miRNA-122-5p stimulates the proliferation and DNA synthesis and inhibits the early apoptosis of human spermatogonial stem cells by targeting CBL and competing with IncRNA CASC7

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ABSTRACT

Epigenetic regulators of human spermatogonia stem cells (SSCs) remain largely unknown. We found that miRNA-122-5p was upregulated in human spermatogonia from obstructive azoospermia (OA) patients compared with non-obstructive azoospermia (NOA). MiRNA-122-5p stimulated the proliferation and DNA synthesis of human SSCs, whereas it inhibited the early apoptosis of human SSCs. CBL was predicted and identified as a direct target of miRNA-122-5p in human SSCs. CBL silencing led to an enhancement of cell proliferation and DNA synthesis and neutralized the effect of miRNA-122-5p inhibitor on the DNA synthesis of human SSCs. The decrease in the early apoptosis of human SSCs was observed after CBL knockdown. By comparing the profiles of IncRNAs between OA and NOA spermatogonia, CASC7 was significantly deficient in OA spermatogonia, and it had a direct association with miRNA-122-5p. LncRNA CASC7 competed with miRNA-122-5p, and it suppressed the inhibition of CBL. Collectively, these results implicate that miRNA-122-5p enhances the proliferation and DNA synthesis and inhibits the early apoptosis of human SSCs by targeting CBL and competing with IncRNA CASC7. Therefore, this study provides novel insights into epigenetic regulation of fate determinations of human SSCs, and it offers new targets for gene therapy of male infertility that is associated with aging.

INTRODUCTION

Spermatogonial stem cells (SSCs) can produce mature and functional spermatids through the precise process, namely spermatogenesis, and male germ cells from SSCs to spermatids are able to transmit genetic information across generations [1]. Notably, SSCs can be reprogrammed to obtain pluripotency, dedifferentiate to become embryonic stem-like (ES-like) cells [2], and directly transdifferentiate to functional cells of other lineages [3, 4] and tissues [5]. As such, SSCs could be utilized in reproductive and regenerative medicine.

In the testis, SSCs undergo self-renewal to maintain stemness of the cells, and they differentiate to spermatocytes and mature spermatids [6]. The self-renewal and differentiation of SSCs are precisely regulated by epigenetic factors and genetic elements [7–10]. Non-coding RNAs, including microRNAs (miRNAs) and long non-coding RNAs (IncRNAs), are
one of the most significant epigenetic factors that determine the fate decisions of the cells, and they perform biological modifications without changing DNA sequences. MiRNAs are endogenous RNA molecules with a length of 18-25 nucleotides (nt) [11, 12], while lncRNAs are transcribed RNA molecules with a length of over 200 nt [13, 14]. MiRNAs complementarily bind to the sequences in the 3’ UTRs of the targeting mRNAs, which results in the degradation of mRNA or translation inhibition [15]. LncRNAs diversely control gene expression, and it has been suggested that lncRNAs interact with miRNAs by acting as endogenous sponges or competing endogenous RNA (ceRNA), which further affects the expression of targeting genes [16–19].

MiRNAs play important roles in regulating the self-renewal, differentiation, and apoptosis of SSCs. It has been shown that miRNA-221/222 is essential for maintaining the undifferentiated state of mammalian spermatogonia through the repression of KIT expression [20]. We have previously demonstrated that miRNA-20 and miRNA-106a enhance the division of mouse SSCs through targeting STAT3 and Ccnd1 [21], while Chen et al. [22] have revealed that miRNA-202 retains the stemness of mouse SSCs by targeting cell cycle regulators and RNA binding proteins. Moreover, miRNA-100 and miRNA-10b stimulate mouse SSC proliferation by regulating Stat3 and Kruppel-like factor 4, respectively [23, 24]. Chdh1-miRNA-486-MMP2 has been shown to be a regulatory axis for the stemness and growth property of SSCs [25], and miRNA-322 is essential for SSC self-renewal by targeting RASSF8 (ras association domain family 8) [26]. MiRNA-17-92 cluster and miRNA-290-295 cluster control the proliferation and/or differentiation of SSCs [27, 28]. Tong et al. [29] have revealed that miRNA-let7 family miRNAs are required for spermatogonial differentiation induced by RA. Deletion of Mir-17-92 cluster in mice leads to an increase in the level of miRNA-106b-25, reflecting the functional cooperation of these two miRNA clusters [30]. MiRNA-146 modulates mouse SSC differentiation by the regulation of RA [31]. MiRNA-34c controls the differentiation of mouse SSCs via targeting Nanos2 [32], and miRNA-17-92 is essential for normal spermatogenesis in mice [33].

Additionally, lncRNAs emerge as the novel determinants of stem cell self-renewal and differentiation [34, 35]. LncRNA033862 has been suggested to be crucial for the maintenance of SSC proliferation and survival by regulating Gfrai [36], while LncRNA AK015322 promotes the proliferation of mouse SSCs and serves as a decoy of miRNA-19b-3p [37]. LncRNA Mrhl negatively regulates the expression of Sox8 and it is important for spermatogonial differentiation [38]. Recently, LncRNA Gm2044 has been shown to inhibit the proliferation of mouse spermatogonia [39].

To date, very little is known about the interaction of miRNAs and lncRNAs in human SSCs. We have previously identified that miRNA-663a is important for the proliferation and DNA synthesis and suppresses the early apoptosis of human SSCs by targeting transcription factor NFIX [40]. It is interesting to unveil the networks among lncRNAs, miRNAs, and their targets in human SSCs. In this study, we have explored the expression, function, and targets of miRNA-122-5p in human SSCs, and we have demonstrated that miRNA-122-5p stimulates the proliferation and DNA synthesis and inhibits the early apoptosis of human SSCs line by targeting CBL. Furthermore, we have found that LncRNA CASC7 competes with the miRNA-122-5p and controls the level of transcription factor CBL. This study on miRNA-122-5p and its functional axis significantly offers novel insights into the epigenetic regulation of human SSCs, and it provides the scientific basis on molecular therapy for treating male infertility.

RESULTS

Isolation and identification of human spermatogonia from OA and NOA patients’ testicular tissues

Human male germ cells were isolated from the testicular tissues of obstructive azoospermia (OA) and non-obstructive azoospermia (NOA) patients using a two-step enzymatic digestion and followed by differential plating. Human spermatogonia were further separated by STA-PUT velocity sedimentation by 0.5-4% BSA from OA and NOA patients [41]. Morphological features and phenotypic identification of human spermatogonia were conducted by our previous work [40].

Differentially expressed MiRNAs in human spermatogonia between OA and NOA patients

MiRNA microarrays were utilized by us to compare global miRNA profiles in human spermatogonia between OA and NOA patients [42]. Among the differentially expressed miRNAs, miRNA-122-5p was expressed at a higher level in human spermatogonia of OA patients compared to NOA patients, which was verified by our real-time qPCR (Figure 1A). These data indicate that miRNA-122-5p plays a potential role in regulating the fate determinations of human SSCs. In addition, we observed that miR-373-3p was expressed at a higher level in human spermatogonia of
OA patients than NOA patients (Figure 1B), whereas the levels of miR-100-5p and miR-145-5p were lower in human spermatogonia of OA patients compared with NOA patients (Figure 1C, 1D).

**Phenotypic identification and overexpression of MiRNA-122-5p in human SSC line**

Human SSC line has been established by us, and phenotypic characteristics of this cell line were verified as SSCs in our recent work [40]. Human SSC line was infected lentivirus with miRNA-122-5p sequences, and as shown by ZsGreen1-labeled miRNA-122-5p lentivirus, the infection efficiency of miRNA-122-5p lentivirus in the human SSC line was over 80% (Figure 1E). After transfection for 24 hr, real-time qPCR revealed that the expression level of miRNA-122-5p in human SSC line was significantly upregulated by miRNA-122-5p lentivirus compared to control lentivirus (Figure 1F).

**MiRNA-122-5p stimulates the proliferation and DNA synthesis and decreases the early apoptosis of human SSCs**

We determined the influence of miRNA-122-5p on the division of human SSC line using several methods. Cell proliferation assays revealed that the proliferation of human SSC line was obviously increased by miRNA-122-5p lentivirus compared to control lentivirus at day 3 to day 5 of culture (Figure 2A). Western blots demonstrated that level of PCNA protein was enhanced in human SSC line at day 5 by miRNA-122-5p lentivirus vs. the control lentivirus (Figure 2B, 2C). Moreover, EDU incorporation assay indicated that the percentage of EDU-positive cells was increased in human SSC line at day 5 by miRNA-122-5p lentivirus vector (Figure 2D, 2E). Collectively, these results suggest that miRNA-122-5p stimulates the division and DNA synthesis of human SSCs. Annexin V and propidium iodide (PI) staining and flow cytometry

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**Figure 1. Differentially expressed MiRNAs of human spermatogonia between OA and NOA patients and transfection efficiency of ZsGreen1-labeled lentivirus in human SSC line.** (A–D) Real-time qPCR revealed the different expression levels of miRNA-122-5p (miR-122-5p) (A), miR-373-3p (B), miR-100-5p (C), and miR-145-5p (D) in human spermatogonia between OA and NOA patients. * indicates statistically significant differences (p< 0.05) in human spermatogonia between human OA and NOA patients. (E) Fluorescence microscope and phase-contrast microscope indicated the transfection efficiency of ZsGreen1-labeled miRNA-122-5p lentivirus compared to the control lentivirus of human SSC line. Scale bars =40 μm. (F) Real-time qPCR showed the relative levels of miRNA-122-5p in human SSCs treated with miRNA-122-5p vector and control lentivirus. * indicated statistically significant differences (p< 0.05) in human SSC line between miRNA-122-5p vector and the control lentivirus.
assays showed that miRNA-122-5p lentivirus reduced the early apoptosis but not the late apoptosis of human SSC line (Figure 2F, 2G), implicating that miRNA-122-5p inhibits the early apoptosis of human SSCs.

CBL is a direct target for MiRNA-122-5p in human SSC line

Using miRNA predict software, including Targetscan and miRDB, we predicted several potential targets of miR-122-5p. We verified these targets using real-time qPCR showing that the expression level of transcription factor CBL was downregulated by miRNA-122-5p lentivirus compared with other targets (Figures 3A–3E). Western blots further revealed that the level of CBL protein was significantly decreased by miRNA-122-5p lentivirus (Figure 3F, 3G). Therefore, CBL is one binding target of miRNA-122-5p in human SSCs. Furthermore, dual luciferase assays were performed to verify the binding cite of CBL mRNA. The predicted

Figure 2. Effect of overexpression of MiRNA-122-5p on the proliferation, DNA synthesis and early apoptosis of human SSC line. 
(A) CCK-8 assays revealed the growth curve of human SSC line treated with miRNA-122-5p vector and control lentivirus in 5 days. (B) Western blots demonstrated PCNA expression in human SSC line of miRNA-122-5p vector and control lentivirus. (C) The relative expression of PCNA in human SSC line of miRNA-122-5p vector and control lentivirus after normalization to the signals of their loading control ACTB. (D) EDU incorporation assay showed the EDU-positive cells in human SSC line of miRNA-122-5p vector and control lentivirus. Scale bars =50 μm. (E) Qualification of EDU-positive cells in human SSC line of miRNA-122-5p vector and control lentivirus. (F, G) APC Annexin V and PI staining and flow cytometry showed the percentages of early apoptosis in human SSC line infected with ZsGreen1-labeled miRNA-122-5p lentivirus and control lentivirus. * denoted statistically significant differences (p< 0.05) in human SSC line between control lentivirus and miRNA-122-5p vector.
protein sequence in 3’UTR of CBL mRNA diminished the luciferase activity of the fusion genes in response to the treatment of miRNA-122-5p mimics (Figure 3H), whereas the mutated target sequence didn’t have any influence on the luciferase activity (Figure 3I). Neither the predicted target sequence in 3’UTR of ZNF668 mRNA nor the mutated sequence had any effect on the luciferase activity (Figure 3J, 3K). Considered together, these data suggest that CBL is a direct target of miRNA-122-5p in human SSCs. The binding of seed

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Figure 3. Identification and verification of the target CBL of MiRNA-122-5p in human SSC line. (A–E) Real-time qPCR revealed the relative levels of ZNF668 (A), PLAG1 (B), MEF2D (C), FOXP1 (D) and CBL (E) in human SSCs of miRNA-122-5p vector and control lentivirus. * indicated statistically significant differences (p< 0.05) in human SSC line between control lentivirus and miRNA-122-5p vector. (F) Western blots depicted CBL protein in human SSC line of miRNA-122-5p vector and control lentivirus. (G) The relative expression levels of CBL protein in human SSC line of miRNA-122-5p vector and control lentivirus after normalization to the signals of their loading control ACTB. * denoted statistically significant differences (p< 0.05) in human SSC line between control lentivirus and miRNA-122-5p vector. (H, I) Validation of the targeting of miRNA-122-5p to wild type CBL and mutated CBL by dual luciferase reporter assays. (J, K) Validation of the targeting of miRNA-122-5p to wild type ZNF668 and mutated ZNF668 by dual luciferase reporter assays. * implied statistically significant differences (p< 0.05) in human SSC line between miRNA-122-5p mimics and miRNA mimics control. (L) Schematic diagram illustrated the binding site of miRNA-122-5p to CBL mRNA.
region (from the 2nd to 8th nucleotides) on miRNA-122-5p to the target 3’UTR sequence of CBL mRNA was illustrated in Figure 3L.

**CBL silencing promotes the proliferation and inhibits the early apoptosis of human SSCs**

We further explored the effect of CBL on the fate decisions of human SSC line. We utilized three pairs of CBL siRNAs, namely CBL siRNA1, CBL siRNA2, and CBL siRNA3, with an aim to obtain sequence-specific siRNAs of CBL. The transfection efficiency of CBL siRNAs in human SSC line was more than 80%, as evidenced by the transfection of FAM-labeled fluorescent oligo [40]. Real-time qPCR showed that three CBL siRNAs reduced the CBL transcript, and notably, CBL siRNA1 had the highest level for CBL knockdown (Figure 4A). Western blots displayed that CBL siRNA1 significantly decreased the level of CBL protein (Figure 4B, 4C).

Proliferation assay showed that CBL siRNA1 enhanced the growth of human SSC line (Figure 4D), and PCNA protein level was increased in human SSC line at day 5 by CBL siRNA1 (Figure 4E, 4F). EDU incorporation assays demonstrated that the number of EDU-positive cells was obviously increased by CBL siRNA1 (Figure 4G, 4H). Annexin V and PI staining and flow cytometry assay indicated that CBL silencing led to the decrease in the early apoptosis of the human SSC line at day 3 (Figure 4I, 4J). Taken together, CBL knockdown stimulates the division and inhibits early apoptosis of human SSCs, which is consistent with the overexpression of miRNA-122-5p.

**Synergetic influence of MiRNA-122-5p and CBL on fate determinations of human SSC line**

We examined whether CBL had the synergetic effect with miRNA-122-5p on human SSC line using rescue assays. As shown in Figures 5A, 5B, the reduction in EDU-positive cells by miRNA-122-5p inhibitor was counteracted by CBL silencing in human SSC line. CCK-8 assays revealed that the proliferation decrease of human SSC line caused by miRNA-122-5p inhibitor was neutralized by CBL silencing at day 4 and day 5 (Figure 5C).

**LncRNA CASC7 is differentially expressed in human spermatogonia between OA and NOA patients**

To further extend the molecular network of miRNA-122-5p in regulating the human SSCs, we compared and analyzed the profiles of lncRNAs in human spermatogonia between OA and NOA patients. As detected by an unbiased lncRNA microarray assay (Figure 6A), there were 6,919 lncRNAs whose expression levels were statistically significant (fold changes ≥ 2). In human spermatogonia between OA and NOA patients, 3,264 lncRNAs were upregulated whereas 3,655 lncRNAs were downregulated (Figure 6B). As predicted by Targetscan and miRDB software, we identified four lncRNAs that potentially had direct association with miRNA-122-5p from the differentially expressed lncRNAs in human spermatogonia between OA and NOA spermatogonia. These candidate lncRNAs were examined by real-time qPCR to validate the results of the microarray assay, and we found that CDKN2B-AS1 (Figure 6C) and XIST (Figure 6D) were expressed at higher levels in human spermatogonia of OA patients than NOA patients, whereas the levels of MAL2 (Figure 6E) and CASC7 (Figure 6F) were significantly lower in human spermatogonia of OA patients compared with NOA patients.

**LncRNA CASC7 competes with the MiRNA-122-5p and counteracts the inhibition of CBL in human SSCs**

It has been reported that lncRNAs communicate with mRNAs [43–45], circular RNAs [46] and miRNAs [19, 47–50], and they act as endogenous miRNA sponges, ceRNAs or the targets of miRNAs. To explore whether CASC7 communicates with miRNAs in human SSC line, we investigated the influence of LncRNA CASC7 on the level of miRNA-122-5p and CBL in human SSC line. Three pairs of CASC7 siRNAs (i.e., CASC7 siRNA 1, CASC7 siRNA 2, and CASC7 siRNA 3) with different binding sites were employed. Real-time qPCR demonstrated that CASC7 siRNA 2 assumed the highest potential for silencing CASC7 among three CASC7 siRNAs (Figure 7A). After CASC7 knockdown by CASC7 siRNA 2, the level of miRNA-122-5p was upregulated (Figure 7B), whereas CBL transcript was decreased (Figure 7C). We also constructed luciferase reporter constructs containing either the wild-type (WT) binding sequence of CASC7 RNA or the mutant forms of the seed sites into the vector. Co-transfection of the miRNA-122-5p mimics and the WT dual-luciferase reporters into 293 T cells decreased about 50% of the luciferase activity (Figure 7D), while there was no significant change in mutation of the seed sequences compared to each control groups (Figure 7D). The seed site for miRNA-122-5p in the CASC7 RNA sequence was illustrated in Figure 7E. Thus, we have revealed a network of miRNA-122-5p-CBL axis that LncRNA CASC7 competes with the miRNA-122-5p and consequently suppresses the inhibition of CBL.
Figure 4. Influence of CBL silencing on the proliferation, DNA synthesis, and early apoptosis of human SSC line. (A) Real-time qPCR showed changes of CBL mRNA by CBL siRNA1, CBL siRNA2, and CBL siRNA3 in human SSC line. (B, C) Western blots revealed changes of CBL protein by CBL siRNA1, CBL siRNA2, and CBL siRNA3 in human SSC line. * denoted statistically significant differences (p< 0.05) of CBL siRNA1-, 2-, and 3-treated human SSC line compared with the control siRNA. (D) CCK-8 assays showed the growth curve of human SSC line treated with control siRNA and CBL siRNA1 for 5 days. * indicated statistically significant differences (p<0.05) in human SSC line between control siRNA and CBL siRNA1. (E) Western blots illustrated the changes of PCNA protein in human SSC line at day 3 after transfection of control siRNA and CBL siRNA1. (F) The relative protein level of PCNA in human SSC line at day 3 after transfection of control siRNA and CBL siRNA1 through normalization to the signals of their loading control ACTB. * showed statistically significant differences (p< 0.05) in human SSC line between control siRNA and CBL siRNA1. (G, H) EDU incorporation assay demonstrated the percentages of EDU-positive cells in human SSC line affected by control siRNA and CBL siRNA1. Scale bars= 50 μm. * indicated statistically significant differences (p<0.05) between CBL siRNA1-treated human SSC line and the control siRNA. (I, J) APC Annexin V and PI staining and flow cytometry depicted the percentages of early apoptosis in human SSC line transfected with control siRNA and CBL siRNA1. * displayed statistically significant differences (p<0.05) between CBL siRNA1-treated human SSC line and the control siRNA.
DISCUSSION

NOA is a multifactorial disorder that is caused by genetic, epigenetic, and environmental factors [51, 52]. Much progress has been made in revealing the molecular mechanisms underlying the spermatogenesis in rodents; however, epigenetic regulators of male germ cell development in humans remains largely unclear. Recently, we have found that miRNA-663a stimulates the proliferation and inhibits the early apoptosis of human SSCs [40]. In addition, we have revealed differentially expressed miRNAs in human spermatogonia between OA and NOA patients [41]. In this study, miRNA-122-5p was found to be expressed at a higher level in human spermatogonia of OA patients compared to NOA patients.

MiRNA-122-5p has been reported as an important modulator in diverse cell types and tissues. IL-22 induces miRNA-122-5p which promotes keratinocyte proliferation by decreasing the expression of Spry2 in the pathogenesis of psoriasis [53]. Bone marrow mesenchymal stem cells-derived exosomes carrying

Figure 5. The effect of MiRNA-122-5p inhibitor and CBL silencing on DNA synthesis and proliferation of human SSC line. (A, B) EDU incorporation assay showed the percentages of EDU+ cells in human SSC line treated with miRNA inhibitor control, miRNA-122-5p inhibitor, as well as miRNA-122-5p inhibitor and CBL siRNA1. Scale bars = 50 μm. (C) CCK-8 assay showed the proliferation of human SSC line treated with miRNA inhibitor control, miRNA-122-5p inhibitor, and miRNA-122-5p inhibitor and CBL siRNA1 for 5 days. * denoted statistically significant differences in human SSC line (p< 0.05) between miRNA-122-5p inhibitor and the inhibitor control as well as the miRNA-122-5p inhibitor and miRNA-122-5p inhibitor plus CBL siRNA1.
miRNA-122-5p reduces Sprouty2 and enhances the activity of receptor tyrosine kinase (RTK), which controls the proliferation and differentiation of rabbit osteoblasts [54]. MiRNA-122-5p stimulates the aggression and epithelial-mesenchymal transition (EMT) in triple-negative breast cancer by negative regulation of CHMP3 [55]. Since the suppression of miRNA-122-5p significantly reduces AFP level and the proliferation in AFP-producing gastric cancer (AFPGC), miRNA-122-5p has been regarded as a potential therapeutic target in AFPGC [56]. The level of miRNA-122-5p in the sperm of patients with oligospermia semen is lower than normal sperm, and thus the deficiency of miRNA-122-5p might affect the proliferation and differentiation of male germ cells, leading to a decrease in the sperm density [57]. Knockdown of ANRIL elevates the level of miRNA-122-5p and suppresses cell proliferation, metastasis and invasion in hepatocellular carcinoma (HCC) [58]. The increase in the expression of miRNA-122-5p suppresses the proliferation, migration and invasion of glioma [59], HCC [60], cervical cancer [61], gastric cancer cells [62], and nasopharyngeal carcinoma [63]. In the present study, we have demonstrated that miRNA-122-5p stimulates the proliferation and DNA synthesis and inhibits the early apoptosis of human SSCs.

![Image of Figure 6](image_url)

**Figure 6. Differentially expressed LncRNAs in human spermatogonia between OA and NOA patients.** (A) The cluster heat map showed LncRNAs with an expression change fold ≥2 from microarray data (p<0.025) in human spermatogonia from OA patients compared with NOA patients. (B) Scatter plot illustrated the differentially expressed LncRNAs with an expression change fold ≥2 (p<0.025) in human spermatogonia from OA patients compared with NOA patients. (C–F) Real-time qPCR revealed the different expression levels of XIST, CDKN2B-AS1, MAL2 and CASC7 in human spermatogonia between OA and NOA patients. * denoted statistically significant differences (p<0.05) in human spermatogonia between human OA and NOA patients.
Characterized by the length of C termini and capability to work as adaptors, the highly conserved ubiquitin ligases CBL families include three mammalian homologs, namely CBL (also known as c-CBL or CBL2), CBL-b and CBL-c (CBL-3). CBL is known as an E3 ubiquitin ligase, and it promotes the degradation of proteins associated with cell growth and migration [64–66]. The main function of CBL is derived from ubiquitination of active receptor tyrosine kinases (RTKs), which results in negative regulation of their signaling and directs them towards lysosomes or degradation [67]. CBL is a tumor suppressor in the pathogenesis of human cancers, and it plays roles in melanoma cell proliferation, migration and invasion [68]. Moreover, CBL restricts myeloid proliferation of human AML1-ETO-induced leukemia [69]. Regulated by Tyr371 phosphorylation, CBL inhibits the tumorigenesis of colorectal cancer via targeting Wnt/β-catenin [70]. However, mutant CBL works as oncogene in myeloid leukemia [71] and myeloproliferative neoplasms [72]. Predicted by bioinformatics and further verified by our real-time qPCR, Western blots and dual-

Figure 7. Influence of CASC7 silencing on level of MiRNA-122-5p and CBL as well as identification and verification of the direct binding of MiRNA-122-5p and CASC7 in human SSC line. (A) Real-time qPCR showed changes of CASC7 by CASC7 siRNA 1, CASC7 siRNA 2, and CASC7 siRNA 3 in human SSC line. * indicated statistically significant differences (P< 0.05) of CASC7 siRNA 1-, 2-, and 3-treated human SSC line compared with the control siRNA. (B) Real-time qPCR demonstrated miRNA-122-5p expression changes in human SSC line at day 2 after transfection of CASC7 siRNA 2 and the control siRNA.* denoted statistically significant differences (p<0.05) between CASC7 siRNA 2-treated cells and the control siRNA. (C) Real-time qPCR showed the change of CBL in human SSC line at day 2 after transfection of CASC7 siRNA 2 and the control siRNA.* showed statistically significant differences (p< 0.05) between CASC7 siRNA 2-treated cells and the control siRNA. (D) Validation of the direct binding of miRNA-122-5p to wild type CASC7 and the mutated CASC7 by dual luciferase reporter assays. * indicated statistically significant differences (p< 0.05) in human SSC line between miRNA-122-5p mimics and miRNA mimics control. (E) The sequences of miRNA-122-5p complement with CASC7.
MATERIALS AND METHODS

Obtaining testicular tissues from OA and NOA patients

Testicular tissues in this study were obtained from OA and NOA patients with microdissection of testicular biopsy at Ren Ji Hospital affiliated with Shanghai Jiao Tong University School of Medicine. OA patients had normal spermatogenesis, whereas NOA patients with hypospermatogenesis and germ cell mature arrest were verified by histological analysis. The consents of testicular biopsies for research only were obtained from the donors.

Separation of human spermatogonia from testicular tissues by STA-PUT velocity sedimentation

Testicular biopsies were washed three times with DMEM/F12 containing 1% penicillin and streptomycin (Gibco). Human male germ cells were isolated from testicular tissues via a two-step enzymatic digestion and followed by differential plating [77]. STA-PUT with 0.5%-4% BSA gradient and velocity sedimentation was utilized to separate human spermatogonia from other germ cells based upon their sizes, mass, and gravities [40]. Cellular fractions of similar size and morphological features with human spermatogonia were pooled together for subsequent analyses.

Real-time qPCR

Total RNA was extracted from human spermatogonia and human SSC line by the RNAiso Plus reagent (Takara, Kusatsu, Japan). Concentrations of total RNA were measured by Nanodrop (Thermo Scientific, MA, USA), and RNA with the A260/A280 ratio of 1.9-2.0 was used for real-time qPCR. Reverse transcription (RT) was conducted using the First Strand cDNA Synthesis Kit (Thermo Scientific, USA), and PCR of the cDNA was performed pursuant to the method [78]. For miRNA real-time PCR, RT reaction was done using TransScript miRNA First-Strand cDNA Synthesis SuperMix Kit (Transgene). Each RT reaction comprised 100 ng RNA, 1 µl miRNA RT Enzyme Mix, 10 µl 2x TS miRNA Reaction Mix, and RNase-free water. Reactions were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems) for 60 min at 37°C and followed by heat inactivation of RT for 5 seconds at 85°C. RT reaction mix was diluted by 5 times in nuclelease-free water and held at -20°C. Primer sequences of miRNAs and mRNAs for real-time qPCR were listed in Table 1. Real-time PCR was performed in triplicate using Power SYBR Green PCR Master Mix (Applied Biosystems, Woolston Warrington, UK) and a 7500
Table 1. The primer sequences for miRNAs, genes, and lncRNAs.

<table>
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<tr>
<th>Name</th>
<th>Primer sequences (5’ to 3’)</th>
<th>Tm (° C)</th>
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<tr>
<td>Has-miR-122-5p</td>
<td>TGGAGTGTGACAAATGGTGTTTG</td>
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<tr>
<td>Has-miR-373-3p</td>
<td>GAAGTGCTTCGATTTTGGGGTTG</td>
<td>60</td>
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<td>Has-miR-100-5p</td>
<td>AACCCTAGATTCAGACATTC</td>
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<td>Has-miR-145-3p</td>
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<td>U6</td>
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<td>ZNF668-5p</td>
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<td></td>
<td>Reverse AGTGAAGACACTCTTGGACTG</td>
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<td>PLAG1-3p</td>
<td>Forward ATCAACTCCACACACGACC</td>
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<td></td>
<td>Reverse GCAGTCACTACACACGCTC</td>
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<td>MED2D-5p</td>
<td>Forward CCCAGGAATCACGGACAGAG</td>
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<td></td>
<td>Reverse GCAGTCACATAGCACGCTC</td>
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<td></td>
<td>Reverse AGTGGTGTTTGT ACTCTC</td>
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<td>CBL-3p</td>
<td>Forward TGGTGCGGTTGTGTCAGAAC</td>
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<td>Reverse GGTAGGGTAGCTTGGGACTG</td>
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<tr>
<td>MAL2-3p</td>
<td>Forward ATGGGAGCAGCGACACACTCC</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Reverse GGAGTGTACGGGGGATCC</td>
<td>60</td>
</tr>
<tr>
<td>CASC7-3p</td>
<td>Forward AACATGGTCTCTTGTGCTGATG</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Reverse CCACGGTAAAGGCGGAGAATC</td>
<td>60</td>
</tr>
<tr>
<td>GAPDH-3p</td>
<td>Forward CAGAGGGCATGGTGCTGATG</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Reverse GAAGGCTGGGGGTTCATTTC</td>
<td>60</td>
</tr>
</tbody>
</table>

Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Melting curve analysis was conducted to validate the specific generation of the expected PCR products. The expression levels of miRNAs were normalized to U6, while the mRNA levels were normalized to GAPDH. Relative levels of genes and miRNAs were calculated using the 2ΔΔCt method [79].

RNA deep sequencing

RNA deep sequencing was conducted pursuant to the method [42], and an Illumina HiSeq 2000 sequencer was utilized for sequencing of miRNA libraries. RNA deep sequencing data were deposited in the Sequence Read Archive (accession no. SRP045287) [42].

Human SSC line and cell culture

Human SSC was established previously by our team [80]. This cell line expresses a number of human male germ cell and SSC hallmarks, and it has been identified as human SSCs [40, 80]. Human SSC line was cultured in DMEM/F12 with 10% fetal bovine serum (FBS) (Life Technologies Inc., Grand Island, NY, USA) and 100 U/ml penicillin and 100 mg/ml streptomycin (Life Technologies Inc.) at 37°C in 5% CO2. Cells were passaged every 2-3 days using 0.05% trypsin (Invitrogen) and 0.53 mM EDTA (Invitrogen).

Transfection of siRNAs and miRNAs and infection of lentivirus

SiRNAs, miRNA mimics and inhibitors were purchased from GenePharma (Shanghai, China). The oligonucleotides of miRNA mimics, inhibitors and siRNAs were shown in Table 2. Human SSC line was seeded at 1 x 10^5/cm^2 density and cultured in DMEM/F12 with 10% FBS overnight.

Transfection of miRNA mimics or inhibitor and siRNAs was performed using lipofectamine 3000 transfection agent (Life Technologies, Carlsbad, CA, USA). After 48 hr of culture, cells were harvested for subsequent analyses.
Table 2. The sequences for miRNA-122-5p mimics, miR122-5p inhibitor, and siRNA oligonucleotides against CBL and CASC7.

<table>
<thead>
<tr>
<th>MiRNAs and siRNAs</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsa-miRNA-122-5p mimics</td>
<td>Sense UGGAGUGUGACAUGGUGUUUG</td>
</tr>
<tr>
<td></td>
<td>Antisense AACACAUUGUCACACUCAUU</td>
</tr>
<tr>
<td>MiRNA mimics control</td>
<td>Sense UUCUCGGAACGUGUACAGGUTT</td>
</tr>
<tr>
<td></td>
<td>Antisense ACGUGACACGUGUACGGAATT</td>
</tr>
<tr>
<td>Hsa-miRNA-122-5p inhibitor</td>
<td>CAACACAUUGUCACACUCCA</td>
</tr>
<tr>
<td>MiRNA inhibitor control</td>
<td>CAGUACUUUUGUGUAGUCAA</td>
</tr>
<tr>
<td>CBL siRNA1</td>
<td>Sense GCCUGAUUGGGCUCAUGAATT</td>
</tr>
<tr>
<td></td>
<td>Antisense UUCAUGAGCCCAAUCAGGCTT</td>
</tr>
<tr>
<td>CBL siRNA2</td>
<td>Sense GGGACAUUCUCCAGCACATT</td>
</tr>
<tr>
<td></td>
<td>Antisense UUGUCGGAAGUUGUCCCTT</td>
</tr>
<tr>
<td>CBL siRNA3</td>
<td>Sense GGACCCUCUGUCACATT</td>
</tr>
<tr>
<td></td>
<td>Antisense AUGUGCAUGAGGUGUCCCT</td>
</tr>
<tr>
<td>CASC7 siRNA 1</td>
<td>Sense GCGUUACAGAUGCACUUTT</td>
</tr>
<tr>
<td></td>
<td>Antisense AAAGUGCAUCUGUAAGCCTT</td>
</tr>
<tr>
<td>CASC7 siRNA 2</td>
<td>Sense GCAAGAGAGAUAUGCAATT</td>
</tr>
<tr>
<td></td>
<td>Antisense UUGCUAUAUCUUCUUGGCTT</td>
</tr>
<tr>
<td>CASC7 siRNA 3</td>
<td>Sense GGUACCACUGUGGUAATT</td>
</tr>
<tr>
<td></td>
<td>Antisense UUAUCCACAGGUGUGUACCTT</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>Sense UUCUCGGAACGUGUACGUTT</td>
</tr>
<tr>
<td></td>
<td>Antisense ACGUGACACGUGUACGGAATT</td>
</tr>
</tbody>
</table>

The lentivirus, namely PGMLV-CMV-MCS-EF1-ZsGreen1, was purchased from Jiman Biotechnology CO., LTD (Shanghai, China). Human SSC line was plated at a density of 5 × 10^5 cells in 100 mm plates, and these cells were cultured with DMEM/F12 and 10% FBS overnight. Culture medium was changed with fresh DMEM/F12 containing 10^8 TU/ml PGMLV-CMV-MCS-EF1-ZsGreen1 and 10 μg/ml polybrene, and the cells were incubated at 37°C in 5% CO₂ overnight. After 24 hr of culture, the medium was changed with fresh DMEM/F12 and 10% FBS, and the expression of ZsGreen1 was detected under a fluorescence microscope (Nikon Eclipse Ti-S, Nikon Corporation, Tokyo, Japan). The infected cells were cultured and expanded in DMEM/F12 medium supplemented with 10% FBS and 1% antibiotic containing penicillin and streptomycin (Gibco).

**CCK-8 assay**

Human SSC line was seeded at a density of 2,000 cells/well in 96-well microtiter plates in DMEM/F12 with 1% FBS. After 5 days of culture, the proliferation potential of human SSC line was examined by CCK-8 assay (Dojin Laboratories, Kumamoto, Japan) according to the manufacturer’s instruction.

**Western blots**

Human SSC line with treatment of lentivirus, miRNA oligonucleotides or siRNAs was lysed with RIPA buffer (Santa Cruz Biotechnology). After 30 min of lysis on ice, cell lysates were cleared by centrifugation at 12,000 x g for 20 min, and the concentrations of proteins were measured by BCA kit (Dingguo, China). Thirty micrograms of cell lysate from each cell sample were used for SDS-PAGE (Bio-Rad), and Western blots were performed according to the protocol [81]. The information of primary antibodies was listed in Table 3. After extensive washes with tris-buffered saline and Tween 20, the blots were detected by chemiluminescence (Chemi-Doc XRS, Bio-Rad, Hercules, CA, USA).

**EDU incorporation assay**

Human SSC line was seeded at a density of 5,000 cells/well in a 96-well plate, and these cells were treated with lentivirus, miRNA oligonucleotides or siRNAs and followed by 50 mM EDU (RiboBio, Guangzhou, China). After 12 hr of culture, the cells were washed with DMEM and fixed with 4% PFA. Cells were neutralized with 2 mg/ml glycine and permeabilized in 0.5% Triton X-100 for 10 min at room temperature.
Table 3. Primary antibodies' information for Western blots.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Vendors</th>
<th>Sources</th>
<th>Working dilutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCNA</td>
<td>CST</td>
<td>Rabbit</td>
<td>1:1000</td>
</tr>
<tr>
<td>CBL</td>
<td>R&amp;D</td>
<td>Mouse</td>
<td>1:1000</td>
</tr>
<tr>
<td>ACTB</td>
<td>Proteintech</td>
<td>Mouse</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

EDU immunostaining was performed with Apollo staining reaction buffer. The nuclei of cells were stained with Hoechst 33342, and the EDU-positive cells were counted from at least 500 cells under fluorescence microscopy (Nikon, Tokyo, Japan).

**Annexin V and PI staining and flow cytometry analysis**

The early and late apoptosis in human SSC line was measured using the APC Annexin V and PI apoptosis detection kit and flow cytometry pursuant to the manufacturer’s instruction. Human SSC line was seeded at a density of 5 x 10^4 cells/well in 12-well plates, and cells were collected by centrifuging at 1,000 rpm for 5 min at day 2 after infection or day 3 after transfection. The cells were simultaneously stained with Annexin V-FITC (green fluorescence) and the non-vital dye PI (red fluorescence), which identified early apoptotic cells (FITC+PI-) and late apoptotic cells (FITC+PI+).

**Dual luciferase assays**

Human SSC line was seeded in a 48-well culture plate and cultured at 37°C in 5% CO_2 for 24 hr. MiRNA mimics were first transfected to the cells using lipofectamine 3000 transfection agent (Life Technologies, Carlsbad, CA, USA). Ten hours later, 500 ng plasmids with the target sequences, Firefly Luciferase (reporter), and Renilla Luciferase (internal control) (pMIR-GIO, Genecreate, Wuhan, China) were transfected by lipofectamine 3000 reagent (Life Technologies, Carlsbad, CA, USA). Cells were lysed after 48 hr of transfection, and luciferase activity was measured using the tube luminometer (Berthold, Germany). Data were normalized to miRNA mimic control-transfected cells.

**LncRNA microarray assays**

Total RNA was isolated from human spermatogonia of OA and NOA patients using RNAiso Plus reagent (Takara, Kusatsu, Japan). DNase I was used to remove potential genomic DNA contamination. The quality of Total RNAs was determined by the NanoDrop ND-2000 (Thermo Scientific), and RNA integrity was assessed using Agilent Bioanalyzer 2100 (Agilent Technologies). The sample labeling, microarray hybridization and washing were performed based upon the manufacturer’s standard protocols. Briefly, total RNA was transcribed to double strand cDNAs and then synthesized cRNAs. Next, 2nd cycle cDNAs were synthesized from cRNAs. Fragmentation and biotin labeling and the 2nd cycle cDNAs were hybridized onto the Affymetrix human lncRNA arrays. After washing and staining, the arrays were scanned by the Affymetrix Scanner 3000 (Affymetrix). Raw data were generated from Affymetrix GeneChip Command Console (version 4.0, Affymetrix) software, and 63,542 lncRNAs were tested using these arrays. Data analysis of gene expression profiling was processed by Genespring software (version 14.9; Agilent Technologies). Differentially expressed lncRNAs were calculated by pairwise combination and error-weighted average. The threshold set for upregulated and downregulated lncRNAs was with a fold change≥2.0 and p value < 0.05, and the false discovery rate (FDR) was adjusted p-values (Benjamini-Hochberg) <0.05.

**Statistical analysis**

All data were presented as mean ± SEM from at least three independent experiments and analyzed by t test using Prism (version 5, GraphPad), and p value< 0.05 was considered statistically significant.

**AUTHOR CONTRIBUTIONS**

F.Z. performed the experiments, wrote the manuscript and helped data analysis; W.C., Y.C., B.L. and Z.L. assisted with the experiments and data analysis; Q.Y. assisted with the experiments and ordered reagents; Z.H. was responsible for the conception and design, supervision of all aspects of the laboratory experiments, data analysis, writing the manuscript and final approval of the manuscript. All authors approved the manuscript.

**CONFLICTS OF INTEREST**

The authors declared no conflicts of interest.

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