BIOLOGY OF REPRODUCTION **85,** 189–197 (2011)
Published online before print 23 March 2011.
DOI 10.1095/biolreprod.110.089458

Expression of *Mirlet7* Family MicroRNAs in Response to Retinoic Acid-Induced Spermatogonial Differentiation in Mice[1]

Ming-Han Tong, Debra Mitchell, Ryan Evanoff, and Michael D. Griswold[2]

School of Molecular Biosciences, Washington State University, Pullman, Washington

ABSTRACT

Spermatogonial differentiation is orchestrated by the precise control of gene expression involving retinoic acid signaling. MicroRNAs have emerged as important regulators of spermatogenesis, and here we show that the *Mirlet7* family miRNAs are expressed in mouse spermatogonia and spermatocytes. Retinoic acid significantly leads to the induction of *Mirlet7* miRNAs through suppression of *Lin28*. We further confirmed both in vitro and in vivo that expressions of *Mycn*, *Ccnd1*, and *Col1a2*, which are targets of *Mirlet7*, were downregulated during spermatogonial differentiation. These results suggest that *Mirlet7* family miRNAs play a role in retinoic acid-induced spermatogonial differentiation.

Lin28, *microRNAs,* Mirlet7 *family, retinoic acid, spermatogonial differentiation*

INTRODUCTION

Spermatogenesis is a highly coordinated and complex process during which mature sperm originate from a common spermatogonial stem cell (SSC) [1]. In the adult mammals, SSCs either enter a self-renewal pathway or undergo repeated mitotic divisions to sequentially produce A paired (A_{pr}) and A aligned (A_{al}) spermatogonia, which are committed to further development [1]. The A_{al} spermatogonia or undifferentiated spermatogonia subsequently differentiate into A_1 spermatogonia in a process that does not include a proliferative division. The A_1 spermatogonia, or differentiating spermatogonia, further form, successively, A_2, A_3, A_4, In (intermediate), and type B spermatogonia through a series of mitotic divisions. The type B spermatogonia divide into preleptotene spermatocytes that subsequently enter into meiosis to yield haploid step 1 spermatids, which undergo spermiogenesis, resulting in the production of spermatozoa [1].

The differentiation of A_{al} into A_1 spermatogonia is a key step in spermatogenesis [2]. Retinoic acid (RA), an active metabolite of vitamin A, is critical in this process [3, 4]. For example, spermatogonial differentiation is blocked at the A_{al} to A_1 transition in vitamin A-deficient (VAD) rats and mice. Administration of retinol or RA to the VAD rat or mouse reinitiates spermatogenesis in a synchronous fashion by releasing the block on spermatogonial differentiation [4]. Several lines of evidence suggest that RA directly induces spermatogonial differentiation via the expression of numerous RA-targeted genes encoding proteins such as *Stra8*, *Kit*, *Ccnd2*, etc. [5–7]. However, the mechanisms by which RA affects spermatogonial differentiation remain largely unclear, and it is likely that the expression of some genes during RA-induced spermatogonial differentiation undergo posttranscriptional regulation.

MicroRNAs (miRNAs) are small noncoding single-stranded conserved regulatory RNA molecules approximately 22 nucleotides long. They are initially generated from long primary transcripts (pri-miRNA) that have an imperfectly matched stem-loop structure [8]. These pri-miRNAs are first processed by the nuclear RNase III DROSHA and its partner, DGCR8, to produce precursor miRNAs (pre-miRNAs), which are then transported into the cytoplasm and further processed by another cytoplasmic RNase III DICER to yield short double-stranded miR-miR* duplexes. One strand of miR is subsequently incorporated into a miRNA-induced silencing complex (miRISC) [8]. The mature miRNAs direct the miRISC to interact with the seed sequence (nucleotides 2–8 at its 5′ end of miRNA) matched recognition sites, generally in the 3′ untranslated region (UTR) of target mRNA. These interactions inhibit the expression of the target genes at the posttranscriptional level through mRNA decay and translational inhibition [8]. A recent study indicated that more than 60% of human protein-coding genes carry 3′ UTR miRNA target sites, suggesting a potential global role for miRNAs in the regulation of gene expression [9]. Indeed, miRNAs have been shown to play critical roles in a wide spectrum of biological processes, including cell proliferation, differentiation, and apoptosis [8]. Emerging evidence has revealed that miRNAs are present in abundance in male germ cells [10–16] and that miRNAs could play an important role during spermatogenesis [17–23].

The lethal-7 (*let-7*) gene is one of the first two miRNAs identified in *Caenorhabditis elegans* [24]. Mature *Mirlet7* miRNAs play an important role in mammals in the differentiation of stem cells and tumor cells [25]. In this current study, we used both an in vitro and an in vivo system to test the hypothesis that the level of *Mirlet7* family miRNAs is dramatically altered in RA-induced spermatogonial differentiation and likely plays a key role in this process.

MATERIALS AND METHODS

Animals and Treatments

Animal experiments were conducted in accordance with the ''Guidelines for the Care and Use of Research Animals of the National Institutes of Health'' and were approved by the Institutional Animal Care and Use Committee of Washington State University. The C57BL/6 (B6) mice were obtained from the Jackson Laboratory and maintained in a standard animal facility with free access to food and water. To generate VAD mice, B6 female mice were fed a VAD diet (Teklad Trucking) for at least 4 wk and were bred with B6 males. The males born to these dams received this diet until they became VAD. At 14 wk of age, when body weight was slightly decreased, animals were injected with all-*trans*-retinoic acid (ATRA; 1 mg of ATRA per animal in 100 μl of 90% sesame oil and 10% ethanol, i.p., twice for 24 h; Sigma) or injected with

[1]Supported by National Institutes of Health grant HD10808.
[2]Correspondence: Michael D. Griswold, Biotechnology/Life Sciences 202, School of Molecular Biosciences, Washington State University, Pullman, WA 99164. FAX: 509 335 9688; e-mail: mgriswold@wsu.edu

Received: 18 November 2010.
First decision: 18 December 2010.
Accepted: 14 February 2011.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

vehicle as controls twice for 24 h before being euthanized. Immediately after the mice were euthanized, testes were removed and were either used for total RNA extraction using Trizol (Invitrogen) or processed for histological studies.

Cell Cultures

Isolation of THY^+ spermatogonia from 5- to 7-day-old mice on a B6 background was accomplished using magnetic-activated cell sorting as described previously [6]. The purity of isolated spermatogonia was identified by POU5F1 staining, and about 85% of cells were POU5F1 positive (data not shown). Cells were cultured under feeder cell-free and serum-free conditions for 24 h with 0.7 μM ATRA or vehicle at 37°C in an atmosphere of 5% CO_2 in air.

Monolayer cultures of P19 cells (mouse embryonal carcinoma cells; ATCC) were grown in α-minimal essential medium (α-MEM; Invitrogen) containing 10% fetal bovine serum. Cells were treated with ethanol or ATRA for the indicated periods of time.

In Situ miRNA Hybridization with Locked Nucleic Acid Probes

In situ miRNA hybridization (ISH) procedure was performed to examine the spatial expression of *Mirlet7a*, *Mirlet7c*, and *Mirlet7e* in 20-day-old and adult mouse testis based on a published protocol with modifications [26]. Briefly, the testes from adult and 20-day-old B6 mice were fixed for 24 h in 10% neutral-buffered formalin or 4% paraformaldehyde and then dehydrated through a graded ethanol series before paraffin embedding and sectioning at 5 μm. Sections were deparaffinized in two consecutive xylene baths for 5 min each, then 5 min each in serial dilutions of ethanol (100%, 100%, 95%, and 75%), followed by an incubation for 20 min in 0.2 N HCl and two washes with diethyl pyrocarbonate (DEPC)-treated water. Slides were digested with 1 μg/ml proteinase K (Roche) at 37°C for 30 min. The reaction was stopped in 0.2% glycine for 10 min, and the slides were washed twice in DEPC-treated water, immersed in 100% ethanol for 30 sec, and air dried. Slides were then hybridized in an incubation chamber overnight at 37°C using 200 nM digoxigenin (DIG)-labeled locked nucleic acid (LNA) probes (Exiqon) diluted with Enzo ISH buffer (Enzo Diagnostic). After hybridization, slides were washed in 0.2× SSCs with 2% bovine serum albumin at 4°C for 5 min. An anti-DIG/alkaline phosphatase (Roche) antibody at a 1:100 dilution in PBS was applied to the slides for 30 min at 37°C. Slides were washed with detection buffer for 5 min at room temperature and incubated with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt solution (Roche) at 37°C under monitoring. Sections were then counterstained with nuclear fast red (Vector Laboratories) for 2 min, washed in water, and rinsed in 100% ethanol for 2 min and xylene for 5 min. Sections were digitally photographed on a Nikon Microphoto-FX microscope (Meridian Instrument Company Inc.) with an Olympus OLY-200 digital camera (Olympus America Inc.). Sections from at least two B6 mice were analyzed for miRNA localization.

Quantitative RT-PCR Assays

Total RNA was isolated using Trizol reagent per the manufacturer's instructions, treated with DNaseI (Ambion) to remove possible contaminating genomic DNA, and quantified in an ND-1000 Spectrometer (Thermo Scientific). Only RNA samples with a value of ≥1.8 on 260:280 ratios were used for subsequent quantitative RT-PCR (qRT-PCR) analyses. For miRNA qRT-PCR, miRNA expression was determined using Mir-X miRNA First-Strand Synthesis Kit and SYBR Advantage qPCR Premix (Clontech) on the Applied Biosystems 7500 Fast system according to the supplier's protocol. Relative miRNA expression was normalized to the U6 small nuclear RNA (snRNA) according to the ΔC_T model [27]. For mRNA and pri-miRNA qRT-PCR, 200 ng of total RNA from each sample was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed with Fast SYBR Green PCR Mastermix (Applied Biosystems) on the Applied Biosystems 7500 Fast system. Relative expression of *Lin28*, *pri-Mirlet7g*, *pri-Mirlet7d/a/f*, *Col1a2*, *Ccnd1*, and *Mycn* was normalized to the ribosomal protein S2 (*Rps2*) in each sample. Quantitative comparison of a given RNA between RA treatment and vehicle was determined using the comparative C_T method ($2^{-\Delta\Delta CT}$) [27]. Primer pairs used are listed in Supplemental Table S1 (all Supplemental Data are available online at www.biolreprod.org).

Western Blot Analysis

Tissues or cells were lysed and homogenized at 4°C in radioimmunoprecipitation assay buffer (50 mM Tris buffer containing 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, and 1 mM ethylenediaminetetraacetic acid [EDTA; pH 8.0]) in the presence of a protease inhibitor cocktail (Roche). The homogenate was centrifuged at 12 000 × *g* for 2 min, and the resulting supernatant was used for Western blot analysis. Concentrations of the protein samples were determined by the DC protein assay kit (Bio-Rad). Proteins were separated on 10% SDS polyacrylamide gels and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked in 5% nonfat milk (Bio-Rad) and then probed with antibodies against LIN28 (1:400; R&D Systems Inc.), N-MYC (1:200; Santa Cruz Biotechnology), and CCND1 (1:200; Santa Cruz Biotechnology). In most cases, the membranes were stripped after use and reprobed with a β-ACTIN antibody (1:5000; Sigma) to confirm equal protein loading. Immunoreactive proteins were visualized using the Western Lighting ECL detection system (PerkinElmer Inc.).

Chromatin Immunoprecipitation

After treatment with ATRA or EtOH for 24 h, P19 cells were cross-linked with 1% formaldehyde/α-MEM for 10 min at room temperature and quenched by adding glycine to a final concentration of 0.25 M for 5 min. Cells were collected and washed twice with cold PBS containing 1× protease inhibitor cocktail (Roche). Cell pellets were lysed in 1 ml of lysis buffer (1% SDS, 50 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 1× protease inhibitor cocktail) for 10 min on ice and sonicated on ice to obtain a chromatin size of 200 to 1000 bp. After preclearing with protein A/G agarose beads (Upstate), an equivalent amount of sheared chromatin was immunoprecipitated with antibody overnight at 4°C, followed by incubation with protein A/G for 1 h. Antibodies used in chromatin immunoprecipitation (ChIP) assays were anti-RARA (kindly provided by Dr. Rochette-Egly, Institut de Génétique et de Biologie Moléculaire et Cellulaire CNRS/INSERM/ULP), anti-RARG (Santa Cruz Biotechnology), and immunoglobulin G (IgG) control (Santa Cruz Biotechnology). Protein-DNA complexes were eluted in fresh 1% SDS/0.1 M $NaHCO_3$. Cross-linking was reversed, and protein was removed. DNA was recovered and purified for PCR using specific primers. The specific primer sequences were the following: *Lin28*, forward 5′-tggagattgaggcatccagt-3′ and reverse 5′-cgctgtccaatcagaaacac-3′; and *Pou5f1*, forward 5′-cctggggtcccgtcc taagg-3′ and reverse 5′-cctggtggaaagacggctca-3′.

Statistical Analyses

For all analyses, data were statistically processed using a Student *t*-test for all pairs computed by SigmaStat 3.0 (SPSS, Chicago, IL). A P value of ≤ 0.05 was considered significant. Data represent mean ± SD.

RESULTS

Expression of Mirlet7 Family miRNAs in Spermatogonia

We performed miRNA ISH with LNA probes to localize the different *Mirlet7* family miRNAs in mouse testis. We chose *Mirlet7a*, *Mirlet7c*, and *Mirlet7e* as representative members of the family. In the adult testis, *Mirlet7a* staining was mainly observed in the cytoplasm of type A spermatogonia, whereas no *Mirlet7a* staining was seen in Sertoli cells (Fig. 1A). Furthermore, *Mirlet7a* was also present in some spermatocytes (Fig. 1A). A few interstitial cells showed a weak *Mirlet7a* staining (Fig. 1A). Similarly to *Mirlet7a*, ISH staining for both *Mirlet7c* (Fig. 1B) and *Mirlet7e* (Fig. 1C) was seen in spermatogonia and spermatocytes. In a testis from a 20-day-old mouse, *Mirlet7a* staining was present in some spermatogonia, and a weaker staining was observed in a few spermatocytes (Fig. 1D). Both *Mirlet7c* (Fig. 1E) and *Mirlet7e* (Fig. 1F) staining was seen in spermatocytes; however, very weak, if any, *Mirlet7c* and *Mirlet7e* staining was seen in spermatogonia. No staining was detected in Sertoli cells for any *Mirlet7a*, *Mirlet7c*, and *Mirlet7e* probes. No staining for the scramble miRNA was seen in either adult or 20-wk-old testis (data not shown). Together, these results indicate that *Mirlet7* family miRNAs are mainly expressed in premeiotic and meiotic germ cells, suggesting that they may play critical roles in spermatogonial differentiation.

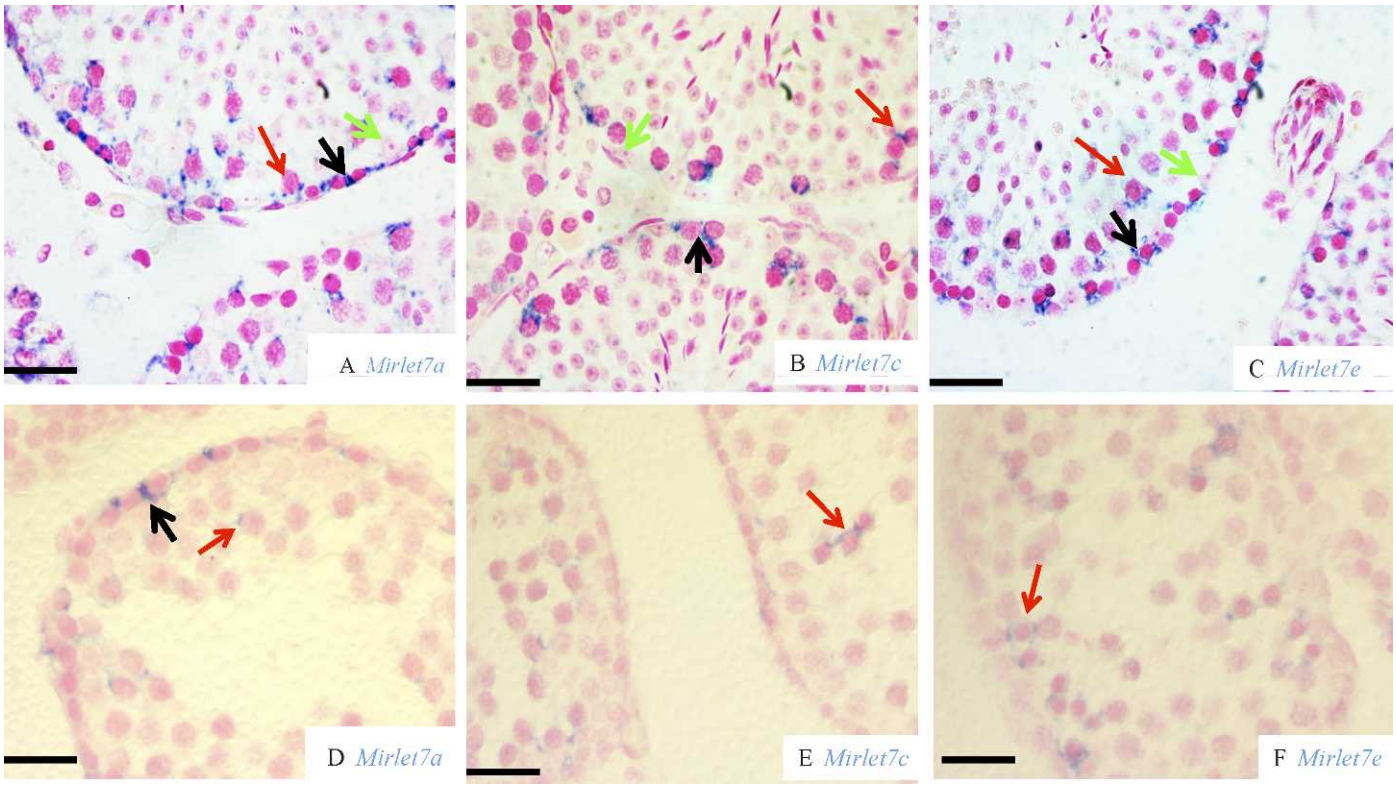

FIG. 1. In situ hybridization of *Mirlet7* family miRNAs in mouse testis. Analysis of *Mirlet7a* (adult [**A**] and 20-day-old [**D**]), *Mirlet7c* (adult [**B**] and 20-day-old [**E**]), and *Mirlet7e* (adult [**C**] and 20-day-old [**F**]) is shown. Cells with positive signal are colored blue. Nucleus was counterstained with fast red. Black arrow, spermatogonia; red arrow, spermatocytes; green arrow, Sertoli cells. Bars = 20 μm.

Expression of Mirlet7 *Family Members Induced by RA Signaling*

Because RA is known to induce spermatogonial differentiation, we next examined whether RA signaling regulates the expression of *Mirlet7* family members. We used both an in vitro model of mouse spermatogonial differentiation and an in vivo model composed of VAD mice. The levels of mature *Mirlet7* family members were determined by miRNA qRT-PCR. Following RA treatment and before miRNA qRT-PCR, we verified that the tissue responded to RA by checking the levels of the RA-responsive gene *Stra8* (also a marker for spermatogonial differentiation). The expected RA induction of *Stra8* was confirmed in all RA-treated samples (data not shown). The melting curve showed a unique peak from the qPCR amplification of each *Mirlet7* family miRNA (Fig. 2a). This demonstrated the specificity of the amplification, whereas the correct product sizes of ~70 bp were verified by gel electrophoresis (Fig. 2b). We found that RA treatment significantly increased the expression of six members of the *Mirlet7* family miRNAs, including *Mirlet7a*, *Mirlet7b*, *Mirlet7c*, *Mirlet7d*, *Mirlet7e*, and *Mirlet7g*, in both in vitro (Fig. 2c) and in vivo (Fig. 2d) experiments. The RA similarly increased the level of *Mirlet7* family miRNAs in P19 cells (Supplemental Fig. S1a). These data strongly indicate that the *Mirlet7* family miRNAs are regulated by RA signaling during RA-induced spermatogonial differentiation.

Posttranscriptional Regulation of RA-Induced Mirlet7 *Family Members by LIN28*

The six members of the mature *Mirlet7* family miRNAs upregulated by RA signaling were produced from six different transcription units (*Mirlet7a-1/7f-1/7d*, *Mirlet7g*, *Mir100/Mirlet7a-2/MiR125b-1*, *Mirlet7a-3/Mirlet7b*, *Mir99a/Mirlet7c/Mir-125b-2*, and *Mir99b/Mirlet7e/Mir125a*). To investigate the mechanisms by which RA signaling regulates these miRNAs, we examined the levels of their primary transcripts in both in vitro and in vivo experimental systems. We chose *pri-Mirlet7a-1/7f-1/7d* and *pri-Mirlet7g* as representative members and found that the abundance of both the *pri-Mirlet7a-1/7f-1/7d* and *pri-Mirlet7g* were not induced by RA (Fig. 3, a and b), suggesting that RA signaling upregulated multiple *Mirlet7* family miRNAs through a posttranscriptional pathway. Recent studies have shown that a conserved RNA-binding protein, LIN28, which is expressed in mouse spermatogonia [28, 29], binds to stem loops of *Mirlet7* precursors and inhibits maturation of the *Mirlet7* family by blocking both DROSHA- and DICER-mediated processing of the *Mirlet7* precursors and accelerating degradation of the *Mirlet7* precursors [30–32]. We therefore examined whether repression of *Lin28* by RA signaling mediated the induction of *Mirlet7* family miRNAs. To test this possibility, we used qPCR and Western blot analysis to examine the mRNA and protein levels of *Lin28* in both in vitro and in vivo experiments with or without RA treatment. Although *Lin28* expression was unchanged in the in vitro experiments 8 h after RA treatment, RA signaling significantly reduced the mRNA levels of *Lin28* in both experimental systems within 24 h of RA treatment (Fig. 3, c and d). As shown in Figure 3, e and f, the protein levels of LIN28 in both RA-treated spermatogonia and VAD testes were significantly decreased compared with that of non-RA-treated spermatogonia and VAD testes (average densitometry ratio of LIN28 and β-ACTIN [mean ± SD] for nontreated spermatogonial cells: 0.175 ± 0.044, n = 4; for RA-treated spermatogonial cells: 0.114 ± 0.008, n = 4; $P < 0.05$ [Fig.

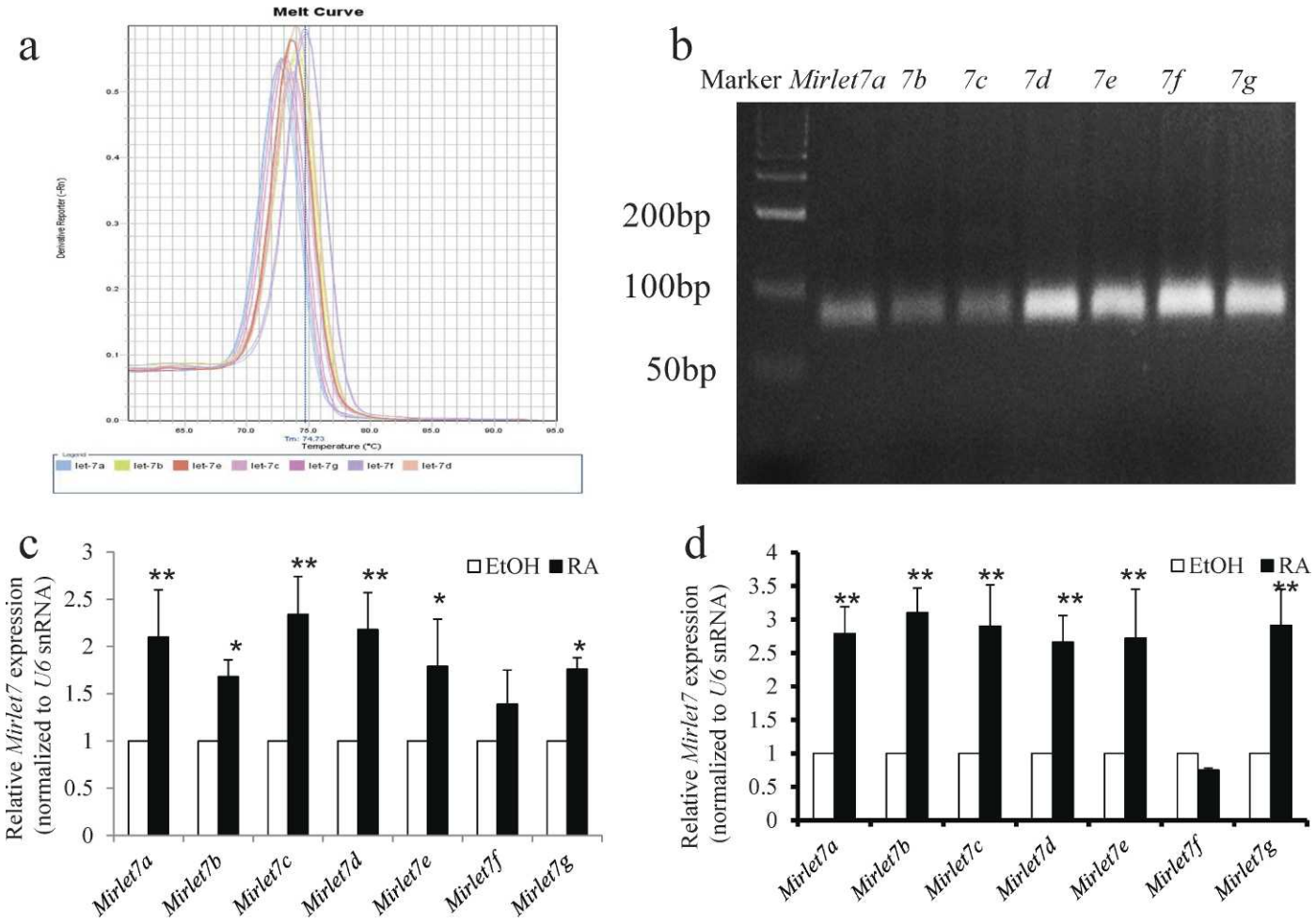

FIG. 2. Retinoic acid signaling induces expression of the *Mirlet7* family miRNAs. **a**) Melt curves of qRT-PCR amplification of *Mirlet7a*, *Mirlet7b*, *Mirlet7c*, *Mirlet7d*, *Mirlet7e*, *Mirlet7f*, and *Mirlet7g*. **b**) The qRT-PCR products for *Mirlet7a*, *Mirlet7b*, *Mirlet7c*, *Mirlet7d*, *Mirlet7e*, *Mirlet7f*, and *Mirlet7g* resolved on 3.5% agarose gel. **c**) Retinoic acid-inducible expression of the *Mirlet7* family miRNAs in isolated THY^+ spermatogonia. THY^+ spermatogonia from 8 to 10 mice in each experiment were pooled and were treated with ethanol or RA (0.7 μM) for 24 h in vitro, and miRNAs were subjected to qRT-PCR. U6 snRNA was used for normalization between samples (mean ± SD, $*P < 0.05$, $**P < 0.001$, ethanol control versus RA treatment for 24 h, n = 3; Student *t*-test). **d**) Treatment of VAD male mice with RA for 24 h significantly increased the expression of the *Mirlet7a*, *Mirlet7b*, *Mirlet7c*, *Mirlet7d*, *Mirlet7e*, and *Mirlet7g* in testes (mean ± SD, $**P < 0.001$, sesame oil control versus RA treatment for 24 h, n = 6; Student *t*-test).

3e]; and average densitometry ratio of LIN28 and β-ACTIN (mean ± SD) for nontreated testes: 0.207 ± 0.015, n = 11; for RA-treated testes: 0.145 ± 0.023, n = 11; $P < 0.01$ [Fig. 3f]). These results suggest that RA could induce the expression of *Mirlet7* family members by repression of *Lin28*. In addition, the level of *Lin28* in P19 cells was significantly decreased by RA treatment, whereas there was no significant difference in the expression of *pri-Mirlet7d/a/f* and *pri-Mirlet7g* between ethanol and RA treatment (Supplemental Fig. S1, b and c).

Retinoic Acid Receptors (RARs) Associated with Conserved Regions Upstream of Lin28

The action of RA on gene expression is mediated by two families of nuclear hormone receptors, the RARs (isoforms α, β, and γ) and the retinoid X receptors (RXRs; isoforms α, β, and γ), which work as RAR/RXR heterodimers. The RAR/RXR heterodimers bind to RA response elements (RAREs), typically composed of two direct repeats of a core hexameric motif, PuG(G/T)TCA, separated by a 5-bp spacer sequence (referred to as DR5) [33]. Promoters of many RA-regulated genes carry RAREs. To examine whether *Lin28* is directly regulated by RA signaling, we performed ChIPs to determine whether RARs associate with a conserved locus upstream of *Lin28*. Inspection of ~2-kb sequences upstream of the transcription initiation site of *Lin28* by TESS (www.cbil.upenn.edu/cgi-bin/tess/tess) [34] revealed the presence of three putative RAREs (designated as RARE1, RARE2, and RARE3; Fig. 4a). We used Vista software [35, 36] to identify several conserved regions ~2 kb upstream of *Lin28* and RAREs with sites showing high conservation among mammals (Fig. 4a). RARE2 (AGGTCAGCGCCA), which exhibits a conserved homology to RARE sequences [37, 38], is located between −647 and −658 relative to the first transcription start site of *Lin28* and is also situated 3′ to an Sp1 consensus binding site. Interestingly, the RARE located closely downstream of the Sp1 binding site in the *Pou5f1* promoter is believed to mediate the RA-induced repression of *Pou5f1* in P19 cells [39, 40]. We therefore designed PCR amplicons within the RARE2 to evaluate RAR binding in ChIP samples. As a positive control, an amplicon was designed within the promoter region of *Pou5f1*. Compared with ChIP samples generated with an IgG, a strong signal of the RARE2 region amplicon in both RARA and RARG ChIP samples was observed in RA-untreated P19 cells, whereas the signal in RARA ChIP samples was greatly reduced by RA treatment, and no signal in RARG ChIP

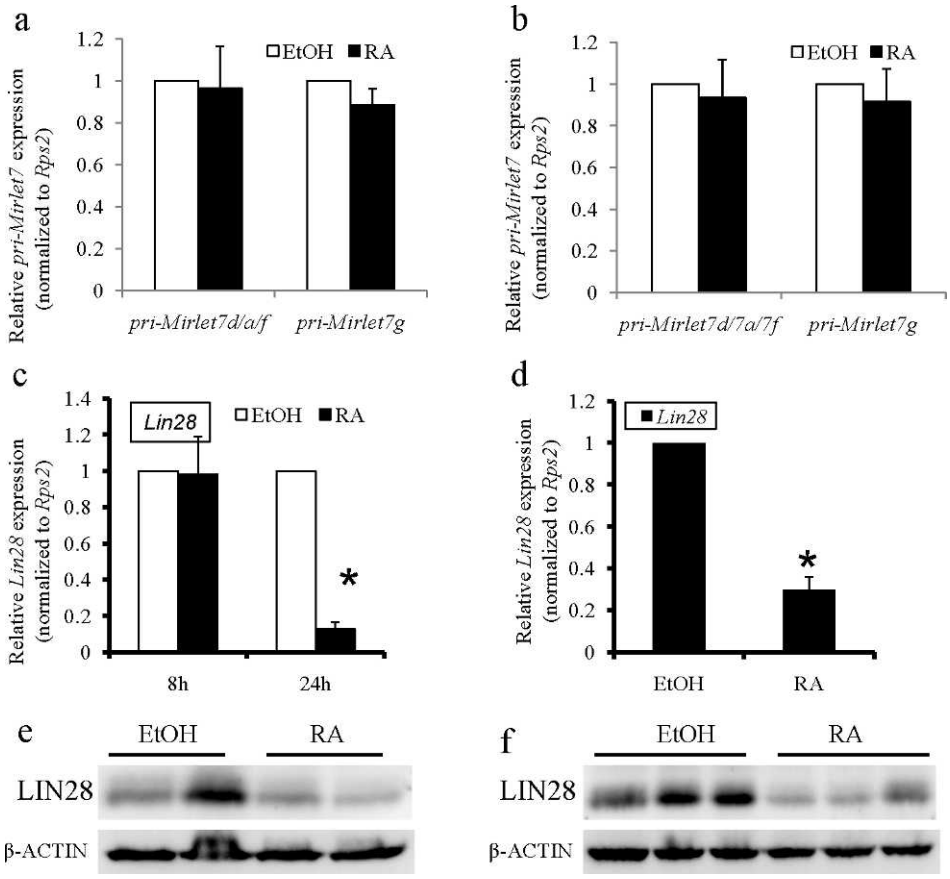

FIG. 3. Posttranscriptional induction of *Mirlet7* expression by RA signaling correlates with *Lin28* repression. **a** and **b**) Quantitative RT-PCR analysis of the abundance of *pri-Mirlet7s* in both the in vitro model (**a**) and in vivo model (**b**) with ethanol vehicle or RA treatment. For the in vitro model, THY^+ spermatogonia from 8 to 10 mice in each experiment were pooled and were treated with ethanol or RA (0.7 μM) for 24 h in vitro. For the in vivo model, VAD male mice were treated with sesame oil or RA for 24 h. Total RNA was subjected qRT-PCR, and *Rps2* was used for normalization between samples. The levels of *pri-Mirlet7d/a/f* and *pri-Mirlet7g* were not significantly different between vehicle and RA treatment in both in vitro ($P > 0.5$ for both, n = 3; Student *t*-test) and in vivo ($P > 0.5$ for both, n = 16; Student *t*-test) model systems. **c** and **d**) Quantitative RT-PCR analysis of the *Lin28* expression in both in vitro (**c**) and in vivo (**d**) models treated with vehicle or RA. Expression of *Lin28* was significantly decreased in both in vitro (*$P < 0.001$, n = 3; Student *t*-test) and in vivo (*$P < 0.001$, n = 16; Student *t*-test) model systems within 24 h of RA treatment, whereas *Lin28* expression was not significantly changed in the in vitro ($P > 0.5$, n = 5; Student *t*-test) model 8 h after RA treatment. **e** and **f**) Western blot analysis of the LIN28 protein in both in vitro (**e**) and in vivo (**f**) models treated with vehicle or RA. The level of LIN28 was decreased in both in vitro and in vivo model systems within 24 h of RA treatment. For the in vitro model, the result shown is representative of two independent experiments. For the in vivo model, the result shown is representative of three independent experiments. Equal protein loading was confirmed by β-ACTIN levels.

samples was obtained in RA-treated P19 cells (Fig. 4b). Thus, RARs are associated with the conserved RARE2 region just upstream of *Lin28* gene, suggesting that RA signaling directly regulates the expression of *Lin28*. We found that both RARA and RARG interact with the *Pou5f1* promoter in untreated P19 cells, but not in RA-treated P19 cells. This result is consistent with the previous observation that in P19 cells, the *Pou5f1* promoter was occupied by RAR/RXR heterodimers before RA treatment, but this occupancy was lost following the treatment [41, 42], demonstrating the specificity of our findings.

Putative Targets of the Mirlet7 *Family miRNAs During RA-Induced Spermatogonia Differentiation*

We used bioinformatics and mRNA microarray analysis to identify *Mirlet7* target genes during RA-induced spermatogonial differentiation. We first investigated whether any genes expressed in mouse spermatogonia are putative targets of *Mirlet7* family miRNAs. Based on computationally predicted target genes found in the database TargetScan (www.targetscan.org) [9, 43], *Mirlet7* family miRNAs are predicted to target 325 mRNAs in mouse spermatogonia (www.wsu.edu/~griswold/microarray.html) [44]. We next determined whether any RA-regulated mRNAs were the targets of *Mirlet7* family miRNAs. Results from a microarray analysis of RA-untreated and 24-h RA-treated VAD mouse testes were used for this purpose (www.wsu.edu/~griswold/microarray.html) [44]. From these data, we found that *Mirlet7* miRNAs potentially target 11 RA-repressed, 20 inducible, and 317 non-RA-regulated genes (Fig. 5a and Supplemental Table S2). Some of the mRNAs (11) in the array that were decreased in RA-treated VAD testis showed conserved *Mirlet7* family miRNA-binding sites in their 3′ UTRs with 7-mer or 8-mer seeds (Fig. 5b). Among these genes, *Mycn*, *Ccnd1*, and *Col1a2* are known to be involved in spermatogonial development [45–48]. We further confirmed by qRT-PCR and Western blot analysis that expression of *Ccnd1*, *Col1a2*, and *Mycn* was significantly downregulated in both RA-treated spermatogonia (Fig. 6, a and c; Fig 6c, average densitometry ratio of N-MYC and β-ACTIN [mean ± SD) for non-RA-treated cells: 0.503 ± 0.055, n = 4; for RA-treated cells: 0.264 ± 0.053, n = 4; $P < 0.05$; average densitometry ratio of CCND1 and β-ACTIN [mean ± SD]: for non-RA-treated cells: 0.130 ± 0.009, n = 4; for RA-treated cells: 0.098 ± 0.007, n = 4; $P < 0.05$) and VAD testes (Fig. 6,

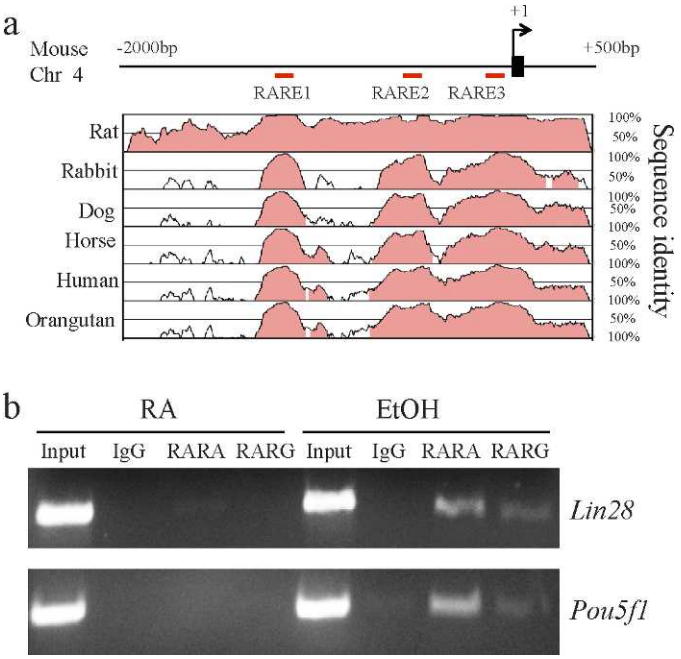

FIG. 4. The RARs associated with the conserved region upstream of *Lin28*. **a**) Vista analysis of phylogenetic conservation encompassing upstream of *Lin28*. Vista was used to generate pairwise alignments between the genomic sequence from mouse and that from the indicated species. Red bars indicate locations of the RAREs, which are present in the conserved regions of *Lin28*. **b**) Chromatin immunoprecipitation analysis showing interaction of both RARA and RARG with the RARE2 (shown in **a**) of *Lin28*. The ChIP DNA samples obtained with RARs antibody and IgG control were subjected to PCR with specific primers encompassing RARE2 upstream of *Lin28* (upper) or the *Pou5f1* promoter as positive controls (lower). The RARs bind to both RARE2 and the *Pou5f1* promoter in RA-untreated P19 cells, whereas only a very weak signal for RARA binding to RARE2 was detected in RA-treated P19 cells. No PCR product was detected in ChIP samples generated with an IgG control.

b and d; Fig. 6d, average densitometry ratio of N-MYC and β-ACTIN [mean ± SD] for non-RA-treated testes: 0.376 ± 0.018, n = 11; for RA-treated testes: 0.305 ± 0.007, n = 11; $P < 0.05$; average densitometry ratio of CCND1 and β-ACTIN [mean ± SD] for non-RA-treated testes: 0.197 ± 0.01, n = 11; for RA-treated testes: 0.171 ± 0.006, n = 11; $P < 0.05$). It is plausible that RA may increase the expression of *Mirlet7* which, in turn, downregulates proliferation-relevant gene expression and promotes spermatogonial differentiation.

DISCUSSION

The role of RA in the initiation of spermatogonial differentiation has been well described [3, 4, 6, 7, 49, 50]. However, the mechanisms underlying spermatogonial differentiation remain to be explored. We proposed in this study that the posttranscriptional regulation of genes by miRNAs may be a molecular mechanism contributing to spermatogonial differentiation. For this study, we focused on determining the role of *Mirlet7* family miRNAs in RA-induced spermatogonial differentiation. We chose this family because: 1) mature *Mirlet7* is highly conserved across metazoans [51]; 2) the *Mirlet7* family processing regulator LIN28 is expressed in mouse undifferentiated spermatogonia [29]; and 3) its role in multiple differentiation events in various tissues has been well established [25, 52].

The testis has a complex miRNA signature, and its role in testis function is just beginning to be examined. We found in this study that the *Mirlet7* family miRNAs are expressed in spermatogonia and spermatocytes. We have presented evidence here that RA-induced spermatogonial differentiation is accompanied by an increase of six members of mature *Mirlet7* miRNAs. The fact that the levels of *Mirlet7* primary transcripts were not controlled by RA was striking and suggested that a common pathway was implicated in the processing of *Mirlet7* family miRNAs. In embryonic stem cells and primordial germ cells, the pluripotency factor LIN28 has been shown to act as a specific inhibitor of all *Mirlet7* family miRNA processing [30, 32, 53]. *Lin28* is expressed in undifferentiated spermatogonia [28, 29], and our microarray experiments and qPCR analysis demonstrate that *Lin28* is repressed within 24 h of RA treatment in spermatogonia. Chromatin immunoprecipitation analyses have further shown that RARs associate directly with a conserved RARE upstream of *Lin28*. These results indicate that inhibition of *Lin28* could account for the RA-driven induction of the *Mirlet7* miRNAs in spermatogonia.

There is extensive evidence to document a potent differentiation and antiproliferation activity of the *Mirlet7* family. The *Mirlet7* miRNAs are associated with cellular differentiation, and enforced expression of *Mirlet7* inhibits self-renewal/proliferation and promotes differentiation [54–56]. These functions are attributable to the capacity of *Mirlet7* to silence the self-renewal program by suppressing many

a.

	Number of gene	Number of *Mirlet7* family miRNAs targeted gene
mRNAs repressed by RA (FC≤1.5)	237	11
mRNAs induced by RA(FC≥1.5)	388	20
Non-RA-regulated mRNAs	10010	317

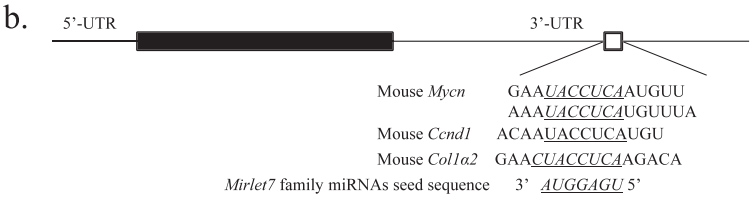

FIG. 5. The RA-regulated genes potentially targeted by *Mirlet7* family miRNAs. **a**) Number of genes potentially targeted by *Mirlet7* family members in RA-treated VAD testes. The mRNAs are subclassified into RA-repressed, RA-induced, and nonregulated by RA but expressed in spermatogonia based on microarray analysis of THY^+ spermatogonia, vehicle, or RA-treated VAD testes (www.wsu.edu/~griswold/microarray.html). FC, fold change. **b**) *Mirlet7* miRNAs target the *Ccnd1*, *Col1a2*, and *Mycn* 3′ UTR. Sequence alignment shows a putative *Mirlet7* complementary site in the *Col1a2* and *Ccnd1* 3′ UTR and two putative *Mirlet7* complementary sites in the *Mycn* 3′ UTR.

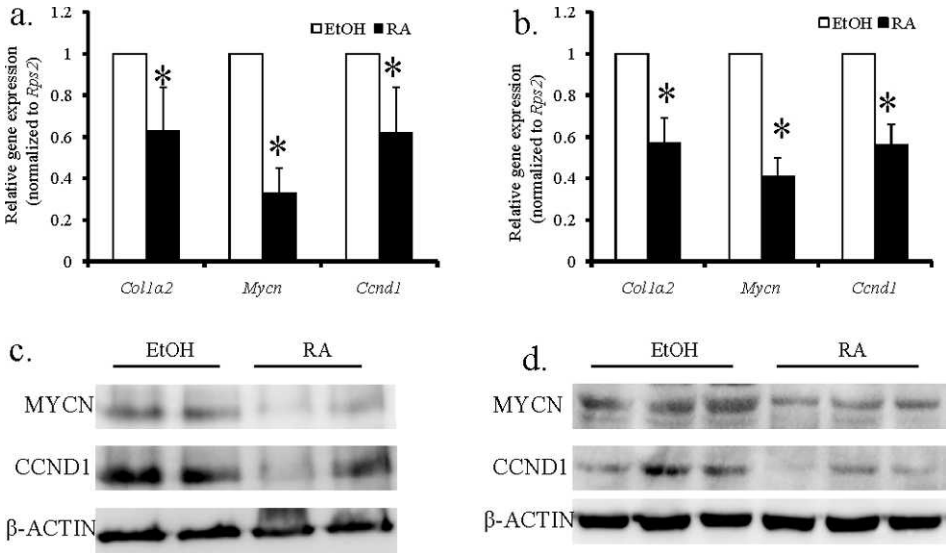

FIG. 6. Quantitative RT-PCR (**a** and **b**) and Western blot analysis (**c** and **d**) of *Mycn, Ccnd1*, and *Col1a2* in both in vitro (**a** and **c**) and in vivo (**b** and **d**) models treated with vehicle or RA. Data from qRT-PCR analyses are expressed as fold differences compared with vehicle-treated samples (mean ± SD, $*P < 0.01$, $n = 3$ for in vitro model, $n = 14$ for in vivo model; Student *t*-test). *Rps2* was used for normalization between samples. Compared with non-RA-treated THY^+ spermatogonia (EtOH; **c**) and VAD testes (EtOH; **d**), RA-treated THY^+ spermatogonia (RA; **c**) and VAD testes (RA; **d**) had reduced expression of MYCN and CCND1. For the in vitro model, THY^+ spermatogonia isolated from 8 to 10 pups were pooled in each group, and the result shown is representative of two independent experiments. For the in vivo model, protein was extracted from VAD testis, and the result shown is representative of three independent experiments. Equal protein loading was confirmed by β-ACTIN levels.

downstream targets, including *Ras*, *Hmga2*, *Myc*, *Mycn, Ccnd1*, and even *Lin28* itself [54, 56–60]. To assess the contribution of *Mirlet7* miRNAs to the regulation of spermatogonial differentiation, we determined whether the expression of their potential targets can be changed as a result of RA in this process. Identification of an miRNA target by using an mRNA transcriptome database is relatively complex because most miRNAs regulate gene expression through translational repression rather than mRNA degradation in animals [61]. To overcome this obstacle, we first focused only on the genes with expression levels that showed a significant inverse correlation with *Mirlet7* miRNAs. By analyzing the expression profile from VAD mice treated with RA, we found a downregulation of 11 genes within 24 h of RA treatment. Of the 11 gene candidates, we chose *Mycn*, *Ccnd1*, and *Col1a2* for further investigation. These genes were selected because 1) *Ccnd1*, a key cell cycle regulator, has been implicated in spermatogonial proliferation, particularly during the G_1/S transition [47, 48]; 2) *Mycn*, which is expressed in undifferentiated spermatogonia, has been shown to be involved in glial cell line-derived neurotrophic factor (GDNF)-induced SSC self-renewal/proliferation [46, 62, 63]; and 3) *Col1a2* is associated with type A spermatogonia [45]. Using a bioinformatics approach and experimental validation, we identified *Mycn*, *Ccnd1* and *Col1a2* as targets of *Mirlet7* in spermatogonia. We found that the expression of *Mycn*, *Ccnd1*, and *Col1a2* is significantly reduced, whereas the level of *Mirlet7* family miRNAs is dramatically increased upon RA-induced spermatogonial differentiation. Moreover, the direct downregulation of *Mycn*, *Ccnd1*, and *Col1a2* by *Mirlet7* miRNAs has been experimentally validated in other systems [52, 54, 64]. These studies suggest that *Mirlet7* miRNAs play a critical role in spermatogonial proliferation and differentiation through targeting key genes.

It is interesting to note that *Lin28* is itself a target gene for *Mirlet7*, suggesting that the regulatory interaction between *Mirlet7* and *Lin28* functions as a feed-forward loop. A possible mechanism could be that RA induces spermatogonial differentiation, resulting in the inhibition of *Lin28* expression. With the decrease of *Lin28*, *Mirlet7* levels rapidly increase. This increase in *Mirlet7* levels could then in turn suppress its negative regulator *Lin28* in spermatogonia. Moreover, repression of *Mycn* and *Ccnd1* by *Mirlet7* miRNAs blocks spermatogonial proliferation and promotes differentiation. Furthermore, *Myc* and *Mycn* have been shown to be a global regulator of miRNA expression and can activate expression of stem cell-associated miRNAs (such as the *Mir-17-92* cluster and *Mir-290* cluster) to stimulate cell proliferation/self-renewal [65]. Like *Mycn*, *Myc* is also a direct target for *Mirlet7* [57]. Interestingly, *Myc* is highly expressed in undifferentiated spermatogonia [63]. The downregulation of *Mycn* and even *Myc* by *Mirlet7* could prevent the expression of stem cell-associated miRNAs in spermatogonia. Therefore, our studies suggest an RA signal-*Lin28-Mirlet7-Mycn* regulatory loop that may contribute to RA-induced spermatogonial differentiation.

Last, our results provide new insight into the role of an RA-regulated miRNA, *Mirlet7*, in spermatogonial differentiation. However, this study does not exclude the possibility that other RA-regulated miRNAs may also play a role in spermatogonial differentiation. Future experiments are necessary to uncover additional *Mirlet7* targets and other RA-regulated miRNAs, and to clarify the role of miRNAs in the regulation of spermatogonial differentiation.

ACKNOWLEDGMENTS

We thank Dr. Cécile Rochette-Egly for providing antibodies. We thank Dr. Cathryn Hogarth for critical reading of this manuscript.

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