Alternations in Mirna Expression in Chronic Stress-Induced Ageing of Leydig Cells

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Abstract

Purpose: Chronic stress represents a critical influence on ageing of Leydig cells in testis. We aimed to investigate expression changes of microRNA (miRNA) associated with stress-induced ageing in Leydig cells.

Methods: Brown Norway rats were separated into three groups, young control group, stress group and age group. Physiological changes, including serum corticosterone and testosterone levels, 11β-hydroxysteroiddehydrogenase (11β-HSD) activity, GSH/GSSG (glutathione/glutathione disulfide) ratio and DNA damage after chronic stress treatment were tested by radioimmunoassay and Immunohistochemistry. Differentially expressed miRNAs in Leydig cells between young control group and stress group were identified by miRNA microarray analysis, followed by target prediction of miRNAs. Functional annotation of predicted targets of miRNA was carried out using GeneSpring software and miRNA-target gene interaction network was built by String software.

Results: Under chronic stress, increased serum corticosterone levels and reduced testosterone levels were observed. Meanwhile, decline of 11β-HSD activity and GSH/GSSG ratio, increase of DNA damage were also shown in stress group. After performed miRNA microarray analysis, three differentially expressed miRNAs were screened out, including mo-miR-31a-5p, mo-miR-192-5p and mo-miR-191a-3p. Targets of them were mainly involved in apoptosis, cell cycle and transport. In miRNA-target gene interaction network, targets of miR-31a-5p (Tp53, Ywhae and Ndel1), and targets of miR-192-5p (Cdk2, Rac2, S6k3) were with higher degree.

Conclusion: These data suggest a central role of miRNA-regulated gene expression in response to chronic stress, especially dys-regulation of mo-miR-31a-5p and mo-miR-192-5p play a vital role in ageing of Leydig cells in testis.

Keywords: Chronic stress; Leydig cell; Ageing; miRNA-target gene

Introduction

Ageing is a complex progress that is still not fully understood. The phenomenon is an aspect of every living organism. Physiological ageing is associated with a progressive decline in cellular functions, an increased risk of major diseases and alteration in gene expression, protein processing and metabolic processes [1]. As evidence indicated in previous studies that accumulation of cellular senescence in some organs or tissues contribute to ageing or several age-related diseases [2,3]. Many different factors have been shown to cause ageing, including lifestyle-related habits, environmental exposures, Reactive Oxygen Species (ROS), and stress. Among the negative factors contributing to aging, the most studied of them is stress [1]. Chronic stress has been widely associated with brain ageing, alterations in neuronal morphology, function and neurogenesis [4]. Epigenetics of ageing has emerged as novel aspect of every living organism. Physiological changes, including serum corticosterone and testosterone levels, 11β-Hydroxysteroiddehydrogenase (11β-HSD) activity, GSH/GSSG (glutathione/glutathione disulfide) ratio and DNA damage after chronic stress treatment were tested by radioimmunoassay and Immunohistochemistry. Differentially expressed miRNAs in Leydig cells between young control group and stress group were identified by miRNA microarray analysis, followed by target prediction of miRNAs. Functional annotation of predicted targets of miRNA was carried out using GeneSpring software and miRNA-target gene interaction network was built by String software.

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Materials and Methods

Animals

Adult male Brown Norway rats, approximately 6 and 21 months old, from the Animal Center of the Chinese Academy of Sciences (Shanghai, China) were used. The rats were habituated to the local environment prior to the start of the experiment and housed under standard vivarium conditions (22 ± 2°C; 12 light/dark cycle, lights on at 9:00 AM with ad libitum access to food and water. Animals care was conducted in accordance with the Institutional Animal Care and Use Committee of School of Medicine Shanghai Jiao Tong University.

Experimental groups

Rats were randomly assigned to one of the following experimental groups: young control group (6-month-old, n=6); chronic stress group (6-month-old, n=6); old group (21-month-old, n=6). The Brown Norway rats in the stress group were subjected to the random chronic unpredictable stress at 9:00 AM each day for 40 consecutive days. But the rats in control and old groups were sampled at the same time-points without stress experiment. The chronic stress exposure was performed as previous described [8].

Briefly, during the experiment, rats in the chronic group were daily subjected to one of the following stressors in an unpredictable order: swimming in ice-cold water (rats were forced to swim in ice-cold water for 5 min), immobilization (rats were restrained in wire restrainers for 3 h), being kept at 4°C (rats were kept in a room at 4°C for 20 min every day) and crowding without food or water (rats were placed into a small cage to simulate crowding without food or water for 24 h). After the last stress exposure rats were asphyxiated using CO2 and then trunk blood was collected immediately. The serum supernatants were sampled by centrifuging at 500 g and stored at -80°C. Testes were rapidly removed.

Isolation and purification of Leydig cells

After testes from rats of three groups, Leydig cells were isolated according to the procedures previously described [9]. In brief, tests were minced and decapsulated with scissors, and then dispersed with collagenase (600 units, Sigma–Aldrich, Oakville, Ontario, Canada) and DNase (750 units, Sigma–Aldrich) in a 50 ml tube that shaken at 60 rpm for 2 h. After 24 h, testes were filtered through a 100-μm pore size nylon mesh to remove seminiferous tubules. The filtrates were transferred into two 50 ml tubes and centrifuged for 10 min at 250 g at 25°C after that supernatant was discarded. Pellets were then resuspended in 35 ml of 55% isotonic Percoll (GE healthcare, UK) with DNase (750 units, Sigma–Aldrich) in 50 ml tube that shaken at 60 cycles/min at 34°C water bath for 15-20 min. When seminiferous tubules were separated, excess medium 199 (Gibco, Carlsbad, California, USA) were added into the tube to terminate enzyme activity.

The suspension was then filtered through a 100-μm pore size nylon mesh to remove seminiferous tubules. The filtrates were transferred into two 50 ml tubes and centrifuged for 10 min at 250 g at 25°C after that supernatant was discarded. Pellets were then resuspended in 35 ml of 55% isotonic Percoll (GE healthcare, UK) with DNase (750 units, Sigma–Aldrich) in 50 ml tube that shaken at 60 cycles/min at 34°C water bath for 15-20 min. When seminiferous tubules were separated, excess medium 199 (Gibco, Carlsbad, California, USA) were added into the tube to terminate enzyme activity.

Physiological testing

Corticosterone and testosterone assay

Rats from young control group and stress group were anaesthetized using CO2 and sacrificed by decapitation, and then trunk blood was collected. Serum supernatants were used to detect hormone levels. Quantiﬁcation of corticosterone and testosterone in serum were performed with corticosterone radioimmunoassay (RIA, ImmuChem Double Antibody Corticosterone 125I RIA Kit for Rats and Mice, MP Biomedicals, Santa Ana, California, USA) Kit and testosterone RIA kit (DSL-4100, Diagnostic Systems Laboratories Deutschland, Sinsheim, Germany). All analyses were carried out according to the supplier’s recommendations.

11β-HSD assay

Immunohistochemistry of 11β-HSD (11β-hydroxysteroid dehydrogenase) was carried out on paraffin-embedded testis slices that subjected to dewaxing process and rehydration in gradient concentrations of ethanol and xylene. Sections were washed with PBS and incubated with anti-11β-HSD antibody (diluted 1:500, Abcam, Cambridge, UK) overnight at 4°C, then incubated with FITC (fluorescein isothiocyanate)-conjugated anti-rabbit IgG (diluted 1:200, Abcam, Cambridge, UK) for 2 h at room temperature. After further washing with PBS, slides were dehydrated stepwise similar as described aforementioned. Randomly chosen spaces bounded by at least 3 seminiferous tubules were evaluated by green fluorescing cytosol with unstained nuclear under Nikon Optiphot-2 microscope (Tokyo, Japan) using X 40 objectives.

GSH/GSSG assay

The GSH/GSSG (glutathione/glutathione disulfide) ratio in Leydig cells was measured using assay kit (371757-1KIT, Merck, New Jersey, USA) according to the supplier’s instructions. The GSH and GSSG levels were assessed respectively to calculate the GSH/GSSG ratio.

Leydig cell counts and number

Rats among three groups were anaesthetized using CO2 and then injected with 0.3% pentobarbital sodium. Next, rats were perfused with 2% glutaraldehyde into the abdominal aorta after which the testes were removed. The Leydig cell numbers were counted according to the procedures described previously [8].

Measurement of DNA damage

Leydig cell DNA damage is estimated by fragmentation that was assessed using single cell gel electrophoresis (Comet) assay. The brief procedure was described by previous study in 2012 [8].

Tissue dissection and RNA extraction

Immediately after collecting the testes, total RNA was extracted from Leydig cells using mirVana™ miRNA Isolation Kit (AM1556, Ambion, Austin, TX, USA) according to the manufacturer's instructions. The extracted total RNA was quantified with NanoDrop ND-2000 (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The quality of total RNA was assessed using Agilent Bioanalyzer 2100(Agilent Technologies, Santa Clara, CA, USA).
Leydig cells from three rats in young (LC-Y1, Y2, Y3) and stress groups (LC-01, 02, 03) were used for miRNA expression analysis. The miRNA microarray analysis was performed by Shanghai OE Biotech Co., Ltd. Total RNA (100 μg) were labeled with miRNA complete Labeling and Hyb Kit (Agilent Technologies) according to the manufacturer's instructions. Aliquots of Cy3-labeled and Cy5-labeled RNA were used for hybridization in a Rat 8’15 k miRNA microarray (Agilent Technologies). Three biological replicates and a color swap for each replicate were analyzed. The hybridized slides were scanned using a DNA microarray scanner G2505C (Agilent Technologies).

Data preprocessing and identification of differentially expressed miRNAs

Signal intensities were preprocessed by Feature Extraction software (version 10.7.1.1, Agilent Technologies). The detected signals were further processed applying the Robust Multiarray Analysis algorithm in GeneSpring software (version 12.5, Agilent Technologies) for background adjustment, quantile normalization and log 2 transformation [10]. Compared with young control group, differentially expressed miRNAs in stress group were filtered based on fold change≥2 and P-value≤0.05 calculating by Student's test.

Principle Component Analysis (PCA) and hierarchical clustering

PCA is an unsupervised clustering and visualization approach for analyzing data from expression array analyses by visualizing microarray data in three dimensions [11]. It displays possible variation in the microarray data with each dimension indicating a component. In the study, the PCA was performed using GeneSpring v12.5 (Agilent Technologies) to all six samples of testes from the control (LC-Y1, Y2, Y3) and stress groups (LC-01, 02, 03). Hierarchical clustering of differentially expressed miRNAs was obtained also using GeneSpring v12.5 (Agilent Technologies).

Target prediction of differentially expressed miRNAs

To search for predicted targets of miRNAs, miRNA.org and TargetScan 6.0 databases were applied.

Statistical analysis

All experiments were carried out with three biological replicates. Data were shown as the mean ± SEM. Error bars represented the standard deviation. Student's t-test was applied to evaluate the differences between experimental groups and identify differentially expressed miRNAs. Fisher's exact test was employed in functional annotation of targets of miRNAs. P<0.05 was considered to be statistically significant.

Results

Serum Corticosterone and Testosterone concentrations

To examine serum hormone levels in rats of young control and stress groups, Corticosterone and Testosterone concentrations were measured by a spectrophotometer. As shown in Figure 1A and 1B, analysis of serum concentration of hormone in control and stress animals showed significant differences after exposure to 40 days chronic stress. Chronic stress induced a significant increase in serum corticosterone levels compared with young control group (P<0.05), which was consistent with the previous indication that excess of corticosterone is the hallmark of stress (Figure 1A) [16]. In addition, an obvious reduction in serum testosterone levels was shown in the stress group compared with control (P<0.05, Figure 1B). In brief, these results indicate that stress model was successfully established.

Effects of chronic stress on 11β-HSD activity

To determine bidirectional 11β-HSD activity in Leydig cells after exposure to chronic stress, 11β-HSD immunohistochemistry was performed on testis sections (Figure 1C). The results showed that the 11β-HSD activity was significantly decreased (P<0.05) when compared with control. It suggests that chronic stress can suppress 11β-HSD activity.

GSH/GSSG ration in Leydig cells

To examine the improvement of the redox imbalance induced by chronic stress, GSH/GSSG ratio was assessed by a spectrophotometer (Figure 1D). After exposure to 40 days stress, Leydig cells showed lower GSH/GSSG than the young control group (P<0.05). It indicates that chronic stress has significant effect on redox unbalance of GSH/GSSG.

DNA damage can be observed in aged cells [17]. In order to examine the effects of chronic stress on DNA damage in Leydig cells, the Comet assay was performed. As shown in Figure 1E, DNA damage in Leydig cells from rats in chronic stress group and aged group were significantly different compared with the young control group. As a result, similar to aging, chronic stress also caused apparent DNA damage in Leydig cells.
miRNA microarray analysis

To examine the expression changes after chronic stress, miRNA microarray analysis was performed using Agilent array. Leydig cells isolated from rats in the young control group and stress group were used for analysis. After normalization, three miRNAs were found to be differentially expressed in stress group compared with the control based on the criteria of |fold change| ≥2 and P-value ≤0.05, including up-regulated rno-miR-191a-3p, rno-miR-31a-5p and down-regulated rno-miR-192-5p.

Hierarchical clustering was used to examine the relationships between young control and alteration under chronic stress in three identified miRNAs (Figure 2A). The distribution of samples was assessed by PCA and shown in Figure 2B.

![Figure 2](image)

**Figure 2:** (A) Principle component analysis for the six samples from young control group (LC-Y1, Y2, Y3) and stress group (LC-01, 02, 03). Each treatment group and replicates are clearly separated. (B). Hierarchical cluster shows changes in the three differentially expressed microRNAs in Leydig cells from young control and stress rats.

These analyses showed that exposure to chronic stress induced significantly different miRNA expression. Similarly, the significant general increase in the miRNA expression was also observed in a nonsymmetrical distribution volcano plot in Figure 3. The plot represented the fold change in miRNA expression plotted against the magnitude of the P value.

![Figure 3](image)

**Figure 3:** Volcano plot shows that significant changes of three miRNAs comparing altered microRNA (miRNA) expression between stress group and young control group. In the plot, X axis represents the log 2 ratio of the normalized miRNA signal, and Y axis represents the –log 10 P value obtained from the Student's t test. Red diamonds represent differentially expressed miRNAs in stress group compared with young control group.

Analysis of predicted miRNA targets

The 2-8 nt at 5’ end region of miRNAs is essential for miRNA function with near perfect complementary binding to target genes. To find miRNA target mRNA, 3'UTR of predicted targets was screened for miRNA binding sites using microRNA.org and TargetScan prediction algorithms. The overlapping genes between the two databases were identified as predicted targets of three differentially expressed miRNAs. As shown in 335 genes were predicted to be targeted by rno-miR-31a-5p and rno-miR-192-5p which were further analyzed. However, targets of rno-miR-191a-3p were not predicted in the two databases.

Functional annotation of differentially expressed miRNAs

To describe the possible role of the changes in three miRNAs, 335 target genes of them were analyzed using Gene Spring software. GO analysis provided a dynamic and controlled vocabulary for the representation of information on biological process, cellular component and molecular function, allowing a coherent annotation of miRNA targets. For biological processes, genes involved in ion transport, cell adhesion and apoptosis were highly represented. For cellular component, cytoplasm was the most represented GO term, followed by nucleus and membrane. Regarding molecular functions, the most represented categories were protein binding and nucleotide binding. Besides GO analysis, KEGG pathway mapping was also carried out for miRNA targets. As shown in miRNA targets were significantly enriched in Leukocyte transendothelial migration, Rheumatoid arthritis and Protein digestion and absorption.

Construction of miRNA-target gene interaction network

Considering the negative regulation of target genes by differentially expressed miRNAs, we built the miRNA-target gene interaction network to study the role of miRNA in ageing underlying chronic...
stress (Figure 4). In the network, there were 81 targets of miRNAs and 73 interactions between target genes. Target genes of rno-miR-31a-5p included Ywhae (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon), Tp53 (tumor protein p53) and Ndel1 (nudE neurodevelopment protein 1-like 1) that with degree more than 5. Targets of rno-miR-192-5p consisted of Cdk2 (cyclin-dependent kinase 2), Rac2 (ras-related C3 botulinum toxin substrate 2), Mapk14 (mitogen-activated protein kinase 14) and Stk3 (serine/threonine kinase 3) that degree more than 4.

![Figure 4: MicroRNA (miRNA)-target gene interaction network of differentially expressed miRNA and predicted targets. Circular nodes represent target genes of miRNA. Bright blue circles represent transcription factors regulated by miRNAs. Orange diamonds represent differentially expressed miRNAs. The size of nodes is proportional to the degree of nodes.](image)

**Discussion and Conclusion**

The present study revealed that the expression of three miRNAs (rno-miR-31a-5p, rno-miR-192-5p, rno-miR-191a-3p) was significantly differential after 40 days of chronic unpredictable stress treatment. Furthermore, target genes of miRNAs were mainly involved in GO terms of apoptosis, cell cycle, transport and GTP-associated functions. Especially, Ywhae, Cdk2, Tp53, Ndel1, Rac2, Mapk14 and Stk3 were with higher degree in miRNA-target gene interaction network.

**Physiological changes under chronic stress**

Chronic unpredictable stress in this study caused obviously elevated serum corticosterone levels and decreased testosterone levels between young control group and stress group, which are in line with previously reported results [8]. Notably, reduced serum levels of testosterone are a characterization of testicular ageing [18]. Meanwhile, the glucocorticoids are essential for supporting testosterone synthesis and regulating testosterone secretion in the Leydig cells [19]. However, there was no significant difference of Leydig cell numbers between two groups.

These findings suggest that chronic stress could induce age-related decline in the synthesis of testosterone in Leydig cell numbers that contributing to ageing of testis. The present experiments also revealed reduced 11β-HSD activity and GSH/GSSG ratio between stress group and young control. As we all know, excessive corticosterones have an effect on steroidogenic enzymes in the testosterone biosynthesis [20]. Thus, 11β-HSD activity plays a vital dual role in inactivating normal levels of corticosterones and meanwhile, promoting testosterone synthesis. When levels of corticosterones arise above the normal level, the capacity of to inactivate corticosterones would be saturated and activity would be reduce, which are consistent with previous data [21].

Moreover, chronic stress can induce ROS production in Leydig cells that causes unrepaired damage to macromolecules. Under oxidative conditions, GSH molecules form GSSG by donating one electron. A low GSH and/or high GSSG concentration and low GSH/GSSG ratio are well-established characterization of the ageing process [22]. Thus, ROS may be an influential factor to reduced GSH/GSSG ratio. Furthermore, subsequently increased DNA damage in Leydig cells induced by ROS also was observed. The increased ROS by chronic stress induces damage to the seminiferous tubules by induction of DNA damage and formation of protein adducts that contribute to decline in male fertility with ageing [1]. Therefore, the results indicate that chronic stress can stimulate increased production of ROS and DNA damage in Leydig cells which are believed to be involved in ageing.

**Chronic stress causes changes in miRNA expression in Leydig cells**

The role of miRNA expression in ageing Leydig cells was explored in rat testis, as a paradigm for the study of ageing-related process. We observed a significant increase in expression of rno-miR-31a-5p and rno-miR-191a-3p, decline in expression of rno-miR-192-5p in Leydig cell miRNA microarray. Differentially expressed rno-miR-31a-5p was down-regulated in Leydig cells under chronic stress. Previous study has shown that the miRNA is dis-regulated in Hepatocellular carcinoma [23]. From the functional annotation results, predicted targets of rno-miR-31a-5p (Tp53, Ywhae, Ndel1) were mainly involved in GO terms of apoptosis and cell migration. It is well known that Tp53 is a tumor suppressor gene and independently regulates apoptosis and cell-cycle arrest [24]. Interestingly, p53 is also considered to promote ageing under some circumstances [25]. Moreover, predicted targets of miR-31a-5p also included Ywhae, and it has been found to be dysregulated in several tumors [26,27]. Thus, up-regulation of miR-31a-5p is believed to contribute to cancer growth by targeting apoptosis-related genes. Increased resistance to apoptosis is a well-established feature of ageing cells [28]. Specially, cancer is a major age-related disease in humans, mice and many other mammals [29]. Therefore, the over-expressed rno-miR-31a-5p observed in Leydig cells could be interpreted as a mechanism that contributes to the prevention of apoptosis in senescent cells. Down-regulated miR-192-5p has been found to be involved in ageing in mouse [30].

Predicted targets of miR-192-5p (Cdk2, Stk3, Rac2, Mapk14) were significantly enriched in apoptosis, cell cycle and GTP-related functions. Cdk2 is known as a member of the cyclin-dependent kinase family of Ser/Thr protein kinases that is essential for the G1/S transition and cell cycle. Notably, selective inhibition of Cdk2 would induce Myc/Ras expressing cells into cellular senescence. As a result, highlighting Cdk2 as a potential target for treatment of ageing-related disease and tumors driven by Myc or Ras [31]. Moreover, another predicted target gene of miR-192-5p was Rac2 which is essential for reconstituted oxidase activity and Rac-dependent NADPH oxidase activation. Additionally, Rac2 is also relevant to the mechanisms of Rac-mediated ROS production that plays an important role in ageing [32].
Therefore, results speculate that rno-miR-192-5p may be essential for ageing of Leydig cells under chronic stress through regulating cell cycle and ROS response by targeting Cdk2 and Rac2. In summary, we suggest roles for differentially expressed rno-miR-31a-5p and rno-miR-192-5p in ageing of Leydig cells after chronic unpredictable stress. The proposed functional attributions for the two miRNAs are based on correlations of predicted target genes that show altered expression in Leydig cells. Although there is still relatively little information about the roles of the two miRNAs, the up-regulation of rno-miR-31a-5p likely contributes to some of the characteristics of ageing cells, such as the increased resistance to apoptosis by targeting tumor suppressor Tp53. Similarly, down-regulated rno-miR-192-5p may contribute to ROS response or function as a cofactor in cellular senescence by targeting Cdk2 and Rac2. Given the extent of the observed changes in miRNA expression, we provide additional evidence that miRNA might play a crucial role in ageing of Leydig cells under chronic stress. Finally, it remains to be verified by experiments in future studies.

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