Cyclin T2: A novel miR-15a target gene involved in early spermatogenesis

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Abstract

MicroRNAs (miRNAs) are posttranscriptional modulators of gene expression that play important roles in various biological processes. Spermatogenesis is a highly regulated process in which diploid spermatogonia eventually differentiate into haploid spermatozoa. In this study, we identified four differentially expressed miRNAs between two premeiotic male germ cells, made predictions about their putative targets, and confirmed cyclin T2 (Ccnt2) as a direct target of miR-15a. We also report that miR-15a inhibited muscle differentiation at least in part by targeting Ccnt2, which represents a novel interaction. Subsequently, miR-15a and Ccnt2 were profiled in developing mice testes to observe their inverse correlations in the postnatal 3-week period to understand their roles in spermatogenesis.

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1. Introduction

MicroRNAs (miRNAs) are a class of widely distributed and highly conserved non-coding RNAs about 22 nucleotides (nt) long. They are thought to play negative regulatory roles posttranscriptionally, causing mRNA cleavage or translation repression, by targeting the imperfect complementary sequences, usually located in the 3' untranslated regions (UTRs) of mRNAs [1]. It has been reported that miRNAs are involved in various biological processes, including the cell cycle, differentiation and tumorigenesis [2–4]. Additionally, the temporal and spatial specificity that is frequently associated with development and differentiation is another characteristic of miRNA expression [5,6].

As a multilevel differentiation process, spermatogenesis functions to perform cyclic gamete production in which diploid spermatogonia eventually differentiate into haploid spermatozoa. Spermatogenesis can be theoretically separated into three phases: the mitotic, meiotic and spermiogenesis phases. For mice, the first generation of spermatogonia emerge at the 6th day post-partum (dpp), and the type B spermatogonia first appear at the 8th dpp, whereas the pre-leptotene spermatocytes emerge around the 10th dpp and the round spermatids at the 20th dpp [7].

In recent years, studies on miRNA regulation in spermatogenesis have rapidly become one of the focal points in studies on male reproduction. For instance, miR-122a was thought to be involved in the direct regulation of Tnp2, a testis-specific, crucial protein involved in chromatin remodelling in postmeiotic germ cells [8]. miR-34c was recently suggested to enhance the late stages of spermatogenesis by reinforcing the germ cell phenotype [9]. Dead end 1 (Dnd1), an evolutionarily conserved RNA binding protein, was shown to be essential for primordial germ cell development and spermatogenesis in zebrafish and mouse by protecting certain mRNAs from miRNA-mediated repression [10–13], and was newly reported to regulate mitotic arrest and differentiation in male germ cells [14]. Additionally, Dicer, a requisite enzyme for miRNA processing, was proved to be crucial for both the male germline and Sertoli cells in spermatogenesis [15–18].

In this work, a miRNA microarray analysis was performed using two lines of mouse germ cells, GC-1 spg and GC-2 spd, which were type B spermatogonia and premeiotic spermatocytes, respectively, to determine the profiles of differentially expressed miRNAs (DEM). During spermatogenesis, these cells are representative of two successive differentiation stages prior to the onset of meiosis, or rather as two transitional stages in the shift from mitosis to meiosis. The profiling of these stages may allow for some insight into
the nascent period of meiosis. We identified cyclin T2 (Cc12) as a novel target of miR-15a and report that by partially targeting Cc12 at least, miR-15a had a negative effect on muscle differentiation, as well as in the early steps of spermatogenesis.

2. Materials and methods

2.1. miRNA microarray

Total RNA was extracted using TRizol Reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA (5 μg) was labelled with Hy3 using the miCURRY™ LNA microRNA Array labelling kit (Exiqon A/S), and the labelled samples were concentrated using the RNeasy Mini Kit (Qiagen) as recommended. miRNA expression profiles between GC-1 and GC-2 cells were produced by the hybridisation of the labelled RNA and the LNA-based capture probes on the microarray slides (miCURRY LNA™ microRNA Array V.8.0, Exiqon A/S). The spike-in miRNA controls (miCURRY LNA™ Array Spike-in miRNA Kit, Exiqon A/S) were applied as on-chip normalisers. Hybridisation and washing steps were performed as instructed. Hybridisation signals were detected using the GenePix 4000B Array Scanner (Axon Instruments) at 635 nm. Images were analysed in GenePix Pro 6.0 (Axon) and saved as Excel files. Hybridisations were performed in duplicate, and the mean values of miRNA expression and the fold changes between GC-1 and GC-2 cells were recorded.

2.2. Cell culture and differentiation

GC-1 spg and GC-2 spd cells were purchased from American Type Culture Collection (ATCC) and cultured in DMEM containing 10% foetal bovine serum (FBS, Invitrogen), HEK 293T, NIH/3T3, C2C12, TM3, TM4, P19 and CT26.WT cells were obtained from the Cell Resource Centre, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (CAMS). NIH/3T3 cells were cultured in DMEM with 10% bovine calf serum (BCS, Invitrogen); TM3 and TM4 cells in a 1:1 mixture of Ham’s F12 medium (Invitrogen), DMEM with 1.2 g/l sodium bicarbonate, 15 mM HEPES supplemented with 5% horse serum (HS, Invitrogen) and 2.5% FBS. P19 cells were maintained in Alpha Minimum Essential Medium with ribonucleosides, deoxyribonucleosides (Invitrogen), 7.5% BCS and 2.5% FBS, and CT26.WT cells were cultured in RPMI-1640 medium with 10% FBS. Other cells were cultured in DMEM with 10% FBS. To induce myodifferentiation [19], growth medium (GM) maintaining logarithmically-grown C2C12 cells was substituted with DMEM containing 2% HS (differentiation medium, DM) at a 70% confluence.

2.3. Reverse transcription and quantitative PCR (RT-qPCR)

miRNAs in 1 μg total RNA were reverse transcribed using specific stem-loop RT primers and the ReverTra Ace-α-First strand cDNA synthesis kit (Toyobo) as instructed by the manufacturer. qPCR was performed following Chen’s methods [20] with Power SYBR Green PCR Master Mix (Applied Biosystems), the RT product, a miRNA-specific forward primer and a universal reverse primer using the StepOnePlus Real-Time PCR System (Applied Biosystems), according to the company’s instructions. The reactions were incubated at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 45 s. Melt curves were analysed using default methods. U6 small nuclear RNA (snRNA) was utilised as an internal normaliser. As for Cc12, 1 μg total RNA was reverse transcribed as instructed, using β-actin as an internal reference gene (all primers are listed in Supplementary materials). The method of the comparative CT (∆∆CT) quantitation was applied as previously described [21]. Three independent experiments were completed, and each PCR reaction was performed in triplicate.

2.4. Target prediction

Putative target genes of the DEMs were generated from three popular on-line algorithms: miRanda, PicTar and TargetScan. Common targets were chosen to undergo subsequent luciferase assays.

2.5. Constructs, transfection and Dual-Luciferase Reporter Assay

The 3’-UTR sequences containing the putative targets of miRNA candidates were cloned immediately downstream of the firefly luciferase coding region in the pGL3-Promoter Vector (Promega), between the XbaI and FseI restriction sites and later between EcoRV and PstI sites added to the vector. Specific mutations corresponding to the potential target site were set up by PCR-driven overlap extension as Heckman described [22] and inserted into the pGL3 vector (primers listed in the Supplementary materials).

Lipofectamin 2000 (Invitrogen) and FuGENE HD (Roche) were used for GC-1 and C2C12 cell transfection, respectively, as instructed. Synthetic miRNA mimic/mimic negative control (NC) and/or inhibitor/inhibitor NC (20 nM each, GenePharma), pGL3 plasmid (0.4 μg) and the Renilla luciferase expression vector, pRL-TK (0.01 μg), were cotransfected into HEK 293T cells in 12-well plates using FuGENE HD for 24 h. Firefly and Renilla luciferase activities were detected using the Dual-Luciferase Reporter (DLR) Assay System (Promega) as instructed, with the latter serving as a normaliser. Each assay was performed independently in triplicate, at least two times.

2.6. Animals, tissue preparation and isolation of spermatogenic cells

Male BALB/c mice were obtained from the Laboratory Animal Centre, CAMS. All animal experiments were approved by the Animal Care and Use Committee at the Institute of Basic Medical Sciences, CAMS. Testes from 1- to 8-week-old male BALB/c mice were collected and divided into two parts. One part was quickly frozen in liquid nitrogen and homogenised in TRIzol Reagent for RNA extraction, while the other half was homogenised in SDS lysis buffer for protein detection.

Developing mice mentioned above were collected for isolation of spermatogenic cells according to a procedure described previously [23]. Briefly, decapsulated testes were incubated in 0.5 mg/ml collagenase (Sigma) at room temperature for 15 min with gentle oscillation to release interstitial cells. After passing through 100 μm copper mesh, the interstitial cells were discarded. Seminiferous tubules were resuspended in 1 mg/ml collagenase at room temperature for 20 min to remove myoid cells. The tubules were then incubated in 0.5 mg/ml hyaluronidase (Sigma) for 15 min with oscillation and pipetting. The dispersed cells were cultured in F12/DMEM with 10% FCS at 32 °C for 24 h. Non-adherent spermatogenic cells were collected.

2.7. Western blotting

Total protein from mouse testes (from 1- to 8-week-old mice) or various cell types was loaded on a SDS–polyacrylamide gel and electrically transferred to a PVDF membrane (Millipore). The membrane was blocked in 5% skim milk for 1 h at room temperature and incubated with the appropriately diluted primary antibodies at 4 °C overnight. After three washes with 0.1% TBS-T, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. The membrane was washed another three times, and the image was visualised by chemiluminescence using the ECL Reagent (Engreen). Primary
antibodies included anti-cyclin T2a/b (Santa Cruz), anti-myogenin (Abcam) and anti-mysin heavy chain (Abcam). As references, anti-GAPDH (Santa Cruz) or anti-β-tubulin (Abcam) were applied.

2.8. Northern blotting

Total RNA (20 μg per lane) was fractionated on a 15% denaturing polyacrylamide gel containing 8 M urea, transferred to a Hybond-N+ nylon membrane (Amersham Biosciences) and fixed by UV cross-linking (GS Gene linker UV Chamber, Bio-Rad) and baking (80 °C, 15 min). Membranes were hybridised with a γ-32P-labelled LNA-modified probe (Exiqon A/S), complementary to miR-15a. Hybridisations were carried out overnight at 37 °C in PerfectHyb™ Hybridization Solution (Toyobo). The membranes were washed twice for 5 min in 2 × Standard Saline Citrate (SSC), 0.1% SDS at room temperature. Autoradiography was performed to visualise the images. For reference, the membranes were stripped for rehybridisation with U6 snRNA with a probe sequence of 5'-GGGCC ATGCTAATCTTCTCTGT-3'.

2.9. Statistical analysis

Data are presented as the means ± S.D. Student’s t-test was adopted to analyse the differences between groups. P values <0.05 were considered statistically significant.

3. Results

3.1. Screening and verification of the DEMs

miRNA microarrays of GC-1 and GC-2 cells were performed (Fig. 1A). Following the output analysis, 28 miRNAs stood out according to the inclusion criteria, i.e., GC-1/GC-2-fold change >2 or <0.5, among which 20 miRNAs were higher in GC-1 cells, while the other 8 were higher in GC-2 cells (Table 1).

We were primarily interested in which genes were upregulated during the transitional period from mitosis to meiosis. Given the reciprocal relationship between a miRNA and its target, the 20 miRNAs that were highly expressed in GC-1 cells were the first batch to be verified. Four miRNAs—miR-15a, miR-125a-5p, miR184 and miR-468—were selected randomly to determine whether they were actually expressed at higher levels in GC-1 cells (Fig. 1B), which would be consistent with the outputs of the microarrays.

3.2. Ccnt2 is a direct target of miR-15a

Target prediction was subsequently performed with the widely used algorithms: miRanda, PicTar and TargetScan. DLR assays were performed using the constructs containing the predicted sites to evaluate the authenticity of the putative targets (Supplementary Fig. 1), among which only the 3'-UTR of Ccnt2, one of the putative targets of miR-15a, caused a marked reduction in luciferase activity (Fig. 2A).

Ccnt2 is composed of two splice variants (T2a and T2b), is a member of the T-type cyclins, which have been identified only in the past decade [24] and are widely expressed among tissues [25]. Coupled with CDK9, Cnt2 forms a complex known as ‘positive transcription elongation factor b’ (P-TEFb), which is generally considered to function in a cell cycle-independent manner [26] and is involved in muscle differentiation [19,27]. Eight mouse cell lines were collected for the detection of Cnt2 and miR-15a. It was observed that an inverse relationship existed between Cnt2 and miR-15a (Fig. 2B and C), which suggested a regulatory relationship between them. Alignments of the potential target site indicated that the sequence was highly conserved across species (Fig. 2D).

We noticed perfect base pair matches in the seed region (2–8 nt, at the 5’ end of the miRNA). Mutation assays were performed as indicated (Fig. 2E and F). In contrast to the negative control, the luciferase activity of the wild-type group decreased significantly (P < 0.01) in the presence of a miR-15a mimic, whereas the mutant groups restored luciferase activity to varying degrees. Especially in mutant-2, which contained a mutation that covered the entire base-pairing region at the 5’ end, the restored activity level was comparable to that observed in the control, suggesting the importance of base pairing beyond the seed region. Conditions in mutant-1 and mutant-3 revealed the intermediate states (P < 0.01), supporting the idea that the seed region of miRNAs is not always dominant enough to target a gene. Conversely, the addition of a miR-15a inhibitor restored luciferase activity to the wild-type, mutant-1 and mutant-3 groups (P < 0.01) but not in the null and mutant-2 groups. Furthermore, transfection of a miR-15a mimic or inhibitor (50 nM each) into GC-1 cells resulted in a marked reduction or increase of Cnt2 protein levels, respectively (Fig. 2G, Supplementary Fig. 2).

3.3. miR-15a inhibits muscle differentiation by targeting Cnt2

Based on the involvement of the CDK9/Cnt2 complex in muscle differentiation [19,27], we hypothesised that miR-15a may have an effect on this process. We observed this process in a differentiation-inducible myoblast, C2C12. A miR-15a mimic or siRNA from Cnt2 (50 nM each) were transfected into C2C12 cells in advance. After a 48-h incubation, miR-15a was exclusively overexpressed in corresponding cells (Fig. 3A), while Cnt2 mRNA was downregulated in both siRNA and mimic treated cells compared with controls (Fig. 3B), suggesting that a cleavage of Cnt2 mRNA was induced by miR-15a. Similar results were obtained at the protein level (Fig. 3C). Myodifferentiation was induced after transfection, with early and late differentiation markers, myogenin and myosin heavy chain (MHC), respectively, being detected at the appropriate times. Following the progress of differentiation, the levels of myogenin and MHC increased continuously, whereas that of Cnt2 decreased gradually. Compared to the negative control, miR-15a led to a truncated build-up of myogenin and MHC (Fig. 3D and E). Morphologically, the formation of myotubes was delayed by miR-15a (Fig. 3F). Briefly, miR-15a negatively affected muscle differentiation by partly targeting Cnt2 at least.

3.4. Cnt2 is important for early spermatogenesis

Do Cnt2 and miR-15a make a difference during spermatogenesis? Cnt2 was detected in developing mice testes at different levels. Cnt2 mRNA rose continually in the postnatal 3-week period, while the larger form (T2a in mice) increased markedly from the 3rd week (Fig. 4C and D), suggesting that a cleavage of Cnt2 mRNA was induced by miR-15a. Based on these results, Cnt2 and miR-15a might be crucial in early spermatogenesis. As for the Cnt2 protein, it was observed that an inverse relationship existed between Cnt2 and miR-15a (Fig. 4E and F). In contrast to the negative control, the luciferase activity of the wild-type group decreased significantly (P < 0.01) in the presence of a miR-15a mimic, whereas the mutant groups restored luciferase activity to varying degrees. Especially in mutant-2, which contained a mutation that covered the entire base-pairing region at the 5’ end, the restored activity level was comparable to that observed in the control, suggesting the importance of base pairing beyond the seed region. Conditions in mutant-1 and mutant-3 revealed the intermediate states (P < 0.01), supporting the idea that the seed region of miRNAs is not always dominant enough to target a gene. Conversely, the addition of a miR-15a inhibitor restored luciferase activity to the wild-type, mutant-1 and mutant-3 groups (P < 0.01) but not in the null and mutant-2 groups. Furthermore, transfection of a miR-15a mimic or inhibitor (50 nM each) into GC-1 cells resulted in a marked reduction or increase of Cnt2 protein levels, respectively (Fig. 2G, Supplementary Fig. 2).

4. Discussion

In this study, we identified 4 DEMs between GC-1 and GC-2 cells and demonstrated that Cnt2 is a novel target of miR-15a.
Notably, our results indicated that the base pairing at the 3' end of miR-15a was more essential for Ccnt2 recognition, which is in disagreement with the popular theory that the 5' seed region of miRNAs acts as a key determinant in recognition [28,29]. In fact, miRNAs tend to be conserved over the full length, therefore the 3' end of miRNAs probably make sense too. Generally, miRNA target sites are classified into three categories [30,31], 5'-dominant canonical, 5'-dominant seed and 3'-compensatory sites. The canonical sites pair well at both 5' and 3' ends of the miRNA, the seed sites have perfect base pairing at the 5' end and require limited or no 3' pairing support, while the 3'-compensatory sites have weak 5' base pairing and depend on strong compensatory pairing to the 3' end of the miRNA. It has been also reported that transcripts with 3'-compensatory sites can discriminate between miRNA family members [30]. None of the above types matched well to the site we identified. An intermediate form between the 5'-dominant seed and 3'-compensatory sites might be more indicative of what we observed.

As a member of the miR-15a/miR-16-1 cluster, miR-15a has long been considered an important tumour suppressor associated with the inhibition of cell proliferation, the promotion of apoptosis and the suppression of tumorigenesis by specifically targeting

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### Table 1

Differentially expressed miRNAs between GC-1 and GC-2 cells, showing as the fold changes of GC-1/GC-2 expression values.

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<th>MicroRNA</th>
<th>Fold change</th>
<th>MicroRNA</th>
<th>Fold change</th>
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<tr>
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* Fold change >2 or <0.5 is considered as an inclusion criteria.
miR-15a directly targets the 3'-UTR of Ccnt2. (A) Normalised luciferase activity from the Ccnt2 3'-UTR in the miR-15a mimic or in the negative control (NC)-transfected HEK 293T cells. The ratio in the NC was set to 100%. (B, C) Detection of Ccnt2 protein and miR-15a among types of mouse cell lines. GAPDH and U6 snRNA were used as references, respectively. In (C), the expression value from GC-1 cells was set to 1. (D) Imperfect complementation between miR-15a and the potential target site in the Ccnt2 3'-UTR, and the alignments of the target site with various species. The red letters represent the sites complementary to the seed region of miR-15a; the blue letters indicate the base differences between species. The evolutionarily conserved nucleotides are listed at the bottom. (E) Mutations corresponding to the target sites. Different mutagenic sites are indicated in corresponding colours. (F) Normalised luciferase activities of wild-type (WT) construct and a series of mutants cotransfected with small RNAs in HEK 293T cells. The empty pGL3-promoter (Null) served as the intergroup reference. The ratios of the NC of each group were set to 100%. Mi-NC, mimic negative control; In-NC, inhibitor negative control; 15a Mi, miR-15a mimic; 15a In, miR-15a inhibitor. * and ** represent \( P < 0.01 \). (G) Detection of Ccnt2 in small RNA transfected GC-1 cells. GAPDH served as the loading control. LO, Lipofectamin Only.
numerous carcinogenic genes since it was identified [32]. miR-15a downregulation has been associated with various cancers [32,33]. Few studies have suggested that miR-15a is involved in cell differentiation, although it has been thought to be associated with development and differentiation [34,35]. Our findings demonstrated that miR-15a decreased continuously before meiosis and probably contributed to the on-going process of spermatogenesis, which is similar to the effect observed with macrophage differentiation [36].

P-TEFb is thought to facilitate the transition from abortive to productive elongation catalytically by phosphorylating the carboxy-terminal domain (CTD) of RNA polymerase II [37]. Genetic analysis has implicated a broad requirement for the CDK9/cyclin T complex in *Caenorhabditis elegans* during early embryonic gene expression [38]. As a regulatory subunit of P-TEFb, cyclin T, including T1, T2a and T2b, is also required for most genes transcribed by RNA polymerase II [39]. However, the genes affected by these cyclins are different, and among them, the pathways important for early embryogenesis and development are specifically under the control of Ccnt2 [25]. Additionally, as reported previously, cyclin T functions in various types of cell differentiation [19,40]. In this work, we confirmed that Ccnt2 was essential for muscle differentiation. The continuous reduction of Ccnt2 during myodifferentiation, described previously [41], suggested that Ccnt2 was important for the initiation of cellular differentiation.

Until now, little was known about how Ccnt2 and miR-15a functioned in spermatogenesis. Our work shows an inverse relationship between them in the developing mouse testes during the

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**Fig. 3.** Effect of miR-15a on muscle differentiation. (A–C) Detection of miR-15a and Ccnt2 in small RNA transfected C2C12 cells. In (A, B), the expression value in the FO group was set to 1. In (C), tubulin served as the loading control. FO, FuGENE HD Only; C-Si, Ccnt2 siRNA. (D–F) Differentiation of C2C12 cells in different time courses after 48 h of transfection. In (D, E), levels of Ccnt2, myogenin and MHC were determined by Western blotting, and tubulin was detected as a loading control. In (F), images under the light microscope were captured. The scale bars indicate 500 μm.
postnatal 3-week period in which spermatogenic cells exist as spermatogonia and spermatocytes. Following the production of a large amount of spermatids, the expression of Ccnt2 and miR-15a in the testis decreases to a relatively low level, suggesting that Ccnt2 and miR-15a are crucial in early spermatogenesis, including meiosis, but not in the postmeiotic steps. Consequently, based on its known function as well as our findings, we rationally speculate that Ccnt2 promotes germ cell differentiation in the early or premeiotic steps of spermatogenesis, whereas miR-15a negatively regulates Ccnt2 by targeting it during this time.

Due to the lack of a spermatogenic differentiation model in vitro, we cannot observe the effects of Ccnt2 and miR-15a on germ cell differentiation directly. Because Ccnt2+/− mice have an early embryonic lethal phenotype [25], the generation of miR-15a genetically inactivated mice might help clarify their effects on this process. Additionally, in light of our results and its tumour suppressor characteristics, miR-15a is quite promising and may be applied in the treatment of male sterility or testicular cancer in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.06.031.

References


Fig. 4. Expression of Ccnt2 and miR-15a in developing mice testes and spermatogenic cells. Ccnt2 in developing mice testes up to 8 weeks old was detected at both RNA (A) and protein (B) levels. miR-15a concentration in developing mice testes was determined by RT-qPCR (C) and Northern blotting (D). In (A, C), the expression value from 1-week-old testis was set to 1. In (B, D), GAPDH and U6 snRNA served as loading controls, respectively. The expression levels of Ccnt2 and miR-15a were quantified by density analysis, then were normalised to their loading controls, shown as the numbers between the two panels of blots. The normalised density value from 1-week-old testis was set to 1. Ccnt2 mRNA (E) and miR-15a (F) in developing mice spermatogenic cells up to 8 weeks old were detected by RT-qPCR. The expression value from 1-week-old spermatogenic cells was set to 1.


