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miR-34c Enhances Mouse Spermatogonial Stem Cells Differentiation by Targeting Nanos2

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ABSTRACT

miRNAs are expressed in many mammalian cells, acting specific roles in regulating gene expression or mediating special mRNAs cleavage by targeting their 3′-untranslated region (3′UTR). Some miRNAs are essential and important for animal development. However, it is still unclear what the relationship is between miR-34c and mammalian spermatogonial stem cells (SSCs). We found that a conserved microRNA-34c through its target-Nanos2, regulating SSCs′ differentiation in mouse. Immunohistochemistry analysis of Nanos2 and miR-34c FISH results revealed the opposite expression trends between them. Seven bioinformatics websites and programs predicted that miR-34c has interaction sites in Nanos2′s 3′UTR. Dual-luciferase reporter vector and mutated dual-luciferase reporter vector analysis validated that they are interacted. After transfection miR-34c mimics into mouse SSCs, or miR-34c lentiviral vector in vitro co-cultivation with seminiferous tubules, and Western blot analysis demonstrated that miR-34c over-expression could suppress Nanos2 expression in post-transcription level. Our experiments identified that miR-34c may promote meiosis process by interacting with Nanos2 leading up-regulation of *Stra*8 in mouse spermatogonial stem cells. J. Cell. Biochem. 115: 232–242, 2014. © 2013 Wiley Periodicals, Inc.

KEY WORDS: miR-34c; NANOS2; SPERMATOGONIAL STEM CELLS (SSCs); DIFFERENTIATION

Spermatogenesis is a highly mediated process of germ cell differentiation. Taking protamine (Prm1) as an example, meiosis is known for their high transcriptional and low translational activities during spermatogenesis in male germline stem cells (mGSCs). Therefore, post-transcriptional regulation is significant for mammalian spermatogonial stem cells (SSCs) [Wu et al., 2011]. MicroRNAs (miRNAs) are involved in nearly every biological process examined to date, but little is known of the identity or function of miRNAs in their potential involvement in spermatogenesis [Hua and Zhang, 2010; Curry et al., 2011]. There are some miRNAs, which play critical roles in the process of spermatogenesis. For instance, miR-383 is associated with male infertility and promoted embryonal testicular carcinoma cell proliferation [Lize et al., 2010]. MicroRNA-184 down-regulates nuclear receptor co-repressor 2 in mouse spermatogenesis [Wu et al., 2011]. Additionally, miR-34 family is conserved among various species, functioning in the processes of proliferation, apoptosis, and differentiation [Corney et al., 2007]. miR-34c could play an essential role in late spermatogenesis process [Bouhallier et al., 2010]. In 2011, Brinster and colleagues found that miR-34c prohibited the most abundantly in SSC-enriched germ cell cultures by small RNA libraries construction and analysis [Niu et al., 2011]. These studies highlighted the importance of miR-34c expression in controlling SSCs′ growth and differentiation. SSCs are necessary for spermatogenesis, although they constitute one in thousand cells in testis [Niu et al., 2011]. Nanos2 belongs to NANOS family which contains evolutionarily conserved zinc-finger motif encoding RNA-binding proteins, required in mouse SSCs for maintaining their self-renewal by preventing differentiation [Shen and Xie, 2010]. In mouse mGSCs, Nanos2 suppresses meiosis and in turn is required in maintaining SSCs [Suzuki and Saga, 2008; Sada et al., 2012]. Stra8 is a proved signature of entering meiosis in both male and female germ cells; Nanos2 can suppress the expression of Stra8 in mouse stem cells [Suzuki and Saga, 2008; Cannell et al., 2010]. miR-34c could up-regulate Stra8 expression in dairy goat mGSCs [Li et al., 2013].

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Nanos2 interacts with Cnot1, a component of CCR4-NOT deadenylation complex, which could be co-localized with processing bodies (P-bodies). NANOS2-interacting RNAs may be recruited to P-bodies and degraded by the enzymes contained therein through NANOS2-mediated deadenylation [Cannell et al., 2010; Liang et al., 2012].

In P-bodies, Dicer enzymes recognize specific double-stranded RNA, producing small fragment RNA whose 3′ end has two prominent bases. Double-stranded miRNAs nuclease combine to form the RNA-induced silencing complex (RISC), miRNAs open double-stranded to activate RISC by base pairing with mRNA combination, then make mRNA decay or translational repression [Olszewska et al., 2012]. However, there were little information on miR-34 effect on mouse SSCs and the real mechanism. To explore the relationship between Nanos2, miR-34c, and Stra8, we investigated the expression patterns of miR-34c and found that miR-34c could play critical roles in regulation of mSSCs′ meiosis differentiation through suppression its target-Nanos2, simultaneously, up-regulation of Stra8, Scp3.

MATERIALS AND METHODS

ANIMALS AND ISOLATION OF mSSCs

ICR mice were purchased from the animal lab center of FMMU (Xian, China) and were maintained under controlled light, temperature (22–24°C) and humidity (60–70%), with a 12 h light/dark cycle. The mice were killed specifically for our experiment and all the procedures were approved by Northwest A&F University and Shaanxi Centre of Stem Cells Engineering & Technology.

The day on the pups were born was marked as 1 dpp and the counting continued to the appropriate age for the experiments. Testes of Day 6 postnatal mice were harvested and the tunica albuginea were peeled under the stereomicroscope. Digested in collagenase IV (Invitrogen, Carlsbad, CA) for 15 min and pipetted 5 min each time, then centrifuged. The seminiferous tubules were isolated from mouse testes as described above. The germ cells were separated using a second enzymatic digestion with 4 mg/ml collagenase IV (Invitrogen), 2 mg/ml trypsin (Invitrogen) and 1 μg/ml DNase I (Invitrogen). The cell suspension was placed into culture dish and incubated for 1 h at 37°C with gelatin and 1 h at 37°C in laminin coated dish in order to eliminate residual adherent Sertoli cells (differential plating). Then the cells were collected by trypsin digesting and centrifuging at 1,400 rpm for 5 min. Cells were seeded at a density of 1.5×10^5 cells per well on 12-well plates without feeders. SSC cultures were maintained in a serum-free α-MEM medium, supplemented with 20 ng/ml glial cell line-derived neurotrophic factor (GDNF) (Peprotech, NJ), and 2 ng/ml basic fibroblast growth factor (FGF2, Millipore, Billerica, MA) 2.5 μM SB202190 (Sigma, St. Louis, MO), 2.5 μM SB216763 (Sigma), 0.5μM PD0325901 (Sigma) and incubated at 37 °C in 5% CO_2 balance air atmosphere for first three passages. After three passages, SSCs were seeded in plates with mitotically inactivated mouse epidermal fibroblasts. The medium was replaced by DMEM/F12 (Invitrogen) supplemented with 15% FBS (Hyclone, Logan, UT) and 1,000 U/ml leukemia inhibitory factors (LIF; Millipore) every 2 days, and cultures were routinely passaged at intervals of 3 days. The in vitro work described in this study was generated from at least three independent cultures from separate groups of mice.

miR-34c DETECTION BY FLUORESCENCE IN SITU HYBRIDIZATION (ISH)

Mmu-miR-34c-5p detection probe, scrambled probe, and detection kits were purchased from Focobio Corporation (Guangzhou, China). Mouse testicular tissue derived from 14, 21, 28 dpp and adult testis were fixed in 4% formaldehyde. The testicular tissues were embedded in paraffin and cut with the thickness of 5 μm. The slides were dried at 65°C for 5–6 h. The sections were dewaxed in Xylenes I and II for 10 min each. Then the slides were put in 100%, 75%, 50% ethanol for 3 min each. After washing using PBS for three times and 10 min each, the slides were incubated in solution A for 20 min at RT and were treated with solution B for 15 min at RT. After that, the slides were washed in PBS for 10 min, fixed in 4% formaldehyde in PBS for 15 min. Before pre-hybridization with 200 μl C solution added into the section at 37°C, the slides were washed in PBS for 10 min. Removed the C solution, added 10 μl hybridization solution containing 1.5–2.0 μM miR-34c probe, the slides were hybridized overnight at 40–42°C in the incubator. In the second day, removed the probe, the slides were washed in washing buffer I for 15 min at RT then rinsed in washing buffer II twice for 15 min each. The slides were washed in 75%, 100% ethanol for 2 min, respectively and air-dried for 10 min, which was followed by adding 10 μl DAPI for 10 min. The slides could be analyzed by Axio Observer Z1 fluorescence microscope (Zeiss, Germany).

BIOINFORMATICS PREDICTION

To predict the targets of miR-34c in the process of spermatogenesis, miRWalk database were utilized for integration information [Dweep et al., 2011]. Then miRDB database [Wang, 2008] was used to identify and find the precise interaction sites.

CELL TRANSFECTION

miR-34c mimics and inhibitors were purchased from Genepharma Co. (Shanghai, China). mSSCs were transfected with miR-34c mimics, miR-34c inhibitor in a 48-well plate, and scrambled oligonucleotides (NC) as a control. miR-34c mimics/inhibitors were diluted to 0.2 ng in 50 μl Opti-MEM (Invitrogen) reduced serum medium. Mixed gently, then added 0.5 μl PLUS™ Reagent (Invitrogen) directly to the diluted RNAs and incubated the mixed medium for 5 min at room temperature (RT). LipofectamineTM LTX Reagent (Invitrogen) was mixed gently before use, then 1 μl was added directly to the diluted DNA. Mixed gently and incubated for 30 min at RT. The 50 μl DNA–Lipofectamine™ LTX complexes were added, and incubated the cells for 4–6 h at 37°C in a CO_2 incubator. The transfection medium was replaced 4 h later by fresh growth medium, and the cells were observed after 48 h under Evos f1 fluorescence microscope (AMG, America).

Nanos2 siRNA ANALYSIS

Nanos2 siRNA duplexes were synthesized by Genepharma Co. (sense: CUGGAUGUCUGCCUACCAUTT; anti-sense: AUGGUAGGCAGAC-

TABLE I. PCR Primers

Gene	Forward primer	Reverse primer	Annealing temp (°C)	Product Size (bp)
β-Actin	GCGGCATCCACGAAACTAC	TGATCTCCTCTGCATCCTGTC	104	58
CD90	GATCCAGGACTGAGCTCTCGG	TCACGGGTCAGACTGAACTCATAC	195	58
Vasa	GCTGGCGTAATAGCGAAG	GCACAGATGCGTAAGGAGAAAA	107	58
c-Myc	CTGGTGGGCGAGATCATCA	CACTGCCATGAATGATGTTCC	304	54
Dazl	CAAGTTCACCAGTTCAGG	GACAACGGAGTTTCTCAGTCTATTC	299	58
Nanos2	AACTTCTGCAAGCACAATGG	CCGAGAAGTCATCACCAG	220	58
Nanos3	CCAGCAAGCCAGCAAGGAAT	GACTCGCCATTGTGTTTGCAG	51	58
Oct-4	CTTTCCTCTGGCCCCAGG	CTCAGTTTGAATGCATRGGAGAGC	152	58
Plzf	CACCGCAACAGCCAGCACTAT	CAGCGTACAGCAGGTCATCCAG	127	58
Stra8	AGCAGCTTAGAGGAGGTCAAGA	TACTCGGAACCTCACTTTTGTC	111	58
5S	CTGGTTAGTACTTGGACGGGAGAC	GTGCAGGGTCCGAGGT	50	58
miR-34c	GCAGCCAGGCAGTGTAGTTAGC	GTGCAGGGTCCGAGGT	50	58

AUCCAGTT), as well as si-NC and mimic NC. The si-Nanos2, miR-34c mimic, si-NC, and mimic NC (all these small RNAs were 0.2 ng) were permutated and combined into four different groups. These combined small RNAs were transfected into SSCs using LipofectamineTM LTX Reagent (Invitrogen) according to the described previously [Zhang et al., 2011; Li et al., 2013].

RT-PCR AND QRT-PCR

Total RNA was extracted with RNAiso reagent (Tiangen, China) from mSSCs. Single strand cDNAs were prepared from 0.5 μg RNA using a reverse transcription Kit (Fermentas). RT-PCR and QRT-PCR were based on our previous study [Cao et al., 2012].

The QRT-PCR reactions were set up in 15 μl reaction mixtures containing 7.5 μl 2× BioEasy SYBR Green Mix (Bioer Technology, Hangzhou, China), 0.3 μl sense primers, 0.3 μl antisense primers, 6.6 μl distilled water, 0.5 μl template, and 0.1 μl Taq DNA polymerase. Reaction conditions were as follows: 94°C for 5 min, and then 40 cycles 94°C for 20 s, 58°C for 30 s, and 70°C for10 s. All expression levels were normalized to β-actin in each well. The double ΔCt method was used to measure the expression alteration [Zhu et al., 2012]. The fluorescence signal was collected every 0.5°C for 10 s. The QRT-PCR primers are listed in Table I.

SEMINIFEROUS TUBULE CULTURING IN VIRUS PARTICLES AND WHOLE MOUNT STAINING

After removing from the tunica albuginea, the seminiferous tubules were untangled surgically. Single seminiferous tubule was picked into culturing plate (12-well or 24-well) and stretched flatly [Chu et al., 2013]. After the tubules adherenced on plate for 2–3 min, culture medium (negative group: DMEM + 10% FBS; positive group: DMEM + 10% FBS + pLL3.7-miR-34c-lentiviral virus particles) was added into the well. The pLL3.7-miR-34c-lentiviral particles were constructed as described in previous work [Liu et al., 2012a]. After culturing for 4–5 days, the seminiferous tubules were prepared for whole mount staining or fluorescence analysis.

Whole-mount staining was processed according to the methods of Sada's paper [Sada et al., 2012]. The tubules were fixed with 4% formaldehyde for 2 h at 4°C. After washing with PBS-T (0.3% TritonX-100/PBS), the specimens were incubated with 3% skim milk/PBS-T for 1 h at RT and subsequently with primary antibodies at RT overnight. The following day, the tubules were washed with PBS-T and were incubated with secondary antibodies at 4°C overnight. After that, they were washed with PBS-T before mounting. The specimens were examined using an eclipse TI-U inverted fluorescence microscope (Nikon, Japan).

Primary antibodies were used at the following dilutions: Oct4 (1:500, Santa Cruz Biotechnology, CA), NANOS2 (1:200, Abcam, Cambridge, MA), DAZL (1: 500, Abcam), NANOS3 (1:200, Santa Cruz), SCP3 (1:200, Santa Cruz), STRA8 (1:300, Abcam). All secondary antibodies were used at a 1:250 dilution: anti-rabbit or anti-mouse IgG antibodies conjugated with either Alexa-488 or Alexa-594 (Molecular Probes, Carlsbad, CA).

ALKALINE PHOSPHATASE (AP) STAINING

The mSSCs were cultured in 48-well plate in normal conditions. To detect the AP activity, the cells were fixed with 4% formaldehyde and were washed three times with PBS. Then the cells were stained with naphtol AS-MX phosphate (200 μg/ml, Sigma) and Fast Red TR salt (1 mg/ml, Sigma) in 100 mM Tris-buffer (pH 8.2–8.4) for 15—20 min at RT. Then, the mSSCs were washed several times with PBS to terminate staining. The results were analyzed by eclipse TI-U inverted fluorescence microscope (Nikon, Japan) [Cao et al., 2012].

IMMUNOFLUORESCENCE STAINING

The cells (transfected with miR-34c mimics or not) cultured in 48-well plate were fixed with 4% formaldehyde 10 min at RT and were washed with PBS for two times, 5 min each. The cells were permeabilized by 0.1% Triton X-100 for 10 min at RT and were blocked for minimum 30 min with 1% BSA at RT. Then the cells were incubated with primary antibodies specific against GFRα1 (1:100, Santa Cruz), NANOS2 (1:200, Abcam), PLZF (1:200, Santa Cruz), CD90 (1:100, BD), VASA (1: 200, Abcam), DAZL (1: 500, Abcam), SCP3 (1:200, Santa Cruz), respectively for overnight at 4°C. The appropriate FITC- or TRITC-conjugated secondary antibodies were used following the manufacturer's manual (1:500, Chemicon, Temecula, CA).

TABLE II. Primers for Constructed Dual-Luciferase Reporter Vectors

	Name	Sequences
Primers for Nanos2-3′UTR	F	ATTgcggccgcTTGAGACCCTGTGAGGTACCTGTC
	R	CCGctcgagGTAGACTAACAACGCTTTATTCAGCAG
Primers for Mut-Nanos2-3′ UTR	Mut-F1	CCGctcgagTTGAGACCCTGTGAGGTACCTGTCA
	Mut-F2	ACTGATCCTGATCGTTAGCGCTA
	Mut-R1	CCAACATCCACTTCTTAGCGCTA
	Mut-R2	ATTgcggccgcAACAACGCTTTATTCAGCAGCAGAC

Concurrently, the negative controls were stained with conjugated secondary antibodies alone: goat anti-rabbit IgG and goat anti-mouse IgG. The nuclei of cells were stained by Hoechst 33342. Images were captured with Evos f1 fluorescence microscope (AMG, America).

LUCIFERASE REPORTER ASSAY

The dual-luciferase reporter gene vectors constructs were generated by cloning the entire 3′UTR or the mutant3′UTR of Nanos2 into pmiR-RB-Report™ vector (Ruibo, Guangzhou, China) at the site which was digested by *Not*I and *Xho*I enzyme. The Nanos2 3′UTR fragment

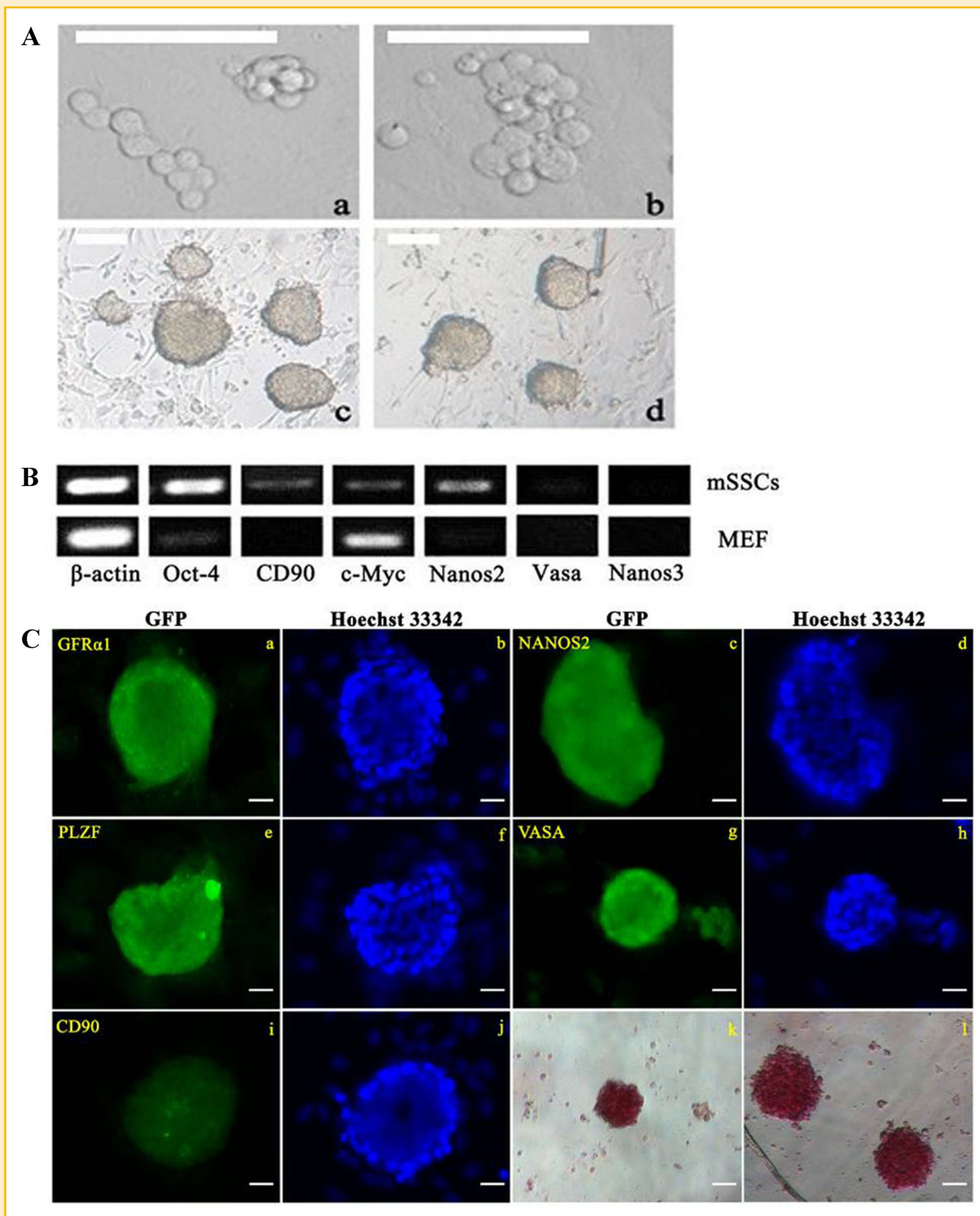

Fig. 1. Characteristics of cultured mouse SSCs. A: The morphology of SSCs formed colonies (scale bar = 100 μm). B: PCR analysis reveals the mRNA level expression of Oct-4, CD90, c-Myc, Nanos2, Vasa, Nanos3 in mSSCs, MEF cells as a negative control. C: Immunofluorescence reveals the expression of GFRα1, NANOS2, PLZF, VASA, and CD90 in mSSCs, nuclei were stained with Hoechst 33342 (blue) (a–j scale bars = 10 μm; k and l scale bars = 5 μm).

cloning was performed using PCR (Table II). According to the predicted interaction sites, miR-34c had seven seed bases paired with Nanos2 3′UTR. Among them, four bases were mutated by PCR and enzyme digestion methods to construct the Mut-Nanos2-3′UTR. The Firefly luciferase vector was used for internal reference. Construction with mutated 3′UTR of Nanos2 (Mut-Nanos2) was used as negative control. A total of 50 ng of pmiR-RB-ReportTM-Nanos2 3′UTR (Nanos2 3′UTR) or pmiR-RB-ReportTM-Mut-Nanos2 3′UTR and miR-34c mimics were co-transfected into HeLa cells in a 48-well plate using Lipofectamine 2000 (Invitrogen). After 48 h, all the target validation assays were performed with the dual-luciferase reporter system (Vigorous Biotechnology, Beijing) according to the manufacturer's instructions and previous work [Li et al., 2013]. The activities were measured by a BHP9504 optical analyze reader (Hamamatsu, Japan).

WESTERN BLOT

Total cell extracts were prepared from mSSCs in the transfected miR-34c or NC, and proteins were extracted in 1× SDS–PAGE sample loading buffer. Total cell proteins were resolved by SDS–PAGE, transferred to 0.22 μm PVDF membrane about 55 min in 80 V, and probed with β-actin (1:1,000, Beyotime, China), Stra8 (1:1,000, Abcam), Scp3 (1:1,000, Santa Cruz). Horseradish peroxidase-conjugated anti-rabbit was used as a secondary antibody (1:1,000, Beyotime). The detection was performed using the Thermo Scientific Pierce ECL Western blot substrate (Thermo Scientific) [Cao et al., 2011]. The results were analyzed by Tanon-410 automatically gel imaging system (Tanon Corporation, China).

STATISTICAL ANALYSIS

The data are presented as mean ± SEM. Differences in the expression of specific markers were evaluated using Student's *t*-test (Excel, Microsoft Corporation). Results of the different treatments were considered significantly different when a P-value <0.05 was obtained.

RESULTS

CHARACTERIZATION OF SSCs DERIVED FROM 6- TO 12-DAY POSTNATAL MOUSE TESTIS

SSCs were derived from 6- to 12-day postnatal Kunming mouse. At first, the cultured SSCs were presented paired or aligned, or 4–8 single cells aggregated colonies (Fig. 1A). PCR indicated that the cultured SSCs were positive for Oct4, CD90, Nanos2, while MEF cells were negative for the SSC's markers (Fig. 1B). They formed typical colonies at second passage. From third or fourth passages, SSCs were plated onto MEF layers. Most SSCs were positive for GFRα1, CD90, NANOS2, PLZF (SSC markers) by immunofluorescence (IF) assay. VASA positive staining demonstrated that SSCs presented male germ cells characters (Fig. 1C).

miR-34c WAS HIGHLY EXPRESSED IN THE ADULT MOUSE TESTIS

To localize miR-34c expression in the developing testis, a miR-FISH probe in 14, 21, and 28 dpp and adult mouse testis were used. A scrambled probe was used as a negative control. The results showed that the miR-34c signal exhibited strongest, and the percentage of miR-34c positive spermatogonia reached the top in adult murine

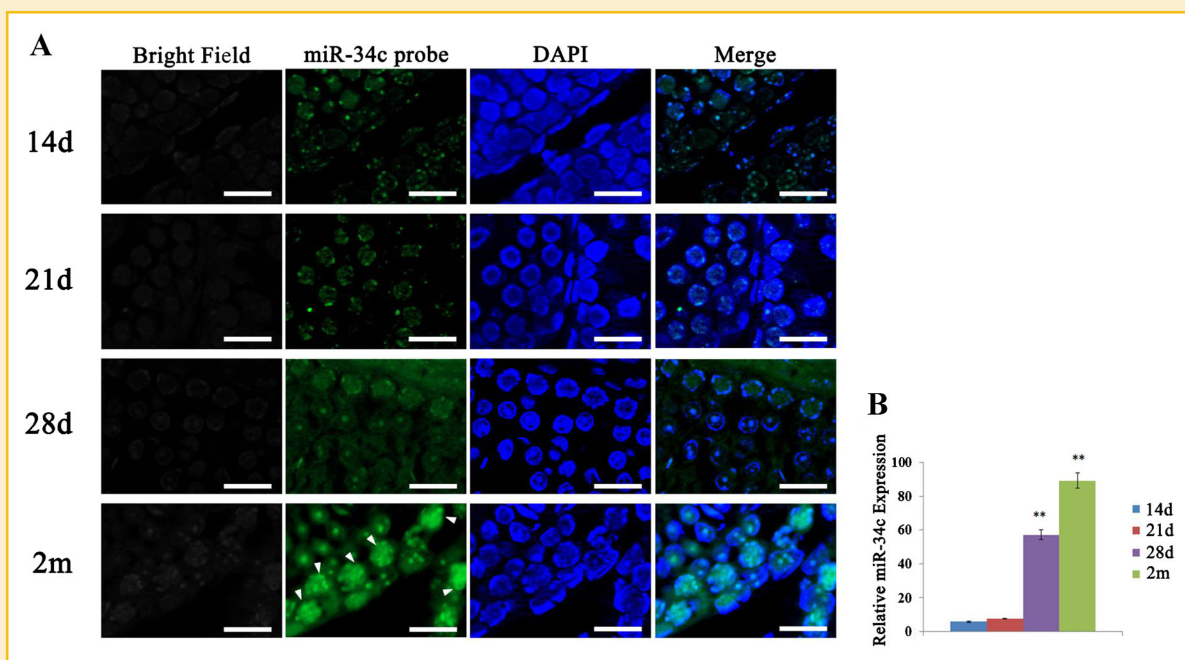

Fig. 2. Expression profiles of miR-34c in mice testis. A: FISH analysis reveals the expression profile of miR-34c. In adult testis, miR-34c probe tests up to the highest among various age testes (scale bar = 20 μm). Arrowheads indicate the leptotene spermatocytes and pachytene spermatocytes. B: Quantification of miR-34c FISH results (Scale bars = 20 μm, error bars indicate ±SD of three technical replicates, $^{**}P < 0.01$).

testis (Fig. 2A,B). In contrast, the signal intensity and the percentage of miR-34c positive in 2 dpp, 7 dpp mouse testis were significantly weaker compared than that in adult testis (data not shown). According to the FISH results, in the adult mouse testis, the hybridization signal for miR-34c was detected in pachytene spermatocytes and round spermatids (arrowheads in Fig. 2A). These results were in consistent with previous studies [Bouhallier et al., 2010; Liang et al., 2012; Zhang et al., 2012].

NANOS2 IS A DIRECT TARGET OF miR-34c

In order to explore how miR-34c regulates mSSCs, we computationally predicted that Nanos2 was the candidate of miR-34c targets from miRwalk database (http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/). Then miRDB (http://mirdb.org/miRDB/) provided the detailed interaction information between miR-34c and Nanos2 (Fig. 3A,B). It was validated that they did have interaction analyzed by luciferase reporter assay. The predicted binding site, 3′UTR of Nanos2 was then

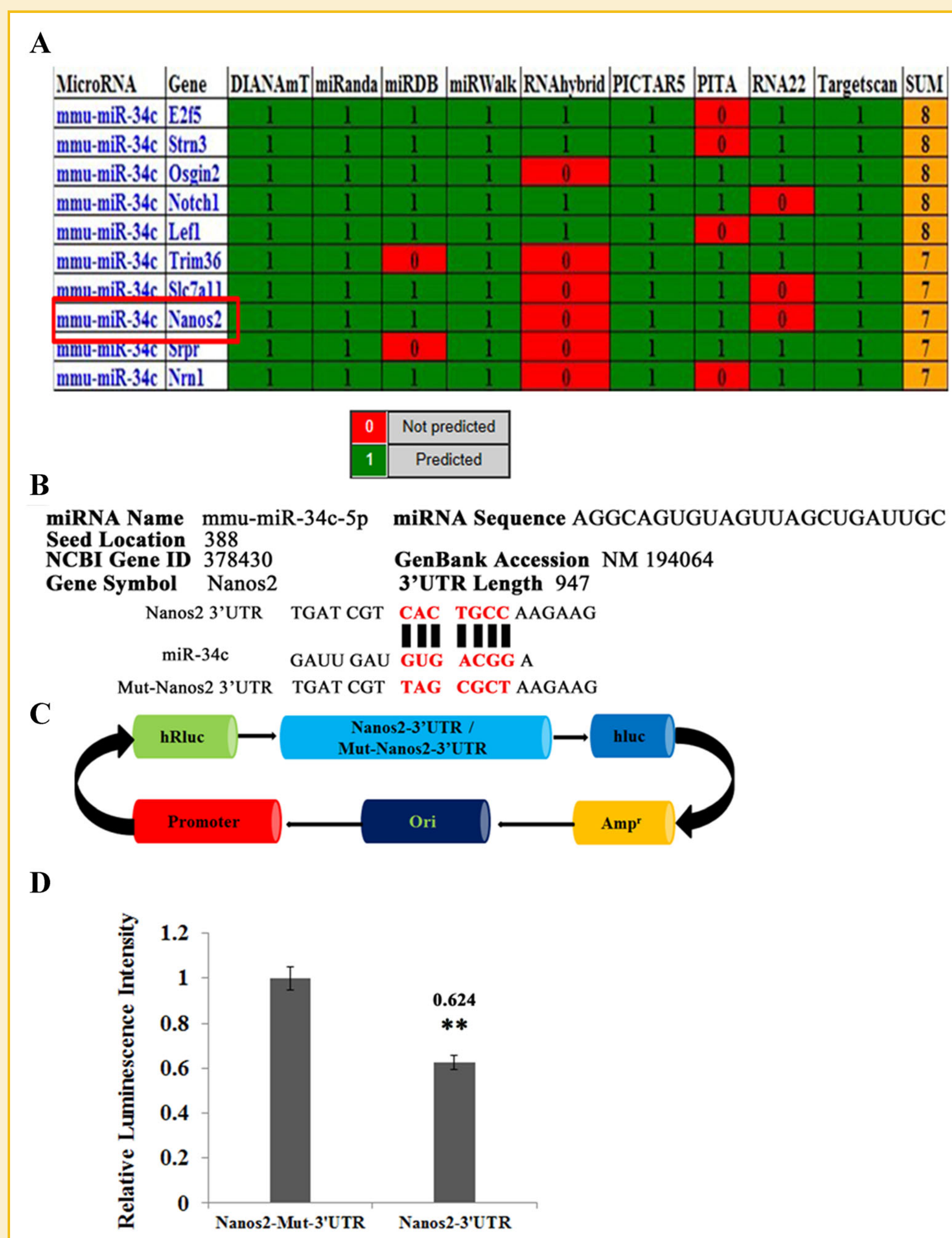

Fig. 3. Target genes of miR-34c was predicted and verified. A: Target genes of miR-34c was predicted by miRwalk website. B: Nanos2 is a target of miR-34c. Seed sequence of miR-34c and the 3′UTR of Nanos2 is correctly base paired. C: The construction schema of pmiR-RB-REPORT-Nanos2-3′UTR and pmiR-RB-REPORT-Mut-Nanos2-3′UTR vectors. D: Relative luminescence intensity detected by a Hamamatsu optical analyze reader after miR-34c mimics and dual-luciferase vectors were co-transfected into Hela cells ($^{**}P < 0.01$). (scale bars = 100 μm, error bars indicate +SD of three technical replicates, $^{**}P < 0.01$).

inserted downstream from the Renilla luciferase coding region in the reporter vector (Fig. 3C). Each reporter construct was separately co-transfected with the miR-34c mimics into Hela cells. Compared to the mut-Nanos2-3′UTR control, the luciferase activity declined by about 37.5% after transfection with miR-34c mimics and Nanos2-3′UTR reporter vector (Fig. 3D). The luciferase analysis showed that ectopic over-expression of miR-34c reduced Nanos2 protein expression via directly binding to Nanos2-3′UTR, indicating that Nanos2 is one target of miR-34c. These results demonstrated that miR-34c directly regulates Nanos2 protein expression through its binding to the 3′UTR region of Nanos2.

miR-34c OVER-EXPRESSION INHIBITED NANOS2, AND PROMOTED MEIOSIS IN mSSCs

To further investigate the effects of miR-34c on mSSCs, negative control small RNAs, miR-34c mimic, miR-34c inhibitor, and in combination with miR-34c mimic and its inhibitor were transfected into mSSCs, QRT-PCR results manifested that miR-34c were transfected efficiently into SSCs (Fig. 4A), and over-expression miR-34c specifically down-regulated its target-Nanos2. Simultaneously, the mRNA expression levels of Nanos3, Stra8 (the pre-meiotic markers), and Scp3 (meiotic marker) were up-regulated by miR-34c over-expression at 48 h after transfection into mSSCs (Fig. 4B). Furthermore, we found that mSSCs transfected miR-34c mimics become irregular edged compared with that transfected NC. IF analysis revealed the expression level of miR-34c′s target-NANOS2 was significantly down-regulated by miR-34c, and the pre-meiotic marker STRA8 and meiotic, germ cell marker SCP3 were significantly up-regulated in over-expression of miR-34c compared with NC. Additionally, germ cell marker-VASA was little up-regulated, however, PLZF (self-renewal marker of SSCs) was down-regulated by miR-34c (Fig. 5A,B).

Nanos2 siRNA analysis showed that the meiosis markers: Stra8 and Scp3 expression levels were specifically upregulated in Nanos2 siRNA group compared with control analysed by QRT-PCR and IF (Figs. S1 and S2).

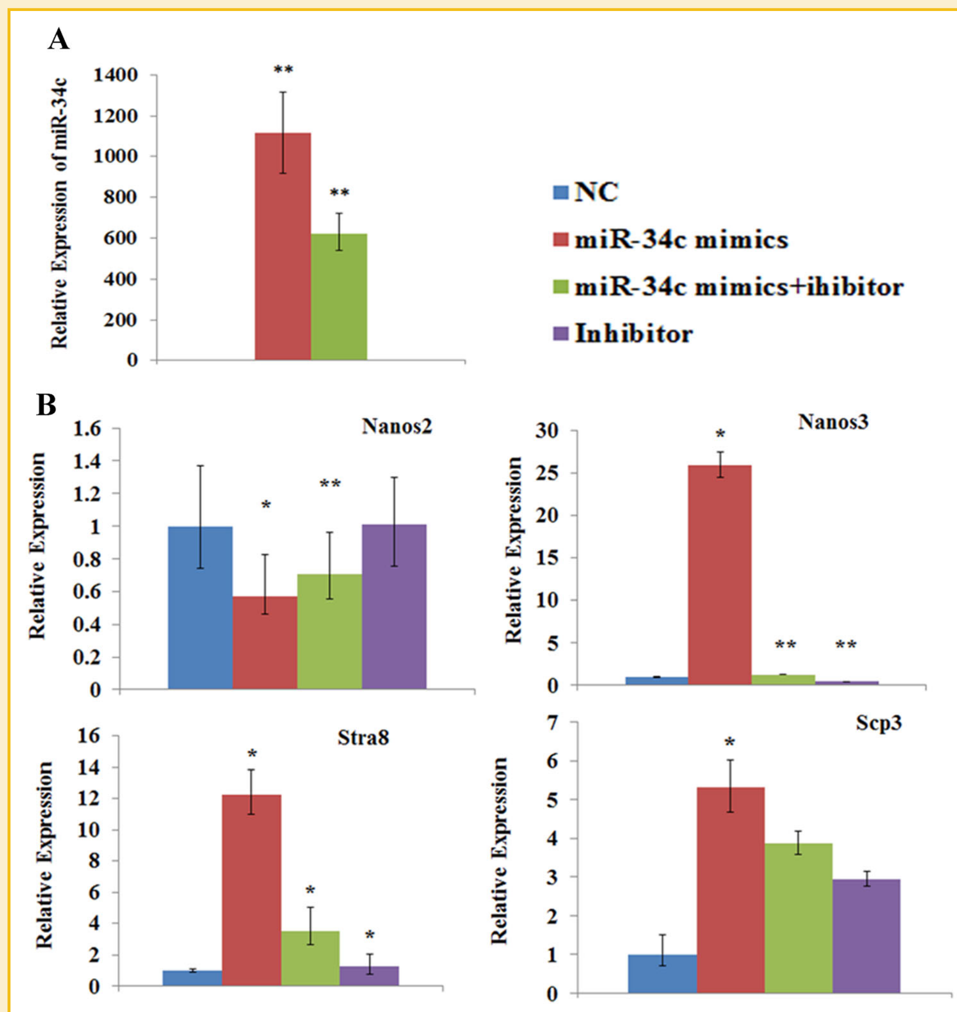

Fig. 4. QRT-PCR analysis about the effects of miR-34c on the expression of Nanos2, Nanos3, Stra8, and Scp3. A: Real-time PCR analysis reveals that miR-34c mimics and inhibitor are effectively transfected into SSCs. B: Real-time PCR analysis reveals that miR-34c mimics inhibit the expression of Nanos2, and promote the Nanos3, Stra8, and Scp3 expression (error bars indicate +SD of three technical replicates, $^{*}P<0.05$, $^{**}P<0.01$).

miR-34c OVER-EXPRESSION EFFECT IN SEMINIFEROUS TUBULES

To assess how miR-34c function in vivo, lentiviral particles of pLL3.7-CMV-34c were cultured with mouse seminiferous tubules [Chu et al., 2013]. Through IF microscope, the transduced GFP positive cells were observed in seminiferous tubules (Fig. 6A). PCR analysis and Western blot showed that the expression of Scp3 and Stra8 in mRNA and protein levels were significantly increased in over-expression of miR-34c. Additionally, expression of Nanos2 was significantly down-regulated by miR-34c (Fig. 6B). Moreover, whole mount staining demonstrated the meiotic related proteins: NANOS3, DAZL, SCP3, and STRA8 were significantly increased by miR-34c, however, NANOS2 and OCT4 were significantly down-regulated, respectively (Fig. 6C,D).

DISCUSSION

Some miRNAs play critical roles in life process by targeting 3′UTR of their specific mRNAs. Studies have showed that miRNAs, as a kind of newly found small RNAs, might play an important role in spermatogenesis in mammals [Ro et al., 2007; Bjork et al., 2010; Luo et al., 2010; McIver et al., 2012a,b; Tong et al., 2012]. miR-146 modulates the effects of RA on spermatogonial differentiation [Huszar and Payne, 2013]. miR-122 expression is associated with abnormal sperm development. miR-122 may influence spermatozoa-like cells by suppressing TNP2 expression and inhibiting the expression of proteins associated with sperm development [Liu et al., 2013]. Expressions of Hsa-miR-34c were regarded differences between immature and mature testes and they regulated a series of gene expression, which is essential for different types of cells (mainly spermatocytes and spermatids) formation and differentiation during primates′ spermatogenesis [Yan et al., 2009]. MicroRNA-34c expressed highly in adult testis, and by transfection of miR-34c into vasa-overexpressed Hela cells, spermatogenesis-related genes (even containing some late-stage expressed genes) were detected in these cells and miR-34c might be involved in the control of the late steps of spermatogenesis [Bouhallier et al., 2010]. Sperm-borne miR-34c is important for the first cell division via modulation of Bcl-2 expression [Liu et al., 2012b]. In our study, miR-34c FISH and QRT-PCR analysis demonstrated that miR-34c was testis-specific and most highly expressed in testis of sexually matured mice, exactly in

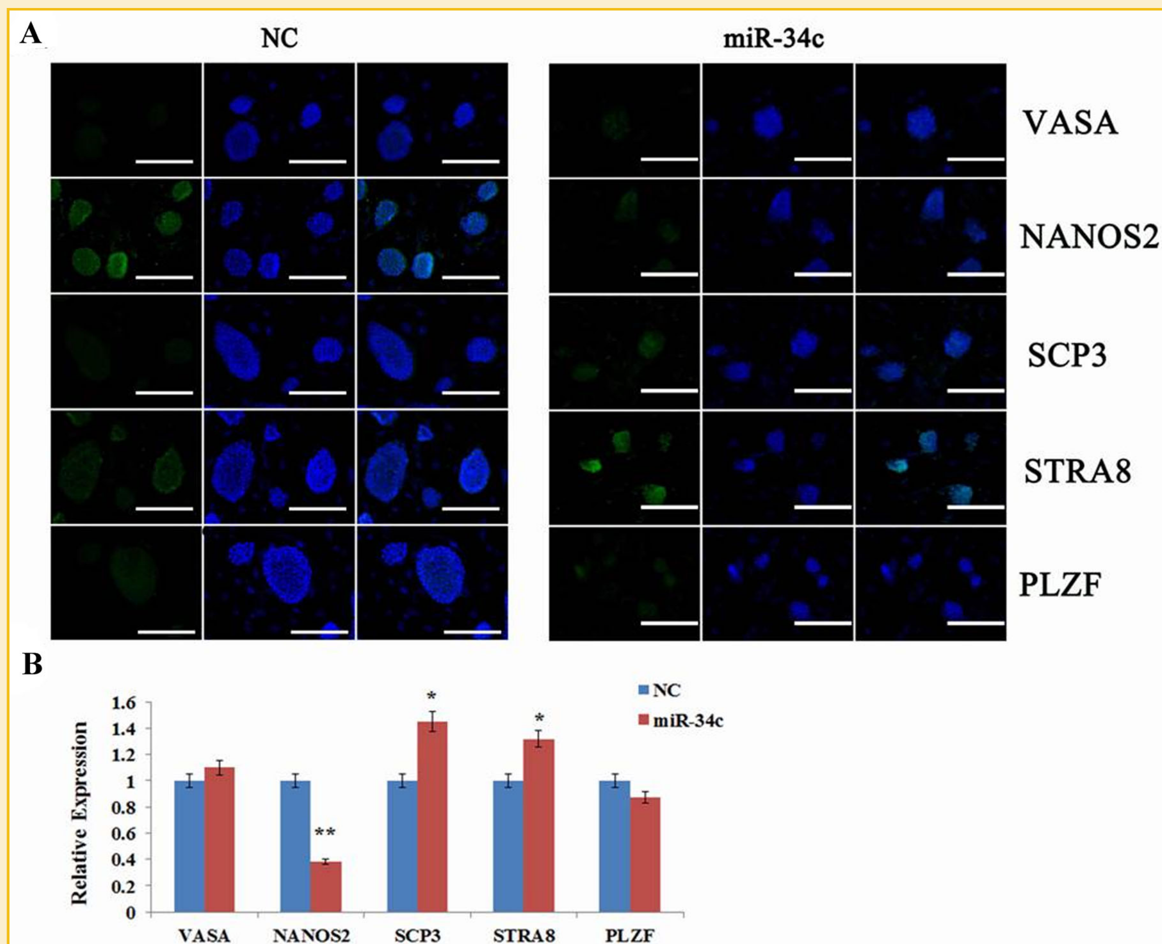

Fig. 5. Effects of miR-34c inhibition on NANOS2, PLZF protein expressions and promotion on VASA, STRA8, and SCP3 protein expression analyzed by immunofluorescence. A: Immunofluorescence analysis of mSSCs at 48 h after transfection negative control and miR-34c mimics. B: Quantification of immunofluorescence analysis results (scale bars = 200 μm, error bars indicate +SD of three technical replicates, $^{*}P < 0.05$, $^{**}P < 0.01$).

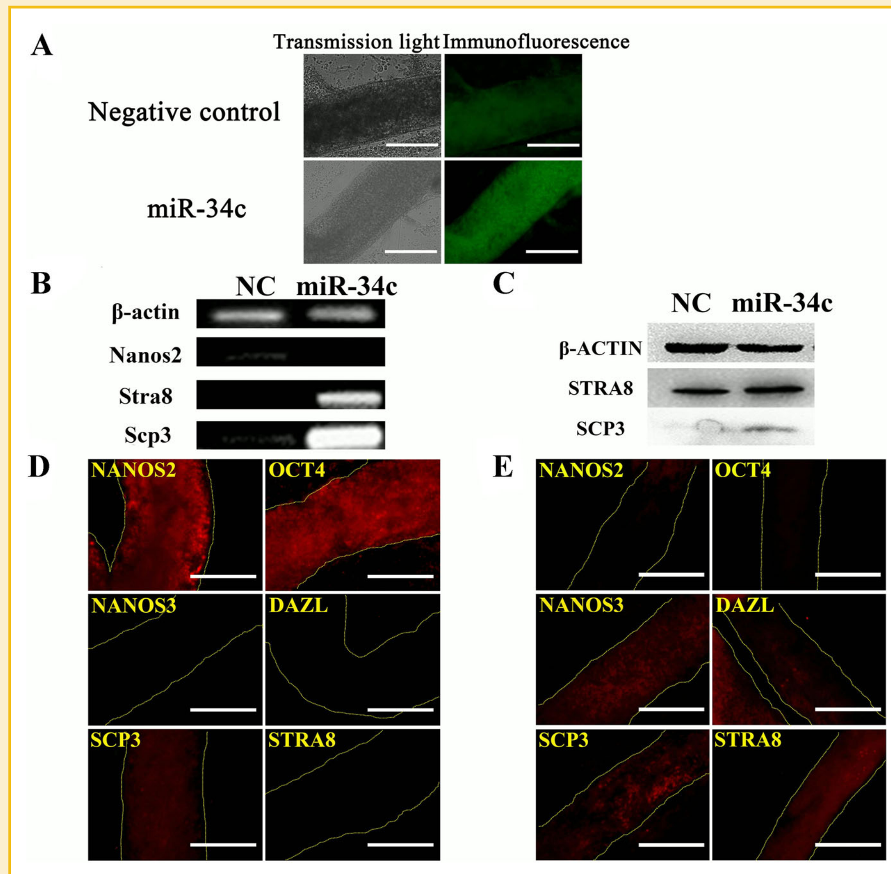

Fig. 6. Effects of miR-34c on the culturing seminiferous tubules. A: The morphology and immunofluorescence of the seminiferous tubules after transduction with pLL3.7-U6-miR-34c lentiviral vectors, pLL3.7-EGFP lentiviral vectors were as control. B: Nanos2 was decreased and Scp3, Stra8 in mRNA level increased after transduction with miR-34c lentiviral vectors. C: SCP3, STRA8 were increased after transduction with miR-34c lentiviral vectors analyzed by Western blot. D: Whole mount staining analysis reveals that NANOS2, OCT4 were decreased and NANOS3, SCP3, STRA8, DAZL increased after transduction with miR-34c lentiviral vectors (scale bars = 200 μm).

spermatogenic cells. miR-34c might play an important role in mammalian spermatogenesis [Bouhallier et al., 2010; Liu et al., 2012a]. These results were almost in consistent with previous studies [Bouhallier et al., 2010; Niu et al., 2011; Zhang et al., 2012].

NANOS2 is a Nanos family protein that mediates a pivotal role in SSC's self-renewal and differentiation [Sada et al., 2009, 2012]. Bioinformatics analysis and Luciferase reporter assay evidenced that Nanos2 3′UTR has a specific miR-34c binding sequence. Further, the morphology of mouse SSCs ectopic over-expressed miR-34c could not maintain the typical colony formation, but promoted SSCs differentiation trend. The expression levels of meiotic prophase marker and germ cell markers in mRNA level were up-regulated, accompanied with the down-regulation of Nanos2. In contrast, these markers exhibited downward expression patterns compared with NC group in treated with miR-34c inhibitor. These results further indicated that Nanos2 is one target of miR-34c, and over-expression miR-34c influenced SSCs′ differentiation by suppressing Nanos2 expression, and promoting the expression genes associated with meiosis, including Nanos3, Scp3, and Stra8.

Stra8 (stimulated by retinoic acid gene 8), which is required for meiotic initiation in both sexes [Koubova et al., 2006; Anderson et al., 2008; Zhou et al., 2008]. miR-34c mimics were synthesized and transfected into mSSCs. Moreover, miR-34c lentiviral vector was constructed, virus particles were collected, and cultured with seminiferous tubules in vitro. RT-PCR, Western blot, and whole mounting of seminiferous tubules demonstrated that miR-34c over-expression promoted the expression of meiosis associated markers, including Nanos3, Scp3, and Stra8, through suppressing its target-Nanos2 expression.

NANOS3 have been directly shown to function in germ cell development across diverse species from flies, worms, frogs, and mice to humans [Julaton et al., 2011]. NANOS3 was expressed in germ cells throughout spermatogenesis and oogenesis [Kee et al., 2009]. DAZL and SCP3 are meiosis regulated genes [Koubova et al., 2006; Anderson et al., 2008; Jørgensen et al., 2013]. In our study, the evidences in vivo and in vitro demonstrated that miR-34c plays a critical role in regulation SSC's differentiation, through NANOS2. Thus, we first summarized the function model for the role of miR-34c in regulating mouse spermatogenesis (Fig. 7). In undifferentiated spermatogonia, NANOS2 play a role in inhibiting NANOS3, SCP3, and Stra8 expression to make SSC or spermatogonia maintain an undifferentiated state. When mouse testis is mature, miR-34c abundance removes the suppression of NANOS3, SCP3, and STRA8 by targeting NANOS2, and promotes SSC or spermatogonia transition

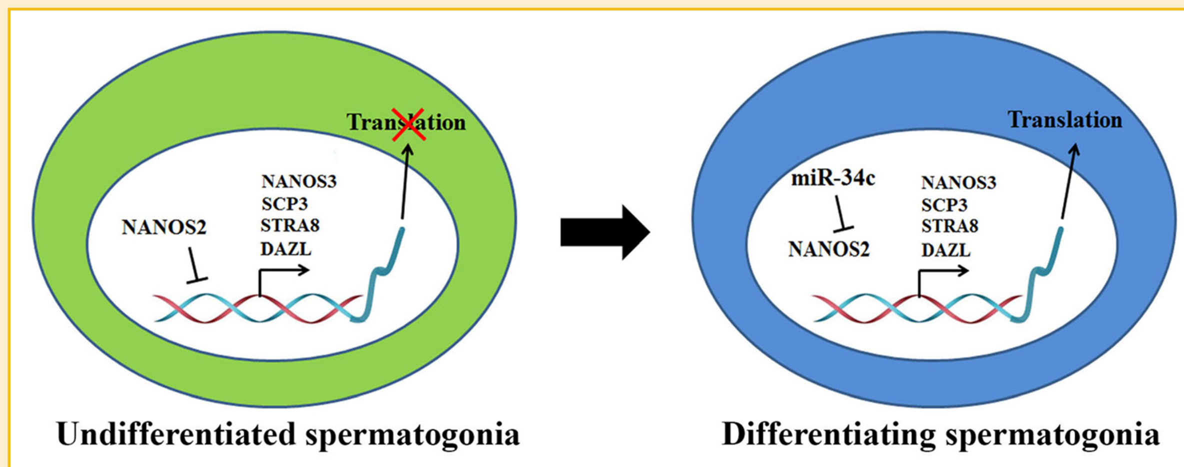

Fig. 7. Models for the role of miR-34c in regulating mouse spermatogenesis. In undifferentiated spermatogonia, NANOS2 play a role in inhibiting NANOS3, SCP3, and STRA8 expression to make SSCs or spermatogonia stay an undifferentiated state. miR-34c abundance removes the suppression of NANOS3, SCP3, and STRA8 by NANOS2, and promotes SSC or spermatogonia transition to a differentiating state.

to a differentiating state. This study further extends the mechanism of meiosis in mammalian spermatogenesis.

Taken together, our study first shows miR-34c functions by targeting the Nanos2 in SSCs meiosis differentiation, providing a novel mechanism with involvement of miRNAs in the regulation of male germ cell differentiation.

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