Impact of miR-223-3p and miR-2909 on inflammatory factors IL-6, IL-1β, and TNF-α, and the TLR4/TLR2/NF-κB/STAT3 signaling pathway induced by lipopolysaccharide in human adipose stem cells

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ABSTRACT

In the current study, we successfully established an acute inflammation model and a chronic inflammation model involving adipose stem cells. We used high-throughput miRNA microarray analysis to identify miRNAs that were significantly ($p<0.05$) differentially expressed during both acute and chronic inflammation. Lipopolysaccharide (LPS) significantly($p<0.05$)reduced the expression of miR-223-3P and miR-2909, while promoting the production of pro-inflammatory cytokines, interleukin (IL) 6, IL-1b, and tumor necrosis factor (TNF)-a via the Toll-like receptor (TLR) 4/TLR2/nuclear factor (NF)-kB/signal transducer and activator of transcription (STAT) 3 signaling pathway in human adipose stem cells. Further, miR-223-3P expression was significantly($p<0.05$) reduced in human adipose stem cells during activation by IL-6 stimulation. The inducible down-regulation of miR-223-3P resulted in the activation of STAT3, which was directly targeted by miR-223-3P. STAT3 directly targeted TLR4 and TLR2, promoting the production of the pro-inflammatory cytokine, IL-6, and formed a positive feedback loop to regulate IL-6 levels. Similarly, TNF-a significantly($p<0.05$) increased the expression of miR-223-3p, with LPS and TLR4/TLR2/NF-kB/STAT3 forming a negative feedback loop to regulate TNF-a levels. In addition, miR-2909, which depends on NF-kB, targeted Krueppel-like factor (KLF) 4 to regulate the levels of pro-inflammatory cytokines, IL-6, IL-1b, and TNF-a. We conclude that miR-223-3p and miR-2909 form a complex regulatory network with pro-inflammatory factors and signaling pathways in adipose stem cells stimulated by LPS. These findings will inform the development of therapies against autoimmune and inflammatory diseases.

1.Establish an acute inflammation model of adipose stem cells

2.Establish a chronic inflammation model of adipose stem cells

3. miRNA microarray and data analysis

4.Target gene prediction, gene ontology (GO) enrichment, and pathway analysis

5.Prediction of target gene binding sites

6.Dual luciferase assay

7.LPS induces STAT3mRNA and protein levels in adipose stem cells, and promotes STAT3 protein phosphorylation, while miR-223-3p regulates STAT3 mRNA

8.miR-223-3p directly regulates TLR2 and TLR4, and the secretion of inflammatory cytokines IL-6, TNF-a, and IL-1b

9. STAT3 promotes phosphorylation of STAT3 protein, and regulates TLR4 and TLR2 expression

10.Treatment of adipose stem cells with IL-6, TNF-a, siIL-6, and siTNF-a affects the miR-223-3p, STAT3, and pSTAT3 levels

11.miR-2909 depends on NF-kB to target KLF4 to regulate IL-6, IL-1b, and TNF-a levels

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

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MATERIALS TEXT

Materials and methods

Cells and reagents

The cells were purchased from Wuhan Hamilton Biotechnology Co., Ltd. (Wuhan, China); they are third-generation frozen human adipose stem cells. LPS(L-2880) was purchased from Sigma. Standard fetal bovine serum(SH30088.03) and Dulbecco's modified Eagle's medium F12(SH30023.01) were purchased from GIBCO. CCK8 kit(PH0534), BCA protein concentration assay kit(NCI-3225), site-directed mutagenesis kit(KM101), SDS-PAGE Sample Loading Buffer(P0015), and horseradish peroxidase-labeled goat anti-rabbit antibody (ORB175845,1:50,000)were from China Biyuntian Biotechnology Co., Ltd. Rabbit polyclonal anti-TLR2 antibody(ab191458，1:1000), rabbit polyclonal anti-TLR4 antibody(ab13556
，1:1000), rabbit monoclonal anti-STAT3 antibody(ab32500，1:1000), rabbit antibody anti-p-STAT3 (Tyr-705,1:2000) monoclonal, rabbit monoclonal anti-NF-κB antibody(ab59238，1:50,000), rabbit monoclonal anti- p-NF-κB antibody(ab16502
，1:10,000), rabbit monoclonal anti-KLF4 antibody(ab80230,1:1000) and mouse monoclonal anti-β-actin antibody (HC201-01，1:200)were purchased from Abcam. Enzyme-linked immunosorbent assay (ELISA) kits (for the detection of IL-6 (E-EL-H0102), IL-1β(E-EL-H0149), TNF-α(E-EL-H0109), etc.), human IL-6–stimulating factor(PeproTech 96-200-06-5), human TNF-α–stimulating factor(PeproTech 96-300-01A-10), p-STAT3 inhibitor (S1155 S3I-201), and NF-κB inhibitor (BAY 11-7082) were purchased from Wuhan Elitet Biotechnology Co., Ltd. Real-time polymerase chain reaction (PCR) special reagent(DRR802A), SYBR® Premix Ex TaqTM II (Perfect Real-Time) (DRR081A)was purchased from TAKARA. Trizol (10296010)was purchased from Invitrogen. P004ECL luminescence detection kit (W028-1)was from Vigorous. Further, miR-223-3p and miR-2909 mimics and mimics controls, miR-223-3p and miR-2909 inhibitor and inhibitor controls, small interfering (si) RNA (STAT3 siRNA, siIL-6, and Si-TNF-α), and control siRNA were purchased from Ruibo Bio (Guangzhou, China). Lipofectamine 2000 （11668019）was from Thermo Fisher.

1 1. Establish an acute inflammation model of adipose stem cells

2 1. Cells were routinely grown in medium under 5% CO2 incubator at 37 °C. For the experiments, cell suspension with a density of approximately 1 × 105 /mL was uniformly seeded in a 96-well plate (100 μL per well). The cells were incubated in a 5% CO2 incubator at 37 °C

3 2. The experiments were started once the cell fusion rate exceeded 80%.

3. 0.0102 g LPS was added to 10 mL PBS to make 1 g/L LPS mother liquor, and then diluted to the corresponding concentration (1 g/mL, 10

4 g/mL, and 100 g/mL) with complete medium.

5 4. After removing the spent culture medium with a sample gun, three different concentrations of LPS (1 μg/mL, 10 μg/mL, and 100 μg/mL; 100 μL/well) were added to stimulate adipose stem cells at three stimulation time-points 2, 6, and 12 h

6 5. The control cells were incubated with 100 μL/well of the complete medium. All treatments were conducted in three replicate wells

7 6. The control group was counted together, a total of four concentrations of LPS-stimulated adipose stem cells at three-time stimulation points, and 12 groups were collected for cell supernatant and pellet.

8 7. The culture supernatant from the treatment and control wells was transferred to a 1.5-mL centrifuge tube, and centrifuged at 4 °C for 10 min (3000 rpm). The supernatant was collected and stored at −20 °C for ELISA analysis. A portion of the cell pellet was immediately used for RNA extraction, reverse-transcribed into cDNA, and stored at −20 °C for real-time quantitative PCR (qPCR). Another portion of the pellet was used for protein determination. The sample was denatured at 100 °C for 10 min and stored at −20 °C before western blotting.

9 8. After 2, 6, and 12 h of treatment, the medium was discarded, and 10 μL of CCK8 reagent and 100 μL of medium were added to each well sample absorbance was measured at 450 nm using a microplate reader. The cell growth curve was plotted with time as the x-coordinate and cell growth as the y-coordinate

10 2. Establish a chronic inflammation model of adipose stem cells

11 1. 1000 adipose stem cells were transferred to 6-well plates and divided into four groups. After 24 h of culturing, the cells were stimulated with LPS (1 μg/mL and 100 μL/well)

12 2. After 5-d stimulation, one of the four groups was selected as the first group, and the supernatant was collected for the first group ELISA analysis. In addition, the pellet was collected for qPCR analysis and protein determination for the first group, 10 μL of CCK8 reagent and 100 μL of medium were added to each well for cell proliferation activity of the first group sample absorbance was measured at 450 nm using a microplate reader. The cell growth curve was plotted with time as the x-coordinate and cell growth as the y-coordinate

13 3. For the other three groups, half of the supernatant was collected, centrifuged, and the cells processed further, and the withdrawn volume was replaced with fresh medium

14 4. After 1-d culture, second LPS-stimulation was performed (the medium was aspirated, half of LPS (1 μg/mL, 100 μL/well) was added to the fresh medium, and then half of the previously collected medium was added]).

15 5. After additional 5 d, one of the three groups was selected as the second group, and then half of the supernatant was collected for ELISA analysis for the second group; the pellet was collected for the second group qPCR analysis and protein determination. 10 μL of CCK8 reagent and 100 μL of medium were added to each well for cell proliferation activity of the second group sample absorbance was measured at 450 nm using a microplate reader. The cell growth curve was plotted with time as the x-coordinate and cell growth as the y-coordinate

16 6. For the other two groups, half of the supernatant was collected, centrifuged, and the cells processed further, and the withdrawn volume was replaced with fresh medium

17 7. After 1-d culture, third LPS-stimulation was performed (the medium was aspirated, half of LPS (1 μg/mL, 100 μL/well) was added to the fresh medium, and then half of the previously collected medium was added]).

18 8. After additional 5 d, one of the two groups was selected as the third group, and then half of the supernatant was collected for ELISA analysis for the third group; the pellet was collected for the third group qPCR analysis and protein determination. 10 μL of CCK8 reagent and 100 μL of medium were added to each well for cell proliferation activity of the third group sample absorbance was measured at 450 nm using a microplate reader. The cell growth curve was plotted with time as the x-coordinate and cell growth as the y-coordinate

9. For the last one group, half of the supernatant was collected, centrifuged, and the cells processed further, and the withdrawn volume was

19 replaced with fresh medium

20 10. After 1-d culture, fouth LPS-stimulation was performed (the medium was aspirated, half of LPS (1 μg/mL, 100 μL/well) was added to the fresh medium, and then half of the previously collected medium was added]).

21 11. After additional 5 d, the last one group was selected as the fouth group, and then half of the supernatant was collected for ELISA analysis for the fouth group; the pellet was collected for the fouth group qPCR analysis and protein determination. 10 μL of CCK8 reagent and 100 μL of medium were added to each well for cell proliferation activity of the fouth group sample absorbance was measured at 450 nm using a microplate reader. The cell growth curve was plotted with time as the x-coordinate and cell growth as the y-coordinate

22 3. miRNA microarray and data analysis

23 1.Based on data analysis, adipose stem cells stimulated with 1 μg/mL LPS for 6 h are the optimal acute inflammation model, adipose stem cells stimulated with 1 μg/mL LPS for 4 weeks are the optimal chronic inflammation model. The best acute inflammation model ,the best chronic inflammation model and the normal control group were subjected to high-throughput sequencing.

24 2.High-throughput miRNA sequencing was performed by Shanghai Lie Bing Information Technology Co., Ltd. A set of the same miRNAs are differentially expressed in the acute and chronic inflammation models.

25 3.Heat map and qPCR verification of miRNA microarray analysis results.

26 4.Target gene prediction, gene ontology (GO) enrichment, and pathway analysis

27 1.Target mRNAs of differentially expressed miRNAs were predicted using 12 predictive databases (miRWalk, Microt4, miRanda, mirbridge, miRDB, miRMap, miRNAMap, Pictar2, PITA, RNA22, RNAhybrid, and Targetscan). To predict the function, target mRNAs whose prediction was supported by nine databases were used for gene annotation (GO analysis) and information enrichment. GO analysis was performed using the Integration Discovery (DAVID) software, version 6.7 (http://david.abcC.ncifcrf.gov) to identify biological processes, cellular components, and molecular functions. Simultaneously, pathway analysis was performed on the basis of pathways generated by the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) to predict the possible signaling pathways of the selected miRNA targeted genes.

28 2.Each miRNA could target thousands of genes. Hence, we used the four most commonly used target gene prediction programs (Target Scan, CLIP-Seq, miRDB, and miRanda) for Venn diagram aggregation analysis

29 3. GO analysis of target genes predicted by differentially expressed miRNAs in the normal group and the acute inflammation model group or in the normal group and the chronic inflammation model group

30 4. Analysis of target gene pathways predicted by differentially expressed miRNAs in the normal group and the acute inflammation model group or in the normal group and the chronic inflammation model group

31 5.Prediction of target gene binding sites

32 After overlapping with TargetScan, CLIP-Seq, miRDB, and miRanda, miR-223-3p was found to bind to the 3′ UTR of STAT3 mRNA and IL6 mRNA, and miR-2909 was found to bind to the 3′ UTR of KLF4 mRNA. Analysis using the bioinformatics database JASPAR (default threshold score of 85.0), revealed seven potential TLR4 gene promoter binding sites in the coding region of the STAT3 gene and four potential TLR2 gene promoter binding sites. It is highly probable that STAT3 regulates the expression of TLR4 and TLR2 genes.

33 6.Dual luciferase assay

34 The wild-type human STAT3 3′ UTR was constructed by amplifying the 3′ UTR of human STAT3 gene, and cloning it into the pMIR-TM reporter luciferase vector. The wild-type human IL6 3′ UTR was constructed by amplifying the 3′ UTR of human IL6 and cloning it into a dual luciferase vector (psi-CHECK2) downstream of the Renilla luciferase open reading frame. The wild-type human KLF4 3′ UTR was constructed by amplifying the KLF4 3′ UTR and cloning it into the pMIR green fluorescent protein reporter vector. All 3′ UTR-binding sites were mutated using a site-directed mutagenesis kit and sequenced. The coding sequence of the STAT3 gene was inserted into the pLVX-IRES-Puro vector; the TLR4 gene promoter sequence and TLR2 gene promoter sequence were inserted into PGL3 vectors. A construct harboring a mutation in the binding site was also generated, and used as a control. The designed miRNA mimic, control, or promoter sequence was then used to co-transfect human adipose stem cells together with the corresponding plasmid. After 24 h, the luciferase activity was determined using a dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. The transfection combinations of the plasmids are shown in Table below.

35 Table . Transfection plasmid combinations of dual luciferase

36 Number Transfection plasmid combinations of dual luciferase

37 7.LPS induces STAT3 mRNA and protein levels in adipose stem cells, and promotes STAT3 protein phosphorylation, while miR-223-3p regulates STAT3 mRNA

38 A 0.5-mL cell suspension containing 2 × 105 cells was inoculated into each well of a 24-well plate and incubated overnight under 5% CO2 incubator at 37 °C. The following were used to transfect human adipose stem cells using Lipofectamine 2000 reagent, according to the manufacturer's instructions. h-miR-223-3p mimic (5′-UGUCAGUUUGUCAAAUACCCCA-3′), h-miR-223-3p inhibitor (5′-UGGGGUAUUUGACAAACUGACA-3′), h-miR-223-3p mimic normal control (NC) and h-miR-223-3p inhibitor NC. The culture supernatant from the treatment and control wells was transferred to a 1.5-mL centrifuge tube, and centrifuged at 4 °C for 10 min (3000 rpm). The supernatant was collected and stored at –20 °C for ELISA analysis. A portion of the cell pellet was immediately used for RNA extraction, reverse-transcribed into cDNA, and stored at –20 °C for real-time quantitative PCR (qPCR). Another portion of the pellet was used for protein determination. The sample was denatured at 100 °C for 10 min and stored at –20 °C before western blotting.

39 8.miR-223-3p directly regulates TLR2 and TLR4, and the secretion of inflammatory cytokines IL-6, TNF-α, and IL-1β

40 A 0.5-mL cell suspension containing 2 × 105 cells was inoculated into each well of a 24-well plate and incubated overnight under 5% CO2 incubator at 37 °C. The following were used to transfect human adipose stem cells using Lipofectamine 2000 reagent, according to the manufacturer's instructions. si-h-STAT3 (5′-GGGUCUGGCUAGACAAUAUTT-3′), si-h-STAT3NC. The culture supernatant from the treatment and control wells was transferred to a 1.5-mL centrifuge tube, and centrifuged at 4 °C for 10 min (3000 rpm). The supernatant was collected and stored at –20 °C for ELISA analysis. A portion of the cell pellet was immediately used for RNA extraction, reverse-transcribed into cDNA, and stored at –20 °C for real-time quantitative PCR (qPCR). Another portion of the pellet was used for protein determination. The sample was denatured at 100 °C for 10 min and stored at –20 °C before western blotting.

41 9. STAT3 promotes phosphorylation of STAT3 protein, and regulates TLR4 and TLR2 expression

42 A 0.5-mL cell suspension containing 2 × 105 cells was inoculated into each well of a 24-well plate and incubated overnight under 5% CO2 incubator at 37 °C. The following were used to transfect human adipose stem cells using Lipofectamine 2000 reagent, according to the manufacturer's instructions. virus-infected STAT3 and p- STAT3 inhibitor were used to transfect adipose stem cells. The culture supernatant from the treatment and control wells was transferred to a 1.5-mL centrifuge tube, and centrifuged at 4 °C for 10 min (3000 rpm). The supernatant was collected and stored at –20 °C for ELISA analysis. A portion of the cell pellet was immediately used for RNA extraction, reverse-transcribed into cDNA, and stored at –20 °C for real-time quantitative PCR (qPCR). Another portion of the pellet was used for protein determination. The sample was denatured at 100 °C for 10 min and stored at –20 °C before western blotting.

43 10.Treatment of adipose stem cells with IL-6, TNF-α, siIL-6, and siTNF-α affects the miR-223-3p, STAT3, and pSTAT3 levels

44 A 0.5-mL cell suspension containing 2 × 105 cells was inoculated into each well of a 24-well plate and incubated overnight under 5% CO2 incubator at 37 °C. The following were used to transfect human adipose stem cells using Lipofectamine 2000 reagent, according to the manufacturer's instructions. si-h-IL-6 (5′-GCCACTCACCTCTTCAGAA-3′), si-h-TNF-α (5′-GGCGTGGAGCTGAGAGATA-3′),si-h-IL-6NC, si-h-

TNF-αNC were used to transfect adipose stem cells. The culture supernatant from the treatment and control wells was transferred to a 1.5-mL centrifuge tube, and centrifuged at 4 °C for 10 min (3000 rpm). The supernatant was collected and stored at –20 °C for ELISA analysis. A portion of the cell pellet was immediately used for RNA extraction, reverse-transcribed into cDNA, and stored at –20 °C for real-time quantitative PCR (qPCR). Another portion of the pellet was used for protein determination. The sample was denatured at 100 °C for 10 min and stored at –20 °C before western blotting.

45 11.miR-2909 depends on NF-κB to target KLF4 to regulate IL-6, IL-1β, and TNF-α levels

46 A 0.5-mL cell suspension containing 2 × 105 cells was inoculated into each well of a 24-well plate and incubated overnight under 5% CO2 incubator at 37 °C. The following were used to transfect human adipose stem cells using Lipofectamine 2000 reagent, according to the manufacturer's instructions. h-miR-2909 mimic (5′-GUUAGGGCCAACAUCUCUUGG-3′), h-miR-2909 inhibitor (5′-CCAAGAGAUGUUGGCCCUAAC-3′), h-miR-2909 mimic NC and h-miR-2909 inhibitor NC. The culture supernatant from the treatment and control wells was transferred to a 1.5-mL centrifuge tube, and centrifuged at 4 °C for 10 min (3000 rpm). The supernatant was collected and stored at –20 °C for ELISA analysis. A portion of the cell pellet was immediately used for RNA extraction, reverse-transcribed into cDNA, and stored at –20 °C for real-time quantitative PCR (qPCR). Another portion of the pellet was used for protein determination. The sample was denatured at 100 °C for 10 min and stored at –20 °C before western blotting.

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