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Original Article

A comprehensive expression profile of micrornas in rat's pituitary

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Abstract: MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules that act as a negative regulator of most mRNAs. miRNAs influence the gene expression as transcriptional regulators and play an important role in many fundamental biological processes. It is generally acknowledged that miRNAs have a very important affection on mammalian pituitary. However, the answers of which role miRNAs play in the development of sexual function or how much they contribute to the pituitary function are not exactly. In our study, we used three female 21-day-old rats and three female 12-month-old rats to analysis the function of miRNAs. By the analyses of microarray data, we finished the stem-loop real-time RT-PCR for the differentially expressed miRNAs. We detected a total of 93 differentially expressed miRNAs between 21-day-old rats' pituitary and 12-month-old rats'. Stem-loop real-time RT-PCR suggests that the obtained data is of high credibility. Among these miRNAs, 7 miRNAs' expression (rno-miR-880, rno-miR-503, rno-miR-125a-3p, rno-miR-3596b, rno-miR-30e, rno-miR-214 and rno-miR-22) are significant different ($P \leq 0.05$). In a word, this study identified a number of specific changes in the expression of miRNAs, in rats by detecting the expression profile of miRNAs in rat's pituitary, and all of that lay the foundation for elucidating the regulatory mechanisms of miRNAs in rat's reproduction process. These differentially expressed miRNAs may play a very important role in rat's reproduction process.

Keywords: MiRNAs, rat pituitary, reproduction

Introduction

MiRNAs are non-protein-coding small RNAs, 19-23 nucleotides in length, which are implicated in the posttranscriptional fine tuning of gene regulation [1]. By base pairing with the 3' untranslated region (3' UTR) of their target mRNA, miRNAs results in repression of the target genes expression or degradation of target genes [2-4]. Since the original discovery in nematodes [5], studies have revealed that miRNAs have key roles in diverse processes such as developmental control, hematopoietic cell differentiation, neural development, apoptosis, cell proliferation and organ development [6]. Recent studies indicated that miRNAs play a direct role in apoptosis of bovine corpus luteum [7], which means miRNAs are involved in the reproductive process.

Since the publication of the Rat Genome Sequence [8], rat is more and more important in scientific research. In the year of 2007, some researchers found rats had possessed metacognition and psychological ability previously only documented in humans and some primates [9, 10]. From then on, there are many studies about rat's brain and intelligence, but only a little about its pituitary.

The pituitary, as the most complex internal secretion gland of the mammal, releases seven kinds of hormones to play a part in the whole life course. Thus profiling pituitary miRNAs may enable us to elucidate not only how miRNAs are involved in regulating the development and function of the organ but also how miRNAs are involved in regulating the development of the individual or species characteristics of an animal [11]. However, there only are a few experiments stepped into the regular pattern of pituitary gland.

Drawing the support from microarray, target Combo and DAVID gene annotation tool, we studied the discipline of miRNAs in rat's pituitary.

Table 1. Group 1 vs Group 2 1.5 fold up regulated miRNAs

Name	Fold change	*P*-value	ForeGround-BackGround	
	Group 1 vs Group 2	Group 1 vs Group 2	Mean of Group 2	Mean of Group 1
Rno-miR-349	2.4485	0.2028	323.1667	809.5000
Rno-miR-218b	1.9746	0.2333	336.8333	1053.5000
Rno-miR-409-3p	1.7240	0.4844	41.8333	125.5000
Rno-miR-672*	4.5245	0.3571	-3.1667	126.1667
Rno-miR-743b	1.6896	0.1930	32.1667	53.3333
Rno-miR-183	1.6504	0.1842	484.0000	493.1667
Rno-miR-431	1.6594	0.4630	2517.1667	4631.3333
Rno-miR-3597-5p	2.1797	0.1497	121.3333	267.0000
Rno-miR-2964	5.7149	0.2536	-52.6667	72.1667
Rno-miR-494*	21.6344	0.3779	-27.5000	121.6667
Rno-miR-3592	2.0292	0.1096	544.5000	1086.8333
Rno-miR-3591	3.2684	0.4549	14.3333	254.6667
Rno-miR-880	2.6260	0.0067	215.6667	572.8333
Rno-miR-351	1.8834	0.1813	198.3333	366.5000
Rno-miR-449c-3p	2.4601	0.3121	409.6667	1109.0000
Rno-miR-874	1.9655	0.3151	53.1667	110.3333
Rno-miR-211	1.5107	0.3192	146.3333	231.5000
Rno-miR-466b-2*	1.7161	0.5069	117.0000	199.1667
Rno-miR-340-3p	1.9902	0.3923	1914.8333	4210.6667
Rno-miR-199a-3p	1.5757	0.4259	123.3333	188.8333
Rno-miR-500	1.9307	0.4332	-6.8333	30.3333
Rno-miR-221	1.6870	0.3220	175.1667	286.0000
Rno-miR-3563-5p	1.7707	0.5996	-7.8333	35.1667
Rno-miR-433	1.6346	0.0770	659.6667	1069.1667
Rno-miR-107	1.5089	0.2541	304.1667	455.0000
Rno-miR-138	1.5057	0.2273	234.0000	345.0000
Rno-miR-377*	1.6294	0.5047	84.6667	154.5000
Rno-miR-192	1.5644	0.4018	88.3333	221.0000
Rno-miR-511*	2.7856	0.2952	-11.1667	148.5000
Rno-miR-3597-3p	1.6560	0.3463	734.6667	1180.0000
Rno-miR-204	1.7792	0.3125	133.3333	245.0000
Rno-miR-186	1.7421	0.1911	130.6667	227.0000
Rno-miR-219-2-3p	1.8049	0.2362	250.0000	435.1667
Rno-miR-503	2.6714	0.0160	70.8333	385.5000
Rno-miR-770*	1.8442	0.1563	211.3333	381.0000
Rno-miR-3581	1.5845	0.3631	-23.0000	36.5000
Rno-miR-136*	2.4591	0.1559	1075.5000	2686.3333
Rno-miR-675*	1.6565	0.5534	110.1667	376.5000
Rno-miR-125a-3p	2.2408	0.0131	153.0000	346.1667
Rno-miR-505*	2.5868	0.1550	141.0000	357.1667
Rno-miR-206	2.0189	0.2072	103.5000	328.5000
Rno-miR-331*	1.6290	0.7458	16.5000	74.0000
Rno-miR-18a*	1.5098	0.5932	-16.1667	40.0000
Rno-miR-3596a	1.9819	0.2679	491.3333	979.8333
Rno-miR-3544	1.9393	0.3228	276.1667	712.3333
Rno-miR-30c-1*	2.3394	0.6563	-14.8333	107.0000

Methods

Tissue collection and RNA extraction

Euthanasia was performed by decapitation following anesthetic injection (chloraldurate, 10%), and pituitary glands from three female 21-day-old rats and three female 12-month-old rats (Wistar) were rapidly dissected, and store in liquid nitrogen. Total RNA was isolated by TRIzol according to the explanatory memorandum of manufacturer. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Jilin University.

Ethics statement

We strictly abided the provisions of laboratory animal center of Jilin University. All animal procedures were conducted following the protocol (2011-036) approved by the Animal Care & Welfare Committee of Jilin University.

The detection of microarray assay

The miRCURY™ Hy3™/Hy5™ Power labeling kit (Exiqon, Vedbaek, Denmark) was used according to the manufacturer's guideline for miRNA labelling. One microgram of each sample was 3'-end-labeled with Hy3TM fluorescent label by using T4 RNA ligase provided in the kit as described following. The RNA mixture (3 μL) with 1.0 μL of CIP Buffer (Exi-

Rno-miR-199a-5p	1.5305	0.5148	46.1667	115.6667
Rno-miR-34b*	1.9660	0.0945	5347.3333	11030.1667
Rno-miR-330*	1.7412	0.1497	206.3333	350.1667
Rno-miR-133b	1.5794	0.2379	207.3333	348.0000
Rno-miR-122*	2.4618	0.4716	35.3333	379.1667
Rno-miR-466d	5.3503	0.2543	3.5000	125.1667
Rno-miR-100	1.7910	0.2371	131.3333	245.3333
Rno-miR-382*	1.6467	0.3734	196.0000	308.6667
Rno-miR-154	1.6600	0.0859	254.8333	443.5000
Rno-miR-3596b	2.3654	0.0479	4769.8333	11446.3333

Table 2. Group 1 vs Group 2 1.5 fold down regulated miRNAs

Name	Fold change	*P*-value	ForeGround-BackGround	
	Group 1 vs Group 2	Group 1 vs Group 2	Mean of Group 2	Mean of Group 1
Rno-miR-200b	0.3483	0.1571	177.5000	69.1667
Rno-miR-30e	0.4015	0.0291	350.1667	142.6667
Rno-miR-665	0.4601	0.6049	24.3333	8.1667
Rno-miR-330	0.2593	0.4208	506.6667	134.6667
Rno-miR-466c*	0.5133	0.5372	1263.6667	634.0000
Rno-miR-29a	0.3096	0.1740	3679.0000	1167.8333
Rno-miR-425	0.4685	0.4351	660.5000	291.8333
Rno-miR-125b-5p	0.5454	0.2492	2974.1667	1652.3333
Rno-miR-540	0.5276	0.2840	25.3333	25.6667
Rno-miR-434	0.4180	0.3902	2510.6667	1071.3333
Rno-miR-352	0.3318	0.3136	335.8333	110.1667
Rno-miR-181a	0.3047	0.4014	1490.0000	457.3333
Rno-miR-30a	0.2803	0.3533	2771.3333	793.1667
Rno-miR-214*	0.1826	0.0257	7.6667	3.8333
Rno-miR-101a	0.2092	0.1388	1368.8333	285.8333
Rno-miR-23a	0.4345	0.1547	202.0000	84.5000
Rno-miR-34c	0.5321	0.3468	542.5000	287.1667
Rno-miR-341	0.4803	0.3416	971.3333	492.0000
Rno-miR-3583-5p	0.6639	0.3414	843.8333	591.6667
Rno-miR-335	0.0466	0.1690	1906.6667	46.5000
Rno-miR-135a	0.1962	0.2150	293.1667	25.3333
Rno-miR-329*	0.6218	0.5528	191.1667	117.3333
Rno-miR-144	0.5820	0.4766	306.3333	173.3333
Rno-miR-200c	0.3558	0.1318	1732.3333	614.5000
Rno-miR-551b*	0.2037	0.1200	1108.1667	219.0000
Rno-miR-296	0.5130	0.4816	80.1667	81.6667
Rno-miR-22	0.4079	0.1896	4353.6667	1814.3333
Rno-miR-127*	0.4520	0.0511	161.6667	71.3333
Rno-miR-375	0.3724	0.0913	938.1667	348.1667
Rno-miR-7a	0.5096	0.2571	14681.1667	7672.8333
Rno-miR-16	0.3363	0.1206	697.0000	232.8333
Rno-miR-141	0.2726	0.2616	2722.6667	455.6667
Rno-miR-22*	0.4228	0.0253	46.8333	43.6667
Rno-miR-339-5p	0.6450	0.5270	314.5000	212.5000

qon) was incubated for 30min at 37°C, terminated by 95°C for 5 min. Then 3.0 μL labeling buffer, 1.5 μL fluorescent label (Hy3TM), 2.0 μL DMSO and 2.0 μL labeling enzyme were added into the mixture. The labeling reaction was incubated for 1 h at 16°C, and terminated by 65°C for 15 min.

The Hy3TM-labeled samples were hybridized on the miRCURYTM LNA Array (v.16.0) (Exiqon) according to array manual after the termination of the labeling step. All the mixture from Hy3TM-labeled samples mixed with 25 μL hybridization buffer were denatured at 95°C for 2 min, incubated on ice for 2 min and then hybridized to the microarray for 16-20 h at 56°C in a 12-Bay Hybridization Systems (Hybridization System-Nimblegen Systems, Inc., Madison, WI, USA), which provides an active mixing action and constant incubation temperature to improve hybridization uniformity and enhance signal. Following hybridization, the slides were achieved, washed several times with Wash buffer kit (Exiqon), and finally dried by centrifugation for 5 min at 400 rpm. Then the slides were scanned by using the Axon GenePix 4000 B microarray scanner (Axon Instruments, Foster City, CA).

Scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicates were averaged and miRNAs that intensities ≥50 in all samples were chosen for calculating normalization factor. Expressed data were normalized using the median

Rno-miR-30b-5p	0.5224	0.1993	777.8333	426.3333
Rno-miR-374	0.3231	0.1900	355.5000	123.0000
Rno-miR-324-5p	0.5221	0.1374	313.1667	163.1667
Rno-miR-551b	0.0159	0.1570	227.1667	-4.3333

Table 3. Group 1 vs Group 2 1.5 fold up regulated miRNAs

Name	Fold change	*P*-value	ForeGround-BackGround	
	Group 1 vs Group 2	Group 1 vs Group 2	Mean of Group 2	Mean of Group 1
Rno-miR-880	2.6260	0.0067	215.6667	572.8333
Rno-miR-503	2.6714	0.0160	70.8333	385.5000
Rno-miR-125a-3p	2.2408	0.0131	153.0000	346.1667
Rno-miR-3596b	2.3654	0.0479	4769.8333	11446.3333

Table 4. Group 1 vs Group 2 1.5 fold down regulated miRNAs

Name	Fold change	*P*-value	ForeGround-BackGround	
	Group 1 vs Group 2	Group 1 vs Group 2	Mean of Group 2	Mean of Group 1
Rno-miR-30e	0.4015	0.0291	350.1667	142.6667
Rno-miR-214*	0.1826	0.0257	7.6667	3.8333
Rno-miR-22*	0.4228	0.0253	46.8333	43.6667

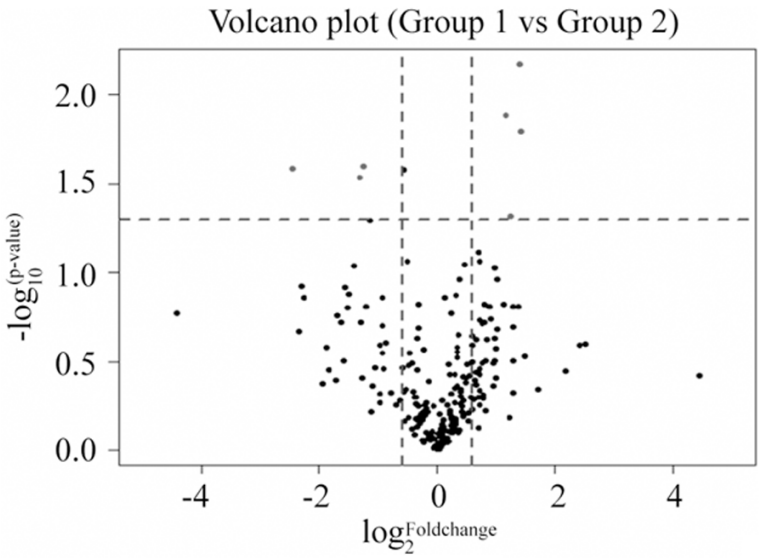

Figure 1. The vertical lines correspond to 1.5-fold up and down, respectively, and the horizontal line represents a *P*-value of 0.05. So the red point in the plot represents the differentially expressed genes with statistically significance. The red refer to the significant differently expressed miRNAs.

normalization. After normalization, differentially expressed miRNAs were identified through Volcano Plot filtering. Hierarchical clustering was performed using MEV software (v4.6, TIGR).

Stem-loop real-time RT-PCR

Total RNA was extracted as the description above. The primers used for Real-time PCR were provided by Jiusheng Corp (Shanghai, China). Our Stem-loop real-time RT-PCR steps followed the previously described [12].

Complementary DNA was reverse transcribed from 1 μg of total RNA mixing with with 1 μg miRNA-specific RT-primers (Jiusheng Corp, Shanghai, China) by incubating 50 min at 42°C and deactivating 15 min at 75°C. Power SYBR green PCR mix was used for the amplification mixture with each primer 1.5 μl and 1 μl cDNA for a total reaction volume [13] of 20 μl. PCR reactions were performed on STRATAGENE Mx3005P sequence detection system as previously described. Briefly, samples underwent amplification by denaturation at 95 C for 1 min, and then cycled 40 times using 95 C for 15 sec, 56 C for 15 sec and 72 C for 40 sec. All reactions were run in triplicate [14]. Last, we recorded the cycle threshold (Ct) for analysis with U6 RNA.

Results

The regular expressions of miRNAs in rat's pituitary detected by microarray assay

We entrusted the microarray analysis with Kangchen Biotech. The 6th generation of miRNA array contains more than 1891 capture probes, covering all human, mouse and rat microRNAs annotated in miRBase 16.0, as well as all viral microRNAs related to these species. In addition, this array contains capture probes for 66 new miRPlus™ human microRNAs. These are proprietary microRNAs not found in miR-

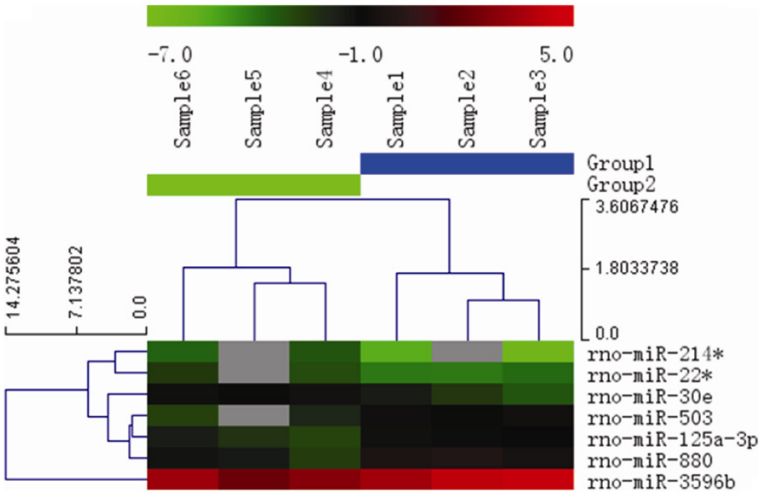

Figure 2. Each row represents a miRNA and each column represents a sample. The miRNA clustering tree is shown on the left, and the sample clustering tree appears at the top. The color scale shown at the top illustrates the relative expression level of a miRNA in the certain slide: red color represents a high relative expression level; green color represents a low relative expression levels.

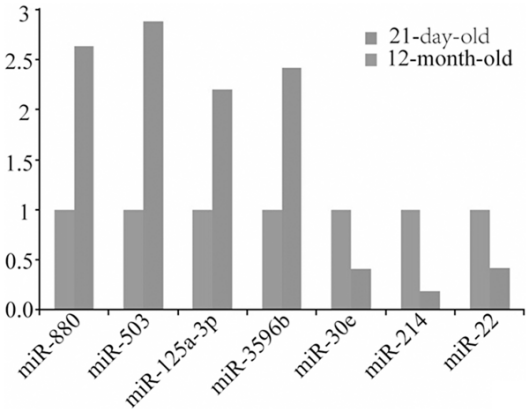

Figure 3. To verify and evaluate the reliability of the results from the microarray data, we selected 7 differentially expressed miRNAs for stem-loop real-time PCR assay. All the miRNAs were successfully detected by the real-time PCR, suggesting that the miRNAs identified by our microarray analysis were reliable for their existence. The expression levels determined by real-time PCR assay were quite consistent with those determined by microarray analysis.

Base. By the analysis of microarray data, we detected a total of 93 differentially expressed miRNAs between 21-day-old rats and 12-month-old rats (**Tables 1** and **2**). Among these miRNAs, 7 miRNAs' with a fold change >1.5 and a p-test value <0.01 are significant different (**Tables 3** and **4**). These 7 miRNAs should be the main regulators between the 21-day-old rats and the 12-month-old rats. Then we used Volcano Plot and Heat Map and Hierarchical Clustering for further testing. The results are as follows (**Figures 1**, **2**).

Real-time RT- PCR for miRNA microarray validation

In order to confirm the correctness and the reliability of the microarray data, we finished the Stem-loop real-time RT-PCR for the 7 miRNAs. The result of real-time RT-PCR suggested that our microarray data are reliable (**Figure 3**).

Discussion

MiRNAs are small, approximately 22 nucleotide (nt), single-stranded non-coding RNA molecules. MiRNAs were first discovered in the nematode Caenorhabditis elegans in 1993 by Lee et al. [5] MiRNAs have been identified in a wide range of species, including bacteria, plants, animals, and even viruses [15, 16]. MiRNAs have been shown to play an important role in regulating many fundamental biological processes, including cell proliferation, differentiation, apoptosis, cell adhesion, metabolism, cell migration, neurogenesis, stress resistance, and hemopoiesis [17].

Under normal circumstances, the mature miRNAs are involved in maintaining normal cell homeostasis by regulating the translation or stability of target gene mRNAs. Therefore, the abnormal expression of miRNAs may lead to the abnormal expression at the level of the corresponding target gene transcript [18]. Currently, the main mechanism of miRNAs is that miRNAs complementarily combine with the 3'UTR of the target gene by RNA-induced Gene Silencing Complexes (RISCs) [19]. MiRNAs perform the function that estimate regulate 10-30% of all protein coding genes in two ways [20]. The first mode is operative in plants, where in miRNAs bind to perfectly complementary base pairs on the target mRNA, thus inducing its cleavage [21, 22]. The alternative and more common method involves the imperfect binding of miRNAs to partially complementary

sites on the 3' UTR of target mRNA leading to some degree of mRNA degradation and inhibition of protein translation [23].

In recent years, some scholars using target scan method predicted 400 miRNAs target genes, which are related to the mammalian development of 13 percent. And a plurality of experiments has shown that miRNAs play an important role in mammalian development. However, the studies of which role miRNAs play in the development of sexual function or how much they contribute to the pituitary function are not sufficient. This study detected the expressions of relevant miRNAs in rat's pituitary by gene-chip. By the analysis of microarray data, it detected a total of 93 differentially expressed miRNAs between 21-day-old rats and 12-month-old rats, including 56 up regulated miRNAs and 38 down regulated miRNAs. These 7 miRNAs should be the main regulators between the 21-day-old rats and the 12-month-old rats. These miRNAs may play an important regulating role in the function of rat's pituitary. And then this study utilized Target Combo to predicting the 7 miRNAs' target genes. It is possible to work through these genes in the development of rat sexual function, and the mechanism will be elucidated by further experiments. In a word, this study identified a number of specific changes in the expression of miRNAs in rats by detecting the expression profile of miRNAs in Rat's Pituitary. It was also preliminarily validated by PT-PCR, and all of that lay the foundation for elucidating the regulatory mechanisms of miRNAs in rat's reproduction process.

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Disclosure of conflict of interest

None.

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