT3; CXCL9; CXCL2; FAS; CSF1; IL15RA; TNFSF10; CXCL11; TNFSF9; IL1RAP; BMP2; IFNAR2; TNFSF14; IL1B; CXCL1; IL18R1; CCL7; CCL20; LIF; CXCL16; CXCL6; CX3CL1; IL6; IL7; CCL2; CCL5; IFNB1; TSLP; CXCL3; CXCL5; CXCL10; CCL4; IFNGR2; PDGFC
hsa04217	Necroptosis	27	4.86	5.04E- 12	RBCK1; FAS; STAT4; TLR3; PLA2G4A; TNFSF10; MAPK8; TICAM1; IFNAR2; TNFAIP3; BIRC3; IL1B; EIF2AK2; MLKL; BIRC2; IRF9; NLRP3; PARP4; RIPK1; JAK2; IFNB1; IFNGR2; STAT5A; STAT2; CASP1; STAT1; CFLAR
hsa04657	IL-17 signaling pathway	19	6.03	1.83E- 10	CXCL8; CXCL2; PTGS2; MMP13; MAPK8; TNFAIP3; IL1B; CXCL1; CCL7; CCL20; CXCL6; TRAF3IP2; IL6; NFKB1; MMP3; CCL2; CXCL5; CXCL3; CXCL10
hsa05162	Measles	22	4.84	5.58E- 10	FAS; OAS1; TNFSF10; MX1; IFNAR2; TNFAIP3; DDX58; IL1B; EIF2AK2; IRF9; MYD88; OAS2; IL6; NFKB1; JAK2; IFNB1; IFNGR2; STAT5A; STAT2; STAT1; OAS3; IFIH1
hsa04620	Toll-like receptor signaling pathway	18	5.11	9.27E- 09	CXCL8; TICAM1; MAP3K8; IL6; RIPK1; NFKB1; IFNAR2; CXCL11; CCL5; IFNB1; MAPK8; IL1B; CXCL9; CXCL10; CCL4; STAT1; MYD88; TLR3

Table S3. Primers of the analyzed genes.

Gene Name	Forward Primer	Reverse Primer
GAPDH	AAGGTGAAGGTCGGAGTCAA	AATGAAGGGGTCATTGATGG
MIR3142HG	TGGCTCAGAACTCCGATT	CCTACTCCTCACAGATACAC
LOC105374444	AGCCCTCTTTGGTCAACTCC	TGGTTGCAGCATTGTCTTGG
LOC105371619	ATGTCTGTAGCATGCGGCTT	TTTCAGAAAACAGGCCCCG
PACERR	CCGTGTCTGGTCTGTACGTC	AACCTTACTCGCCCCAGTCT
LOC105375914	ACGCAAGCTGGGTTATTGGA	AGCAACTGTGGTTGTCAGAT
CXCL1	CTCTTCTTCCCTAGGAGCGTC	GATGCAGGATTGAGGCAAGC
CXCL8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC
CXCL10	GTGGCATTCAAGGAGTACCTC	TGATGGCCTTCGATTCTGGATT
CXCL11	GACGCTGTCTTTGCATAGGC	GGATTTAGGCATCGTTGTCCTTT
CCL20	TGCTGTACCAAGAGTTTGCTC	CGCACACAGACAACTTTTTCTTT
TNF- α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
CXCL9	TGAGAAAGGGTCGCTGTTCC	GGGCTTGGGGCAAATTGTTT
PDL1	GCTGCACTAATTGTCTATTGGGA	AATTCGCTTGTAGTCGGCACC
IL-1 β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCTGTAGCTGGA
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTTCAGGTTG
INOS	TTCAGTATCACAACCTCAGCAAG	TGGACCTGCAAGTTAAATCCC

Figures

Figure 1

The immunoregulatory function of TLR4-primed AS-MSCs. A: To determine the best duration and concentration of TLR4 ligand pre-stimulation, we examined the level of p38 phosphorylation by western blotting. AS-MSCs were exposed to LPS at four different concentrations (0 $\mu\text{g/ml}$, 0.1 $\mu\text{g/ml}$, 1 $\mu\text{g/ml}$, and 10 $\mu\text{g/ml}$) for 4 hours or treated at the concentration of 1 $\mu\text{g/ml}$ for the indicated time (0 hours, 2 hours, 4 hours, 8 hours, 12 hours or 24 hours). The upregulation of the phospho-p38 level was highest at 4 hours with the concentration of 1 $\mu\text{g/ml}$ LPS and declined thereafter. B: AS-MSCs were pre-stimulated with or without 1 $\mu\text{g/ml}$ LPS for 4 hours and then co-cultured with PBMCs at a ratio of 1:10 (MSCs: PBMCs) for 5 days. All PBMCs were then collected for assessment by flow cytometry to determine the positive percentage of CFSE-diluted cells (gated) to evaluate proliferation. AS-MSCs inhibited the proliferation of PBMCs, and this effect was strengthened by the activation of TLR4. C: The gene expression of cytokines and chemokines in AS-MSCs after LPS stimulation was detected by qRT-PCR. The symbol “**” represents $P < 0.05$.

Figure 2

Identification of DE lncRNAs and mRNAs. A: Heatmaps of DE mRNAs between TLR4-primed AS-MSCs and unstimulated AS-MSCs. B: Heatmaps of DE lncRNAs between TLR4-primed AS-MSCs and unstimulated AS-MSCs. C: Volcano plots of DE mRNAs between TLR4-primed AS-MSCs and unstimulated AS-MSCs. D: Volcano plots of DE lncRNAs between TLR4-primed AS-MSCs and unstimulated AS-MSCs.

Figure 3

Validation of DE mRNAs and lncRNAs. A: DE lncRNAs were confirmed by qRT-PCR. B: DE mRNAs were confirmed by qRT-PCR.

Figure 4

GO analysis and KEGG analysis of DE mRNAs. A: The top 10 terms identified by GO analysis of molecular functions, biological processes and cellular components are shown. B: DE mRNAs were clustered by KEGG analysis, and the top 30 pathways are shown.

Figure 5

Advanced analyses of DE lncRNAs and mRNAs. A: Interactions among mRNAs. Red and green represent upregulated and downregulated mRNAs, respectively. The size of the ellipse represents the fold change of DE mRNAs. B: Co-expression network of DE lncRNAs and DE mRNAs. Triangle and ellipse represent lncRNA and mRNA, respectively. The size of the ellipse represents the fold change of DE mRNAs.