Original Article

MiR-200a is involved in rat epididymal development by targeting *β-catenin* mRNA

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The expression of 350 microRNAs (miRNAs) in epididymis of rat from postnatal development to adult (from postnatal days 7–70) was profiled with home-made miRNA microarray. Among them, 48 miRNAs changed significantly, in which the expression of miR-200a increased obviously with time, in a good agreement with that obtained from northern blot analysis. The real-time quantitative-polymerase chain reaction result indicated that temporal expression of rat *β-catenin* was exactly inversed to that of miR-200a during rat epididymal development, implying that miR-200a might also target *β-catenin* mRNA in rat epididymis as reported by Saydam *et al*. in humans. The bioinformatic analysis indicated that 3′ untranslated region of rat *β-catenin* mRNA did contain a putative binding site for miR-200a. Meanwhile, it was found that the sequence of this binding site was different from that of human *β-catenin* mRNA with a deletion of two adjacent nucleotides (U and C). But the results of luciferase targeting assay in HEK 293T cells and the over-expression of miR-200a in rat NRK cells demonstrated that miR-200a did target rat *β-catenin* mRNA and cause the suppression of its expression. All these results show that miR-200a should be involved in rat epididymal development by targeting *β-catenin* mRNA of rat and suppressing its expression.

Keywords   miRNA microarray; template switching; miR-200a; β-catenin; rat epididymis

Introduction

The epididymis, a long convoluted tubule, is a highly ordered and segmented organ that performs a variety of functions including sperm protection, maturation, concentration, and storage. This convoluted tubule consists of a pseudostratified epithelium of several cell types including principal, narrow, clear, halo, basal, and apical cells at rat adult epididymis [1]. But the epididymal epithelium is only characterized by the presence of low columnar cells in postnatal day 7 (P7) old rats. The epididymal epithelium does not show differentiation at P7, while halo cells and narrow cells emerge from P14 and P15, respectively, principal cells and basal cells from P28, and clear cell from P35. The epididymal epitheliums are fully differentiated by P49, followed by the expansion phase of epididymis at P70. The most significant changes observed during this period are the appearance of spermatozoa within the epididymal lumen as well as an increase in the size of the epididymis due to both an increase in organ length and weight [1–3]. This suggests that the epididymal epithelium undergoes a series of cell differentiation that happen from P14 to adult. However, this highly regulated event during the development of the epididymis is not yet completely understood despite considerable efforts. Thus, it is an important significance to explore the molecule mechanism of gene regulation during rat developing epididymis to elucidate the development and function of epididymis.

MicroRNAs (miRNAs) are a family of 18–25-nucleotide, endogenous, non-coding small RNAs that negatively regulate the expression of target mRNAs through incomplete base pairing with the 3′ untranslated region (3′UTR) of mRNA at the post-transcriptional level [4,5]. Over the last decade, many evidences have indicated that miRNAs play important regulatory roles in embryonic development, cell differentiation, cell apoptosis, and the pathogenesis of diverse diseases such as cancer, diabetes, and cardiomyopathy [6–11]. However, as a group of critical regulators of development and physiology, few researches focus on the roles of miRNAs in regulating the mammalian epididymal development. Given the important effect of miRNAs on development and differentiation, it is important to compare the expression profiles of miRNAs in postnatal developing epididymis. MiRNA microarray offers a high throughput tool to facilitate the studies of the biological roles of miRNAs.

In this study, 350 miRNAs expressed in the rat epididymis from P7 to P70, which was a vital period for epididymal differentiation, were profiled with our home-made

miRNA microarray. It was found that 48 miRNAs changed significantly, in which the time dependence of miR-200a expression is different from miR-200c reported in our previous paper [12]. Previous studies reported that miR-200a inhibited epithelial-to-mesenchymal transition (EMT), an initial step in tumorigenesis, by directly reducing the expressions of *ZEB1* and *ZEB2*, which negatively regulated the expression of *E-cadherin* [13–15]. Meanwhile, Saydam *et al*. [16] reported that miR-200a targeted human *β-catenin* mRNA and suppressed its expression. All these results suggested that miR-200a might be involved in the epididymal differentiation and be worthy of further study in details. Here, we validated the role of rat *β-catenin* as target gene of miR-200a by luciferase targeting assay and the overexpression of miR-200a, although a putative binding site for miR-200a in 3′UTR of rat *β-catenin* mRNA had a deletion mutation of two adjacent nucleotides (U and C) comparing with the orthologous sequence of human *β-catenin* mRNA.

Materials and Methods

Animals and cell lines

Sprague Dawley rats at P7 ($n = 20$), P21 ($n = 8$), P35 ($n = 4$), P49 ($n = 3$), and P70 ($n = 3$) old age were purchased from Shanghai Laboratory Animal Co. Ltd (SLAC, Shanghai, China). HEK 293T cells and rat NRK cells (obtained from Shanghai Cell Resource Center, Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (Gibco, Gaithersburg, USA) at 37°C in 5% CO_2.

Enriching of the low-molecular weight RNAs

To obtain epididymal total RNAs at different developmental stages, whole epididymides from 3–20 male rats at P7, P21, P35, P49, and P70 were pooled, respectively, and homogenized in Trizol (Invitrogen, Carlsbad, USA). Total RNAs were measured on the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Total RNAs (20 μg) of epididymal tissues were separated on 15% acrylamide/8 M urea denaturing gel until the faster dye moves to the middle of gel and then 15–30 nt (nucleotide) RNAs were recovered.

Cloning and labeling of miRNAs

The cloning of miRNAs of rat epididymis was obtained with a smart switch method. The products diluted with ddH$_2$O by 1:5 are amplified by polymerase chain reaction (PCR) for 30 cycles (30 s at 94°C, 20 s at 60°C, and 10 s at 72°C) with Ex TaqHS DNA polymerase (TaKaRa, Dalian, China), the 3′ primer (P9, 5′-GCAGATCGTCAG AATTCCAG-3′) and the 5′ primer (P11, 5′-Cy5-ACCG AATTCACAGTCAGACC-3′). The resulting amplicons were separated by electrophoresis on an agarose gel to ensure the right size of the amplicons, and then cloned into pGEM-T Easy vector (Promega, Madison, USA) for DNA sequencing. The resulting amplicons were precipitated, washed, and resuspended in 1× hybridization buffer for hybridization of miRNA microarray. As a test case, 20 ng miRNAs at different developmental ages (P7, P21, P35, P49, and P70) were performed according to this procedure.

Fabrication of miRNA microarray

The miRNA microarrays were fabricated in our lab as described in our previous papers [12,17]. The 350 rat miRNA DNA probes were perfectly matched for all miRNAs that were registered and annotated in the miRBase at Wellcome Trust Sanger Institute (miRBase release 11.0). Each oligonucleotide probe was modified with a free amino group linked to its 5′ terminus through a 10-deoxyadenosine (A$_{10}$) spacer to minimize the spatial obstacle in hybridization and detection. All rat miRNA probes were spotted and immobilized on amine-reactive glass slides activated with glycidoxypropyltrimethoxysilane. Each microarray consisted of six submicroarrays. Besides, we also printed spots with 21 negative control probes and 7 same internal control probes on each microarray, which were located in different submicroarrays. Each miRNA probe or control probe had three replicated spots printed on a microarray. **Table 1** shows part of oligonucleotide probes used in the microarray.

Hybridization and detection of miRNA microarray

Hybridization and detection of microarray were carried out as described previously [12,17]. The labeled PCR products of miRNA obtained from epididymis at P7, P21, P35, P49, and P70, respectively, were precipitated, washed, resuspended in 20 μl of hybridization buffer [4× SSC/0.1% sodium dodecyl sulfate (SDS), pH 7.0] that had been heated to 45°C. The products were heated at 100°C for 2 min to denature, chilled on ice immediately, and then injected into the hybridization chamber on the microarray slide. Hybridization was carried out at 45°C for 16 h. After hybridization, the microarray slides were washed (2× SSC/0.02% SDS, 2 min; 0.2× SSC, 2 min; and 0.1× SSC, 30 s) at 25°C and then scanned with Innoscan700 (Innopsys, Carbonne, France).

Data analysis of miRNA microarray

The images were quantified by MAPIX (Innopsys) using the fixed circle quantification methods. The signal intensities of each spot were calculated by subtracting the background from the total intensities. Further analyses were completed by visual basic for application in Microsoft Excel. All hybridizations were normalized by the total

Table 1 Sequences of part of oligonucleotide probes used in the miRNA microarray

miRNAs	Oligonucleotide probes
Rno-miR-200a	Amino-5′-(A$_{10}$)ACATCGTTACCAGACAGTGTTA-3′
Rno-miR-29a	Amino-5′-(A$_{10}$)TAACCGATTTCAGATGGTGCTA-3′
Rno-miR-411	Amino-5′-(A$_{10}$)CGTACGCTATACGGTCTACTA-3′
Rno-miR-743a	Amino-5′-(A$_{10}$)TCTACCCAGTTTGGCGTCTTTC-3′
Rno-miR-539	Amino-5′-(A$_{10}$)ACACACCAAGGATAATTTCTCC-3′
Negative control 1	Amino-5′-(A$_{10}$)CGTAATACGACTCACTATAGGG-3′

intensity and the analysis of variance was performed for the values. The intensity of each miRNA at each time point used for analysis was the arithmetic mean value of the six replicates from two independent hybridizations. To narrow the range of miRNA candidates the miRNAs with intensity <3000 at all time points (P7, P21, P35, P49, and P70) were filtered out. Then the miRNAs whose *P* value was <0.05 and whose intensity was ≥10,000 and changed ≥2.5-folds at least at one time point were selected for further study. The following cluster analysis was performed using the Cluster/Tree View software [18].

Northern blot analysis of miRNAs

To validate the results of miRNAs expression detected by microarray, northern blot analysis was performed for miR-200a. Total RNA (20 μg) was separated on 15% acryl-amide/8 M urea denaturing gel until the faster dye moves to the bottom of gel and then transferred onto a nylon membrane (Pall, Ann Arbor, USA) with *trans*-blot SD semi-dry transfer cell (Bio-Rad, Hercules, USA). Following transfer, the membrane was dried and cross-linked with ultraviolet light at 125 mJ/cm^2. The DNA probes that were complementary to the miRNA sequences were end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham, Buckinghamshire, UK) using T4 polynucleotide kinase (TaKaRa). The unincorporated [γ-^{32}P]ATP was removed by filtration through a Sephadex G-25 column. Pre-hybridization and hybridization were carried out using PerfectHyb hybridization solution (TOYOBO, Osaka, Japan) at 37°C. The membrane was washed three times using 2× SSC/0.1% SDS at 37°C for 5 min each time according to the user manual. The membrane was exposed to a PhosphorImager (Amersham) and scanned with GE Storm 860 (Amersham). The quantification of the bands was performed by Quantity One Image Software (version 4.6; Bio-Rad). Rat 5S rRNA was used as the control. The DNA probes used in northern blot were as follows: N200a: 5′-ACATCGTTACCAGACAGTGTTA-3′; and rat 5S rRNA: 5′-TTAGCTTCCGAGATCA-3′.

Real-time quantitative polymerase chain reaction

For mRNA analysis of target genes, 2 μg of total RNA from epididymis was reverse transcribed with 20 μM oligo(dT)$_{20}$ using M-MLV (Moloney murine leukemia virus) reverse transcriptase (TaKaRa) for each time point (P7, P21, P35, P49, and P70) at 42°C for 60 min. Real-time quantitative-polymerase chain reaction (qRT-PCR) was subsequently performed with SYBR GREEN PCR Master Mix (TaKaRa) on a Rotor gene 6500HRM machine. *GAPDH* was used as a control. The primers used were as follows: *rβ-catenin*: 5′-CCTCAC TTGCAATAATTACAAAAAC-3′ and 5′-GTGCAGGAG TTTAACCACAACA-3′; *rGAPDH*: 5′-CAAGGAGTAAG AAACCGTGGAC-3′ and 5′- TATTATGGGGTCTGGGA TGG-3′.

Luciferase reporter assay

For luciferase reporter experiments, the portion of 3′UTR of *β-catenin* containing the predictive binding site for miR-200a was amplified by PCR from reverse transcription product of rat epididymis with KOD-Plus-DNA polymerase (TOYOBO) and inserted into the *Xho*I and *Not*I sites of psiCHECK-2 vector (Promega) immediately downstream from the stop codon of *Renilla* luciferase gene (hRluc). HEK 293T cells were seeded into 24-well plates one day before transfection containing 0.4 ml of DMEM medium. Total 100 ng of psiCHECK-2-β-catenin-3′UTR vector were transiently co-transfected into HEK 293T cells with 25 pmol of miR-200a mimics or miR-200a-mut mimics or negative control mimics (Gene Pharma, Shanghai, China), and 100 ng psiCHECK-2-β-catenin-3′UTR-mut vector were transiently co-transfected into HEK 293T cells with 25 pmol of miR-200a mimics using 8 μl lipofectamine 2000 and 100 μl Opti-MEM I reduced serum medium (Invitrogen). Two days later, luciferase activities were measured consecutively using dual-luciferase reporter assay system (Promega). *Renilla* luciferase activity was normalized to *Firefly* luciferase activity. All experiments were performed in triplicate. The primers used were as follows: *rβ-catenin-wt*: 5′-GGCCTCGAGGAAACGGCTTTCGGTTG A-3′ and 5′-AATTGCGGCCAGCGGCCGCCCATTTTTA TAACCGCGTCTG-3′; *rβ-catenin-mut*: 5′-CAGTAACTG TTAGTTTTTTAAGTCTCGTTTACAACTGTTCTAGTGA ACCTGCTACAGC-3′ and 5′-GCTGTAGCAGGTTCACT

AGAACAGTTGTAAACGAGACTTAAAAACTAACAGT
TACTG-3′.

Western blot analysis

Rat NRK cells were added directly into 35 mm culture dish containing RNAiMAX-miR-200a mimics (100 pmol; Invitrogen) or negative control mimics (NC) complexes and transfection occurred while cells were attaching to the well. NRK cells were incubated at 37°C in a CO_2 incubator and harvested 72 h after transfection. Total proteins were extracted from rat NRK cells with western and immunoprecipitation cell lysis buffer (Beyotime, Haimen, China). Each protein sample was separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to BioTrace poly(vinylidene difluoride) membrane (Pall) using Bio-Rad mini trans-blot system. The membrane was blocked with 5% non-fat milk powder/phosphate-buffered saline (PBS)/0.01% Tween-20 for 1 h at room temperature. Then, the anti-β-catenin (Santa Cruz Biotechnology, Santa Cruz, USA) and anti-β-actin (Abmart Biotechnology, Shanghai, China) primary antibody were diluted 1:1000 in 5% non-fat milk powder/PBS/0.01% Tween-20 and applied to the membranes, respectively, which were incubated for 1 h at room temperature. After washing, the protein−antibody complexes were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (Beyotime) and HRP-labeled goat anti-mouse IgG (Beyotime), respectively, in a 1:1000 dilution for 1 h at room temperature. The protein contents were determined with SuperSignal West Pico Trial Kit (Thermo Scientific, Waltham, USA).

Results

Expression profiles of miRNAs in the developing rat epididymis

Figure 1 shows the images of miRNA microarray of rat epididymis at different developmental stages (P7, P21, P35, P49, and P70). Green box in the figure displays concrete locations of miR-200a in the images of microarray. Microarray analysis revealed that the 48 miRNAs selected according to the standard mentioned above show significantly time-dependent changes in juvenile rat epididymis. These miRNAs are grouped using hierarchical clustering algorithm (Fig. 2) and are classified their expression patterns in Table 2. Some miRNAs, such as miR-200a, were in a lower level at P7 and increased obviously with time during epididymal development [Fig. 3(A)] that is consistent with that of northern blot analysis [Fig. 3(B)]. Some, such as miR-29a, -29c, -742, and -743a, were almost undetectable up to P21 but were increased markedly at P35 and thereafter. Some, such as miR-369-5p and -411, were only expressed at an early stage but are almost undetectable
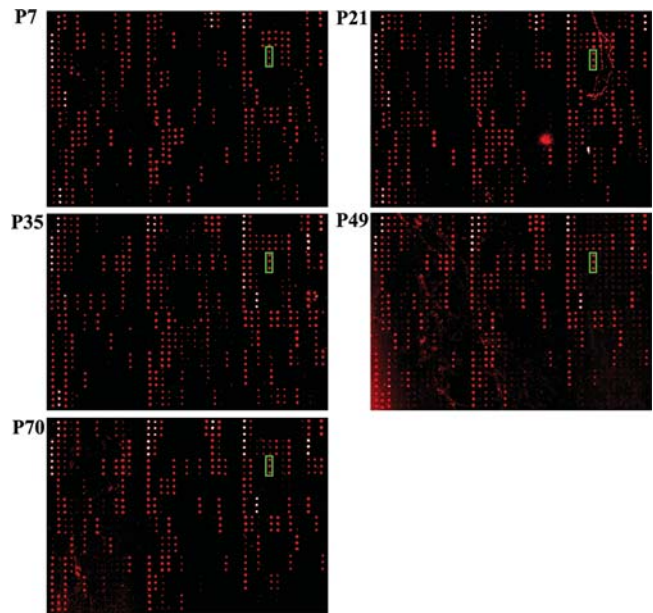

Figure 1 Images of miRNAs microarray during postnatal development of rat epididymis (P7, P21, P35, P49, P70) Green box displays concrete locations of miR-200a in miRNAs microarray.

at P35 and thereafter. We assessed the expression of a subset of miRNAs including four up-regulated (miR-200a, -141, -742, and -743a) and two down-regulated (miR-411 and -369-5p) miRNAs. The relative changes of miRNA expressions assayed using northern blots were consistent with those of microarray analysis (data not shown). Here northern blot analysis of miR-200a was only shown in Fig. 3(B). The similar patterns of miRNAs expressions suggested that the microarray data were reliable to warrant further analysis.

mRNA expression of *β-catenin* during rat epididymal development

Figure 4 shows the expressions of *β-catenin* mRNA at different development stages in rat epididymis obtained from qRT-PCR. The result indicates that the mRNA level of *β-catenin* is higher in the epididymis of 7-day-old rat and then gradually reduced with time, apparently inverse to the expression pattern of miR-200a. The result suggests that rat *β-catenin* might also be a target gene of miR-200a and the increase of miR-200a causes the down-regulation of *β-catenin* mRNA.

Identification of putative target genes of miR-200a

The bioinformatic analysis for the target sites of miR-200a in 3′UTR of *β-catenin* in rat and human is shown in Fig. 5, indicating that the 3′UTR of rat *β-catenin* mRNA contains a potential binding site (612−631 nt) for miR-200a. The detailed observation indicates that the two adjacent nucleotides (U and C) corresponding to positions 869
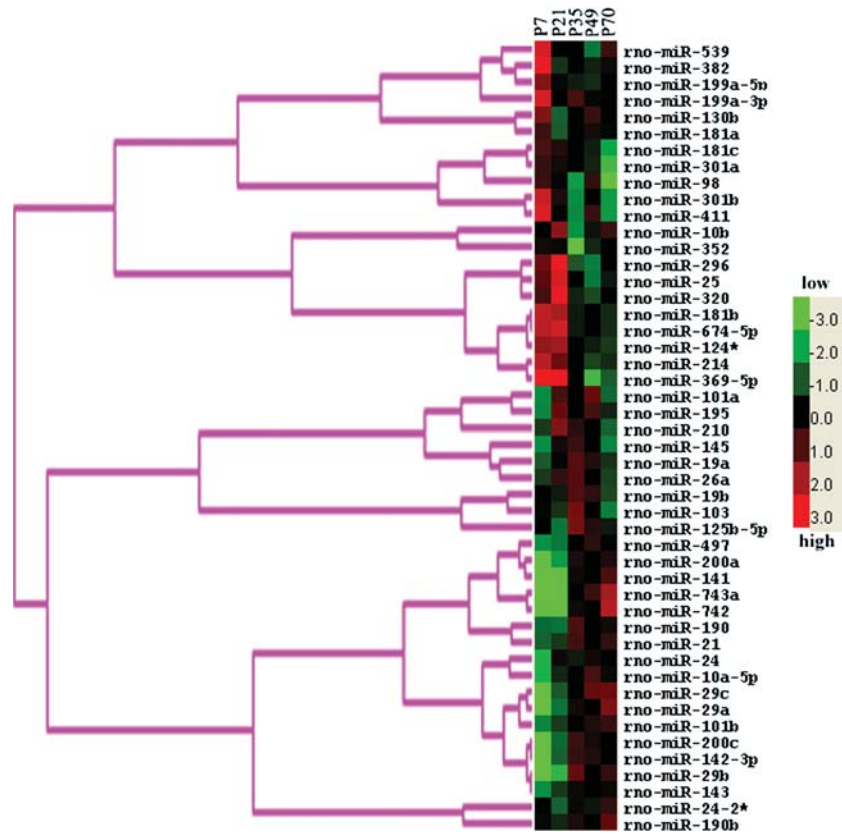
Figure 2 Cluster analysis of 48 miRNAs during postnatal development of rat epididymis Relative level expressions of 48 miRNAs that changed significantly are shown in five columns corresponding to the five time points on post-natal day of epididymis. The miRNA expression profile was sorted using a hierarchical clustering method. The miRNA clustering tree is shown on the left. Pseudocolors indicate expression levels below, equal to, or above the mean (green, black, and red, respectively).

Table 2 Expression patterns of the 48 miRNAs during post-natal development of rat epididymis

Expression patterns	miRNAs
Up-regulated miRNAs	miR-497, miR-200a, miR-141, miR-742, miR-743a, miR-24, miR-29a, miR-29c, miR-101b, miR-143, miR-190b
Down-regulated miRNAs	miR-539, miR-382, miR-199a-3p, miR-199a-5p, miR-181c, miR-301a, miR-369-5p, miR-411, miR-124*, miR-181b, miR-214, miR-674-5p
Other miRNAs	miR-352, miR-29b, miR-200c, miR-26a, miR-10a-5p, miR-130b, miR-181a, miR-301b, miR-320, miR-125b-5p, miR-25, miR-24-2*, miR-296, miR-195, miR-19a, miR-19b, miR-101a, miR-142-3p, miR-10b, miR-21, miR-103, miR-145, miR-210, miR-190, miR-98

During typical microRNA (miRNA) biogenesis, one strand of a 22 nt RNA duplex is preferentially selected for entry into a silencing complex, whereas the other strand is known as the passenger strand or miRNA strand.

and 870 of human *β-catenin* 3′UTR is deleted in rat. This raises a question whether miR-200a still targets rat *β-catenin* mRNA or not. So the wild-type or mutant 3′UTR of rat *β-catenin* mRNA was constructed in the luciferase reporter plasmid (psiCHECK-2) to perform the luciferase target report assay. As shown in **Fig. 6(A)**, the luciferase activity of psiCHECK-2-β-catenin-3′UTR plasmid is significantly suppressed in HEK 293T cells transfected with miR-200a mimics, but is not suppressed transfected with miR-200a mutant. Furthermore, the luciferase activity of

psiCHECK-2-β-catenin-3′UTR-mut plasmids is not inhibited by miR-200a mimics. This result indicates that miR-200a directly targets rat *β-catenin* mRNA by binding the target site. The expression of the endogenous β-catenin protein with overexpression of miR-200a is detected further in rat NRK cells. **Figure 6(B)** shows that miR-200a inhibits significantly the endogenous expression of β-catenin protein in rat NRK cells transfected by miR-200a mimics. All results validate that rat *β-catenin* mRNA is also a target gene of miR-200a.
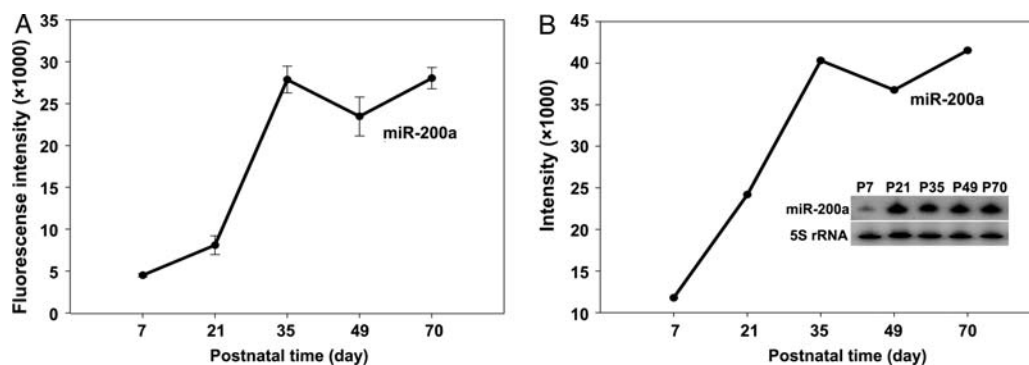
Figure 3 Expression of miR-200a in rat epididymal development (A) The expression pattern of miR-200a in miRNAs microarray. (B) Northern analysis of miR-200a.
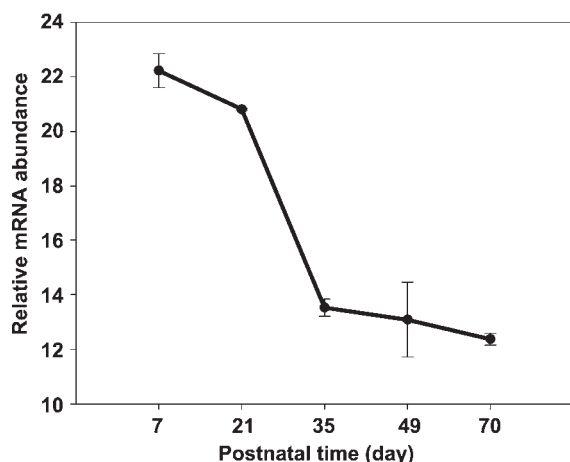

Figure 4 Expressions of *β-catenin* in rat epididymis at different developmental ages by qRT-PCR The result shows that the temporal expressions of *β-catenin* mRNA decease gradually, exactly inverse to that of miR-200a during rat epididymal development.

Discussion

In recent years, miRNA became a focus of many researches as it functions in many important physiological activities. These microarray data obtained in this study indicated that many miRNAs were significantly expressed during postnatal epididymal development. Some miRNAs, such as miR-411, -369-5p, -199a-3p, -539, -382, etc., show higher expression levels in the early development of epididymis, but lower or not at all in the late stage. Meanwhile, some miRNAs like miR-200a, -497 -29a, -29c, -742, -743a, etc., show gradual increase with the development. These results suggest that miRNA be involved in rat epididymal differentiation.

This study focused on rat miR-200a and its target genes for its specific expression pattern during rat epididymal development. There are no reports on the expression of miR-200a in rat epididymal development so far. The recent evidences show that miR-200a can inhibit EMT by simultaneously targeting of mRNAs for *ZEB1* and *ZEB2* (also known as *TCF8* and *SIP1*, respectively), which negatively regulate the expression of *E-cadherin* [13–15]. It is recently reported that miR-200a targets human *β-catenin* mRNA and suppresses its protein expression [16]. But the analysis of rat *β-catenin* mRNA revealed that the annotated Reference Sequence: NM_053357.2 lacks 3′UTR sequence in Nucleotide Database of National Center for Biotechnology Information. Cloning and searching the complete 3′UTR (∼1014 bp) by manual inspection revealed that rat *β-catenin* mRNA did contain a putative binding site for miR-200a with deletion of two adjacent nucleotides (U and C) that did not participate the binding of hydrogen bond with miR-200a comparing with human orthologous sequence (**Fig. 5**). But the current work also demonstrates that this deletion mutant does not influence miR-200a to target rat *β-catenin* mRNA.

Many evidences showed that β-catenin regulated numerous events in development, homeostasis, metabolism, regeneration, and carcinogenesis [19]. It has been shown to have dual functions in epithelial cells, depending on the intracellular localization. β-Catenin is necessary for cadherin–catenin interaction as part of the adhering junctions and E-cadherin associate shortly after the synthesis of E-cadherin in the cytoplasm, and that this complex is then transported to basal-lateral membrane of cells to form adhering junctions [20]. But β-catenin is also an important signaling effector in the Wnt signaling pathway, which is involved in the cell survival, proliferation, embryo patterning, organogenesis, cell migration, cell polarity, and carcinogenesis. Activation of the Wnt pathway facilitates the translocation of β-catenin into the nucleus through binding and derepression of T-cell Factor family transcription factors to promote the target gene expression and cell growth [21]. In brief, free cytosolic β-catenin is quickly turned over, unless the Wnt signaling cascade is activated. The published evidences have shown that β-catenin participated in the formation of adhering junctions, not activated the Wnt pathway during rat epididymal development. Previous results showed that all three catenins (α-, β-catenin, and p120^{ctn}) participating in the formation of adhering junctions were already expressed and localized along lateral
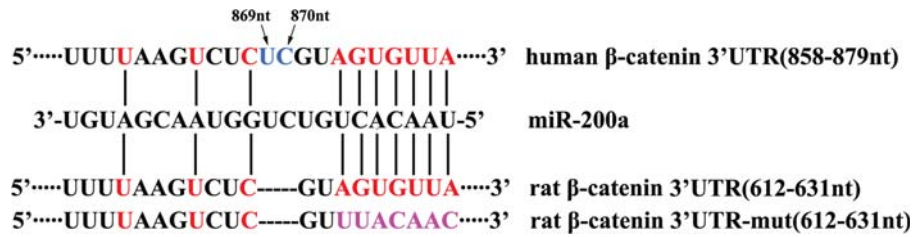
Figure 5 Bioinformatic analysis for the target site of miR-200a in rat *β-catenin* mRNA   Alignment of the binding sites for miR-200a and potential target sites in *β-catenin* 3′UTR in rat and human. The nucleotides of *β-catenin* 3′UTR participating the binding of hydrogen bond with miR-200a are shown in red. The two adjacent nucleotide of deletion (U and C) in 3′UTR of rat *β-catenin* mRNA is shown by the blue letters in 3′UTR of human *β-catenin* mRNA. Sequence of the mutant miR-200a binding site in rat β-catenin 3′UTR-mut is shown by the purple letters.
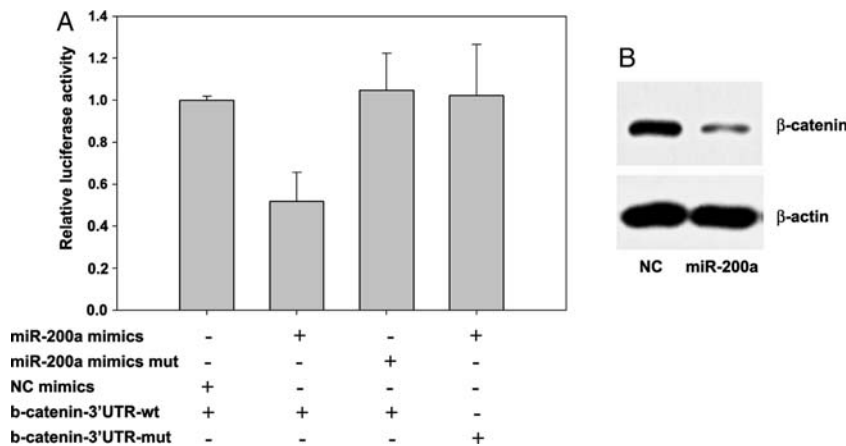

Figure 6 MiR-200a targets β-catenin   (A) The results of luciferase reporter experiments transiently co-transfected with miR-200a mimics or miR-200a mimics mut or NC mimics and psiCHECK2 vector containing the wild-type (wt) or mutant (mut) binding site of miR-200a from rat *β-catenin* mRNA into HEK 293T cells. *Renilla* luciferase activity was measured and normalized to *Firefly* luciferase activity. The presence (+) or absence (−) of mimics or psiCHECK2-β-catenin-3′UTR vector was as indicated above each lane. (B) Western blotting analysis of β-catenin protein in rat NRK cells transfected with miR-200a mimics or NC mimics were shown at the right lane and the left lane, respectively. The result normalized to β-actin levels shows that the expression of β-catenin protein is inhibited significantly by miR-200a.

plasma membrane between the adjacent undifferentiated epithelial cells in postnatal epididymis of 7-day-old rat, while any immunolocalization of β-catenin was not observed in the nuclei of epididymal epithelial cells [22].

The current study revealed that the expression levels of rat *β-catenin* mRNA was high at the epididymis of 7-day-old rat and gradually reduced during the rat epididymal development, apparently inverse to that of miR-200a paralleling the occurrence of adhering junctions between the adjacent epididymal epithelial cells. So we consider that miR-200a inhibits Wnt/β-catenin signal and regulates proliferation of epididymal epithelium and the formation of adhering junctions by targeting *β-catenin* mRNA in the development of rat epididymis.

In summary, the results from our home-made miRNA microarray and northern blot revealed that the expression of miR-200a increased from P7 to adult in rat epididymis. Further studies including the luciferase targeting assay in HEK 293T and the overexpression of miR-200a in rat NRK cells indicated that rat *β-catenin* is also a target of miR-200a, suggesting that miR-200a could be involved in rat epididymal development by targeting *β-catenin*. However, the epididymis is a high complex organ, the particular regionalization and regulation of gene expression and protein secretion, further analysis using miR-200a knock-down rat is necessary to clarify the *in vivo* role of miR-200a during rat epididymal differentiation.

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