Differential expression of miR-672-5p and miR-146a-5p in osteoblasts in rats after steroid intervention

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Abstract

Background: Apoptosis of osteoblasts and osteocytes is one cause of steroid-induced osteonecrosis of the femoral head; however, the molecular mechanism of steroid affecting osteoblasts at the genetic level is unclear. The aim of the present work is to examine differential expression of osteoblasts in rats after steroid intervention and to verify expression by real-time polymerase chain reaction (RT-PCR).

***Methods*:** Primary culture, passaging and identification of osteoblasts of SD neonatal rats were conducted; osteoblasts were divided into two groups, the control group, and the steroid group. Total RNA was extracted separately, and quality control was performed; by means of RNA labeling and microarray hybridization, data were collected and then standardized to ascertain differences in miRNA expression between the two groups. The gene expression spectrum was analyzed. Obvious differential expression of miR-672-5p and miR-146a-5p was verified by RT-PCR. Miranda，microcosm and mirdb bioinformatics software were used to perdict target genes.

Results: Compared with the control group, morphologically, the osteoblasts in the

steroid group were more irregular and showed various shapes. The number of miRNAs (fold change >2) in the steroid group was six. Four miRNAs were upregulated and two miRNAs were downregulated. In particular, upregulated miR-672-5p expression and downregulated miR-146a-5p expression were significant. RT-PCR results showed that the $2^{-\triangle\triangle}$ CT value of miR-672-5p in the steroid group was 3.743-fold of that in the control group, and the $2^{-\triangle\triangle}$ CT value of miR-146a-5p in the steroid group was 0.322-fold of that in the control group. Angptl4, Ccdc51, Ssbp3 and RGD1306991 were perdicted as the target gene of miR-672-5p,while Hrp12 was that of miR-146a-5p.

***Conclusion*:** Expression profiles of miR-672-5p and miR-146a-5p had the most significant changes in the osteoblasts of rats with steroid intervention, which may provides a new viewpoint to pathogenesis of osteonecrosis of the femoral head.

Keywords: steroid-induced osteonecrosis of the femoral head; osteoblasts; miRNA expression

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Introduction

Steroid-induced osteonecrosis of the femoral head is a clinical orthopedic disease that is difficult to treat. Based on the incidence of occult osteonecrosis of the femoral head, once collapse of the femoral head occurs, patients present with hip joint pain and limited joint movement, severely affecting qualify of life. The pathogenesis of osteonecrosis of the femoral head is unclear though apoptosis of osteoblasts and osteocytes has been demonstrated to be one of the causes[1]. Steroid-induced apoptosis of osteoblasts and osteocytes can bring about activation, expression, and regulation of a series of genes[2]. Micro RNAs (miRNAs) are a class of short-stranded non-coding RNAs measuring 19-25 nucleotides in length, and most have a high degree of sequence conservation and temporal sequence of expression and tissue specificity, able to identify specific target mRNAs and exert their regulatory roles in modulating mRNA expression after transcription[3]. miRNA functions in bone homeostasis, as well[4.5]; however, it is unknown whether differential expression of miRNAs takes place in osteoblasts after steroid intervention. In the present study, the aim was to study differential expression of miRNAs in osteoblasts of normal rats and in rats with steroid intervention to determine the differential expression spectrum. Moreover, significantly upregulated and downregulated miRNAs were verified by RT-PCR and the target genes were predicted.

1. Materials and Methods

Primary culture, passage and identification of osteoblasts

All procedures involving animals were approved by the Guangzhou University of

Chinese Medicine Animal Study Committee and were carried out in accordance with the guide for the humane use and care of laboratory animals. Five neonatal rats were obtained from the Experimental Animal Center, Guangzhou University of Traditional Chinese Medicine (Registration number: SCXK (yue) 2013-0034). They were soaked in 100% ethanol. After being washed with PBS (Hyclone), the skulls were removed under sterile conditions and cut into pieces, then transferred into a 25cm^2 culture flask. 10% calf bovine serum (FBS) (Gibco) and DMEM culture medium (Gibco) with 100 μg/ml penicillin/streptomycin (Hyclone) was added to the flask which was turned over andincubated at 37 ºC in a 5% CO_2, saturated and humidified CO_2 incubator (SHEL LAB). 4 hr later, the flask was turned over and the skull pieces were continuously incubated. Cell growth was observed and the medium was changed after 4-5 days of incubation. Once primary culture cells reached confluence on the entire bottom of the flask, they were deemed ready for passage. The culture medium was discarded and the cells were washed with PBS, after which trypsin (Gibco) was added and the cells observed under a reverse microscope. When the cytoplasm became shrunken, and the interstitial substance was enlarged, DMEM culture medium was added to terminate digestion. The cell suspension collected was centrifuged for 4 minutes at a speed of 1000 rpm/minute; passaging was conducted in sub-bottles (1:3) and cultured in culture flasks.

1.1.2 The third passage of osteoblasts was used for further experiments. The culture medium was initially discarded. After the cells had been washed with PBS, they were fixed in 95% ethanol for 30 minutes, washed with ultrapure water, stained with 0.1% Alizarin Red staining solution (LG pH 8.3) for 5 minutes, and then incubated in CO_2 culture medium for 30 minutes. The cells were finally observed under a microscope after staining solution was removed.

1.2 Establishment of osteoblast cell model of steroid intervention

The third passage of osteoblasts was inoculated in two 25cm^2 culture flasks and divided into two groups: the control group and the steroid group . The cell medium was changed when the osteoblasts reached confluence. The cells in the control group

were continuously cultured with normal rat serum and L-DMEM culture medium with 100 μg/ml penicillin/streptomycin, and cells in the steroid group were cultured with methyl-prednisone (Pfizer), normal rat serum and L-DMEM culture medium with 100 μg/ml penicillin/streptomycin. Cell growth in the two groups was observed and 3 days later, the cells from the two groups were digested with trypsin and collected and stored at -80 ℃.

Total RNA extraction, RNA labeling, hybridization and microarray chip scanning

1.3.1 Total RNA extraction and quality control

Total RNA was extracted from tissue cells using Trizol Reagent (Invitrogen life technologies) according to the manufacturer's instructions; total RNA was extracted from whole blood using TRI reagent BD (MRCgene, TB-126). Total RNA was extracted from plasma and serum exosomes using TRIzol LS. RNAsey Mini Kit (QIAGEN) was used for RNA purification; NanoDrop ND-1000 was employed for measurement of RNA concentration after purification. RNA integrity was detected by electrophoresis.

1.3.2 RNA labeling and hybridization using the Exiqon method

(1) After extraction, RNA samples underwent quality control and miRNA was labeled using miRCURYTM Array Power Labeling kit (Cat #208032-A, Exiqon). The detailed steps are described as follows:

One microgram RNA sample was added to 2 μL water, and then 1 μL CIP buffer and CIP enzyme (Exiqon) were added to the RNA sample solution, and the mixture solution was incubated at 37 °C for 30 min.

The reaction in the sample solution was terminated at 95 ° C for 5 min, and then 3 μL labeling buffer, 1.5μL fluorescent label (Hy3™), 2.0 μL DMSO, and 2.0 μL labeling enzyme were added and the solution was incubated at 16 °C for 1 h.

The reaction in the samples was terminated at 65° C for 15 min.

(2) After labeling, the sample was hybridized with miRCURYTM LNA Array (v.18.0) (Exiqon), and the subsequent procedures were conducted according to Exiqon's

experimental methods:

25 μL of sample was mixed with 25 μL hybridization buffer and denatured at 95 °C for 2 min and then kept on ice for 2 min.

The sample was hybridized at 56 ° C for 16-20 hr; the hybridization system used was the Nimblegen system (Nimblegen Systems Inc., Addison, WI, USA).

The microarray chip was rinsed with Wash buffer kit (Exiqon) after hybridization.

1.3.3 *The microarray chip was scanned by the Axon GenePix 4000B Microarray Scanner.*

GenePix Pro V6.0 was utilized to read the microarray scan images and the probe signals were selected. The median was taken and combined for the same probes. Probes >=30.0 for the samples were kept and the median was standardized in all microarray chips. Differentially expressed probes were selected. Differentially expressed miRNAs between two samples (no repetition) were chosen using fold change.

1.4 Verification of significantly differentially expressed miR-672-5p and miR-146a-5p by RT-PCR

1.4.1 cDNA synthesis of RNA samples

20 μL of RT reaction mixture was prepared, then all cDNA samples were separately set up in the RT-PCR System (Applied Biosystems); The solution was mixed up to 8 μL and centrifuged at 5000 rpm for a short period.

1.4.2 The mixture was added to each well of a 384-well PCR plate, and then 2 μL corresponding cDNA was added to each well. The plate was sealed with sealing film and centrifuged.The 384-well PCR plate was placed in the RT-PCR amplification instrument to carry out the PCR reaction. U6 (internal reference) and all parameters were conducted 1.4.3 The RT-PCR reaction was performed for the target miRNA in all samples and the internal reference gene (U6) separately.

statistical analysis

Fold change means the ratio of normalized intensities between two conditions (use

normalized data, ratio scale). SPSS 20.0 (IBM) was employed for statistical analysis of the data.RT-PCR data analysis was performed using $2^{-\triangle\triangle CT}$ method.

2. Results

2.1 Results of differential expression of miRNAs in the two groups

Compared with the control group , the number of miRNAs (fold change >2) affected was six. Four miRNAs were upregulated and two miRNAs were downregulated in the steroid group. In particular, the upregulation of miR-672-5p expression and the downregulation of miR-146a-5p expression were significant ,which represents 3.90 and 0.21 in fold change respectively(Table 1).

Table 1. Compared with the control group , the number of differential expression of miRNAs (fold change >2)in steroid group

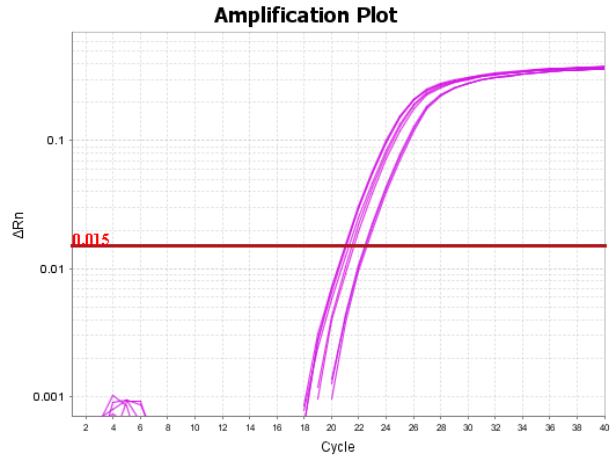
miRNA	Fold change	ForeGround		ForeGround-BackGround		Normalized	
	Steroid group vs control group	control group	Steroid group	control group	Steroid group	Control group	Steroid group
miR-672-5p	3.90495	60.5	84	15.5	34	0.188144	0.163265
miR-3559-3p	2.88429	117	285.5	78	241.5	0.402062	1.159664
miR-98-3p	2.24160	73	196	36.5	153	0.082474	0.734694
miR-92a-1-5p	2.04345	58	86.5	16	38.5	0.079897	0.184874
miR-142-3p	0.36616	309.5	150.5	273.5	107.5	1.409794	0.516206
miR-146a-5p	0.20981	94.5	60	55.5	12.5	0.286082	0.060024

Note: ForeGround: the foreground intensity of each probe. ForeGround-BackGround: the signal of the probe after background correction. Normalized: the normalized ratio of the microRNA. Median Normalization Method was adopted. Normalized Data=(Foreground-Background)/median*

2.2 Results of RT-PCR

Expression of miR-672-5p and miR-146a-5p, which showed the most significant

changes, was verified by RT-PCR. Results of the levels of the two miRNAs in the control group and steroid group showed that the $2^{-\Delta\Delta CT}$ value of miR-672-5p in the steroid group was 3.743 times that of the control group, and the $2^{-\Delta\Delta CT}$ value of miR-146a-5p was 0.322 times that of the control group. Comparison of the results from RT-PCR and the microarray data also indicated that miR-672-5p expression was upregulated and miR-146a-5p expression was downregulated. Amplification and melting curves were obtained(figure 1.2).



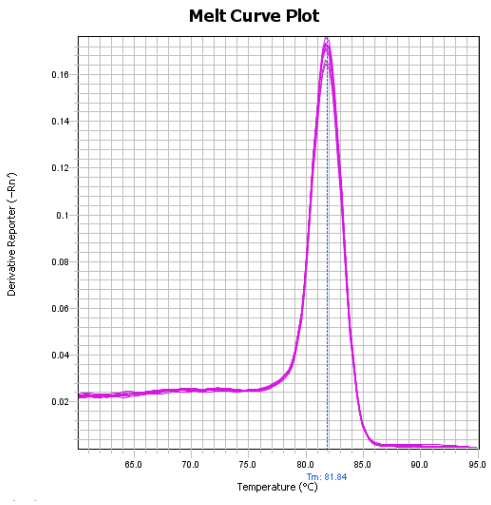


Figure 1.Amplification and melting curves of miR-672-5p.

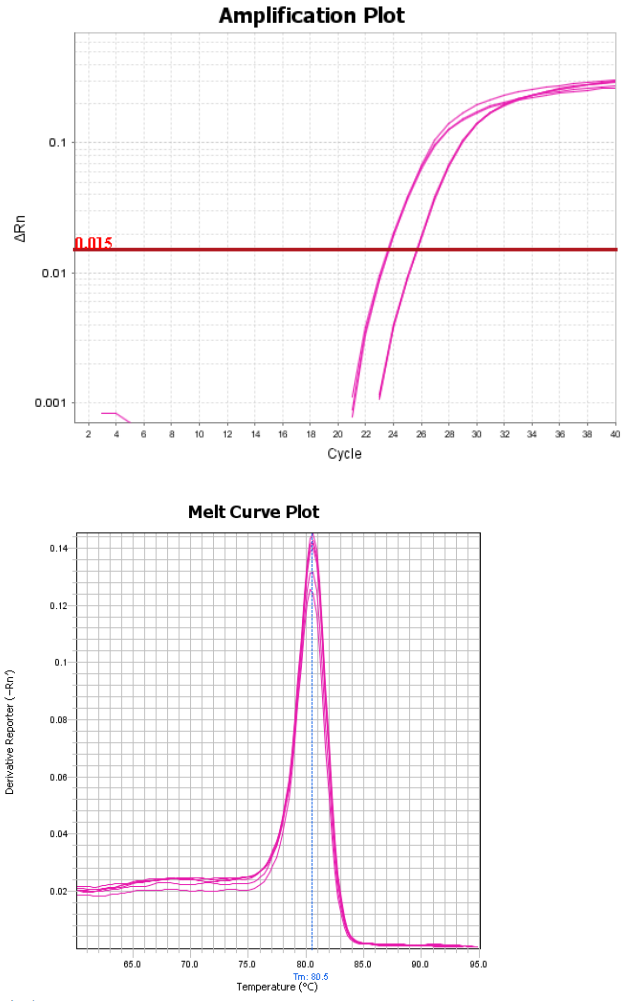


Figure 2.Amplification and melting curves of miR-146a-5p.

2.3 target genes

Miranda，microcosm and mirdb bioinformatics software were used to perdict target genes. Angptl4, Ccdc51, Ssbp3 and RGD1306991 were perdicted as the target gene of miR-672-5p,while Hrp12 was target gene of miR-146a-5p.(figure 3a.3b)

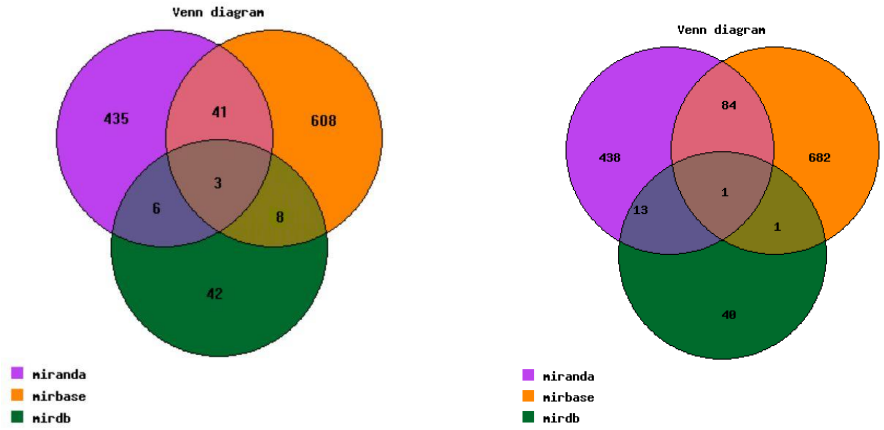


Figure: 3a. Venn diagram of target gene of miR-672-5p; 3b.Venn diagram of target gene of miR-146a-5p.

3 Discussion

In recent times, genetic studies have been progressively more applied in the diagnosis and treatment of steroid osteonecrosis of the femoral head[6, 7]. Many researchers have studied abnormal microRNA expression in local tissues or serum in patients with osteonecrosis of the femoral head[8, 9, 10,11, 12]]and different expressions of microRNA were investigated However, almost no investigations have been conducted to completely understand the mechanisms behind the physiopathology and main pathways of osteonecrosis of the femoral head. Moreover, it has been rat models of osteonecrosis of the femoral head established[13] as well as the protective role of miRNA-29a in a rat bone loss model[14] examined. but there have been no studies performed to detect miRNA expression in the osteoblasts of normal rats or in rat osteoblasts after steroid intervention. Osteoblasts is crucial to bone modeling, which indicates something involved with bone metabolism balance may relate to osteoblasts..Also the functions of osteoblasts have a significant impact on osteonecrosis of the femoral head, attention has been paid to miRNA regulating metabolism of osteoblasts[15.16]. Studies in steroid-induced osteoporosis showed that the steroid may motivate different miRNA through different pathways, thus regulating osteoblast differentiation, lead to osteoblast dyfunction or apoptosis[17.18]. Furthermore, studying miRNA function initially involves application of bioinformatics software in

prediction of target genes, and a single miRNA often has potential to regulate multiple target genes. Meanwhile, multiple miRNAs can simultaneously regulate a single target gene, as well. Investigating differential expression of miRNA in osteoblasts after steroid intervention provides novel experimental ideas for further inquiry into the pathological mechanisms of steroid-induced osteonecrosis of the femoral head.

In the present study, to our knowledge, this is the first time the differential expression of miRNAs in osteoblasts of normal rats and rats with steroid intervention was examined. Compared to normal osteoblasts, certain miRNAs demonstrated higher or lower expression profiles in the osteoblasts of rats that underwent steroid intervention. It is possible that elevated expression of miRNA promotes apoptosis of osteoblasts, while reduced expression of miRNA could inhibit apoptosis.. Lu Y et al found that miR-672-5p was significantly up regulated in GHS(genetic hypercalciuric stone-forming) rats and represented a tendency to lose calcium from the bone[19], which may indicates breakdown of osteoblasts. Dijk et al. theorized that Angpt14 was under sensitive transcriptional control by fatty acids and relevant activated receptors, and its expression was upregulated during differentiation of adipocytes[20], consistent with the pathological changes of necrosis of the femoral head, that is, empty bone lacuna and increased adipocytes.

On the other hand, miR-146 is primarily associated with regulation of inflammation and the functioning of other innate immune systems. It has been reported that miR-146a influences cartilage height in osteoarthritis and may be part of the pathological mechanism behind osteoarthritis. Li et al. reported that downregulation of miR-146a-5p was linked to greater incidences of stomach cancer[21]. Waki et al. described miR-146a-5p being highly expressed in the first 14 days after bone fracture, and then becoming downregulated, related to bone non-union[22]. Additionally, Lu et al. found that upregulation of miR-146a-5p reduced neuropathic pain via inhibiting TRAF6 signaling pathway[23]. Therefore, the hypothesis of the various mechanisms underlying steroid-induced apoptosis of osteoblasts is summarized may as follows:

miR-672-5p was upregulated under hormonal stimulation, regulating the Angpt14 target genes that inhibit angiogenesis and increases the number of adipocytes, disrupting the balance of osteocytes. Downregulation of miR-146a-5p regulates inflammation while inhibiting bone growth, causing pain. The limitation of the present study was that repeated experiments were not performed based on the limited sample size, potentially affecting the accuracy of the results. In the future, although a hypothesis has been proposed, as functional tests were not performed nor were the signaling pathways analyzed, further studies are needed to confirm the pathways and regulation mechanisms of the involved signaling transduction.

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Competing interests

The authors declare that they have no competing interests.

Acknowledgements

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Abbreviation：

miRNAs：Micro RNAs

RT-PCR：real-time polymerase chain reaction

GHS：genetic hypercalciuric stone-forming