ChemistryOpen

Research Article
doi.org/10.1002/open.202100219

Chemistry Europe
European Chemical Societies Publishing

www.chemistryopen.org

NBMA Promotes Spermatogenesis by Mediating Oct4 Pathway

Jinfei Yang[+],* Dengfeng Lin[+], Weiwei Yao, Damin Yun, Liwei Zhou, Sheng Gao, and Fei Sun*[a]

Non-obstructive azoospermia is one of the most common causes of male infertility, but there is still no specific treatment drug. Given that the Oct4 (Octamer-binding transcription factor 4) has an important regulatory effect on spermatogenesis, activating it can effectively promote spermatogenesis, so it is of great value to develop Oct4-targeted drug design and elucidating its mechanism of action. Here, we screened out the Oct4-targeted drug molecule NBMA (*N*-benzyl-4-methoxy-2-(1-(4-(trifluoromethyl)phenyl)vinyl)aniline) by computer-assisted technology, and found that it has a significant promoting effect on spermatogenesis in the established mouse azoospermia model. Subsequently, through transcriptome sequencing and enrichment analysis, real-time fluorescent quantitative PCR (qPCR) and western blot experiments revealed that NBMA promotes the differentiation of spermatogonial stem cells by activating the Oct4 pathway, thereby promoting spermatogenesis. This study proves that NBMA is a molecule with great potential to be developed as a therapeutic drug for azoospermia. It also shows that computer-assisted, chemical and biological multidisciplinary methods play a very important role in innovative drug discovery.

Introduction

In recent years, the infertility rate caused by factors such as food safety, environmental pollution, drug side effects and increased life pressure increased very quickly.[1,2] According to the World Health Organization (WHO), the incidence of infertility among couples of normal childbearing age is nearly 15%, and 50% caused by male factors, and the proportion is increasing year by year.[3,4] Many studies have shown that the quality of human semen has decreased significantly in the past half a century, and the sperm density has dropped by half. Among them, the quality of semen in China dropped by 1% every year. As the number and quality of human sperm decline year by year, the proportion of male infertility will become higher. WHO predicts that male infertility will become the third most serious disease after tumours and cardiovascular and cerebrovascular diseases in this century.[5] It is one of the biggest challenges facing the field of reproductive health research. Azoospermia is currently the most common cause of male infertility. It is clinically divided into obstructive azoospermia and non-obstructive azoospermia. Patients with obstructive azoospermia can be treated by surgery or assisted reproductive technology. However, the cause of non-obstructive azoospermia is unknown and cannot be treated with surgery,[6–8] and this part of the proportion of infertility patients is very high.[9] Although some drugs can be used for empirical treatment, the efficacy of these drugs is still uncertain.[10] Therefore, the development of a highly effective and broad-spectrum drug for the treatment of such non-obstructive azoospermia patients has important research significance.

Octamer-binding transcription factor 4 (Oct4) is a protein encoded by the POU5F1 gene in humans.[11] It is located on human chromosome 6p21.3 and is a member of the transcription factor family POU (The POU domain takes its name after the first four transcription factors in which it was found: mammalian Pit-1, Oct-1, Oct-2 and *Caenorhabditis elegans* Unc86).[12] It has multiple transcription start sites, transcribes different mRNA subtypes (Isoform), and translates into multiple proteins. Oct4 includes 3 domains: N-transcription domain, POU-binding domain and C-transcription domain, and mainly expressed in embryonic stem cells, germ stem cells and embryo/germ cell tumors.[13–17] Although Oct4 is very important on germ stem cells, only few related studies, especially on its regulation of spermatogonial stem cells, have been published to date.[18–20] Since Oct4 plays an important role in the proliferation and differentiation of germline stem cells, we plan to design an Oct4-targeted drug molecule that will mediate Oct4 to promote the differentiation of spermatogonial stem cells, thereby promoting spermatogenesis. This will be of great significance for the treatment of non-obstructive azoospermia. Herein, we used computer-assisted drug molecular design and chemical synthesis strategies to obtain the Oct4-targeted drug molecule NBMA (*N*-benzyl-4-methoxy-2-(1-(4-(trifluoromethyl)phenyl)vinyl)aniline) that has a strong effect on promoting

[a] *Prof. Dr. J. Yang,[+] D. Lin,[+] W. Yao, D. Yun, L. Zhou, S. Gao, Prof. Dr. F. Sun*
Medical school
Institute of Reproductive Medicine
Nantong University
Nantong 226019 (China)
E-mail: sunfei@ntu.edu.cn
jfyang@ntu.edu.cn

[+] *These authors contributed equally to this work.*

Supporting information for this article is available on the WWW under https://doi.org/10.1002/open.202100219

spermatogenesis, and then through transcriptome sequencing and enrichment analysis, real-time fluorescent quantitative PCR and western blot experiments to initially clarify its molecular role mechanism.

Results and Discussion

Computer-aided Drug Design and Chemical Synthesis

In order to find a pharmaceutical molecule that can specifically treat non-obstructive azoospermia, we use computer-aided drug design methods for the precision design of Oct4-targeting drug molecules. Here, we designed dozens of drug molecules containing alkenyl arylamine skeletons, and carried out molecular docking experiments with these molecules and Oct4 using the AutoDock Hyperchem MOE InsightII program. Finally, we designed a molecule NBMA that shows a strong interaction with Oct4. The molecular docking results show that the Oct4's ARG A73 and NBMA's methoxy group show a strong hydrogen bond interaction (Figure 1).

To obtain a target molecule NBMA as soon as possible, based on previous work,[21] we screened many reaction conditions including temperature, solvent and time, and finally developed a $MnBr_2$-catalysed *ortho*-alkenylation of aromatic amines (for details, see Figure S1, Supporting Information). We can get the target product in one step by this strategy, and the synthesis of NBMA in the conventional strategy usually requires two steps to synthesise it (Figure 2; also see Supporting Information in Ref. [20]).[22]

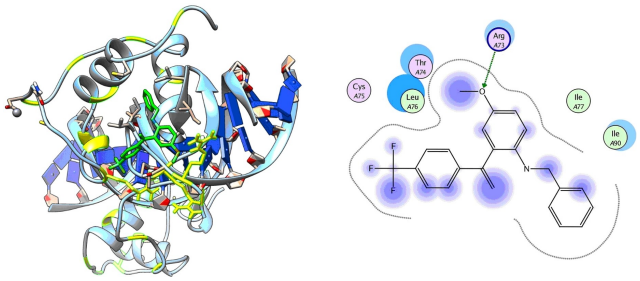

Figure 1. Virtual screening of Oct4-targeted drugs based on computer-assisted molecular simulation docking technology (PDB: 3 l1p).

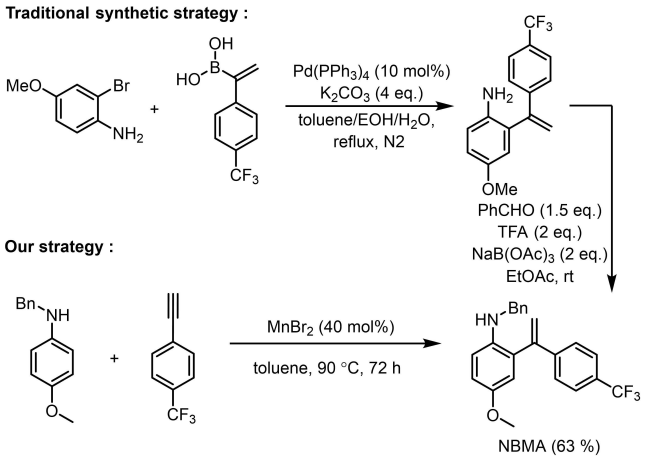

Figure 2. Chemical synthesis of NBMA. *N*-benzyl-4-methoxyaniline (0.2 mmol), 1-ethynyl-4-(trifluoromethyl)benzene (0.4 mmol), $MnBr_2$ (0.08 mmol), toluene (2.0 mL), at 90 °C for 72 h.

The Effect of NBMA on Spermatogenesis

Subsequently, animal-level experiments were carried out using the established mouse azoospermia model (Figure 3). The results of animal experiments showed that there were nearly no sperm in the testis and epididymis of the control group two weeks after the administration. In contrast, a small amount of sperm was produced in the testis and epididymis of mice after administration of 1 mg kg^{-1} NBMA, and the size of the testis increased significantly. As the dose increased, the sperm count in the testicles and epididymis continued to increase, and the size and weight of the testicles increased at the same time. When the drug concentration was increased to 30 mg kg^{-1}, the sperm counts in the testis and epididymis of azoospermic mice were almost close to the normal level, and the size and weight of the testes further increased. Four weeks after administration, there was almost no sperm production in the testis and

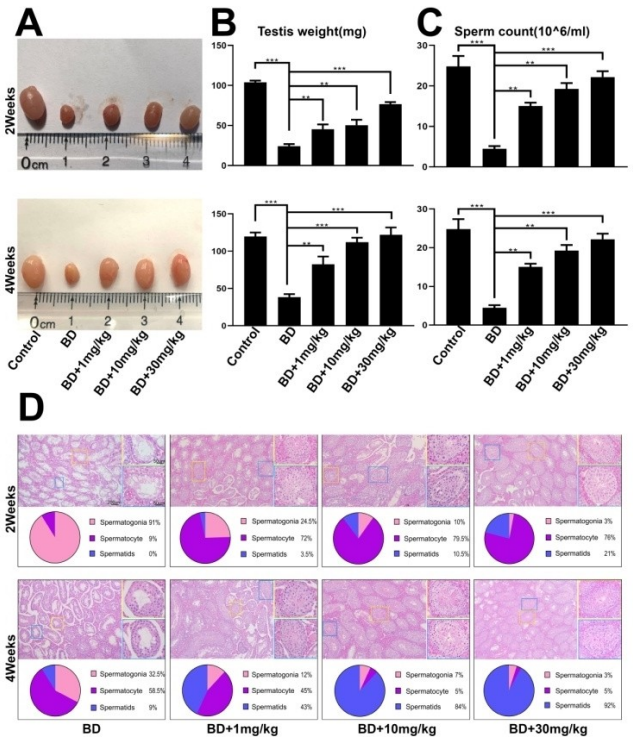

Figure 3. NBMA improved testicular size, weight and sperm concentration in a busulfan-induced testis toxicity mouse model. (A) Morphology of the testes after treatment of busulfan with DMSO, NBMA and control at week 2 and 4 after NBMA gavage. (B) Testis weight index of all groups of mice is presented as a bar graph. (C) Epididymal sperm concentrations in mice is presented as a bar graph. (D) Micrographs of mouse testis sections were obtained by HE staining in three groups 2 week and 4 weeks after NBMA gavage. Scale bar = 200, 50 μm. The results are presented as means ± SD, with n = 6 per group. *p < 0.05, **p < 0.01, and ***p < 0.001 (compared to the BD group).

epididymis in the control group. In contrast, the sperm count and testicular weight in the drug group increased significantly. When the drug concentration was 30 mg kg^{-1}, the sperm count in the testes and epididymis basically returned to the normal mouse level, and the size and weight of the testicles also returned to normal. The results of this study show that the Oct4-targeted drug molecule NBMA obtained by our computer-aided drug design method has achieved the results we expected and has a good effect on promoting spermatogenesis.

Inquiry Experiment of Molecular Mechanism

After obtaining good apparent experimental results, we began to study the molecular mechanism of NBMA to provide a basis for subsequent drug molecular design that is closer to clinical translation. Studies have shown that Lin28a and Sall4 are two important interrelated genes of Oct4.[22] Lin28a can promote primordial germ cell (PGC) development from embryonic stem cells in vitro and in chimeric mice. Knockdown of Lin28a will inhibit PGC development in vitro. Sall4 plays a critical role in maintaining embryonic stem cell (ES cell) pluripotency and self-renewal. Sall4 can activate Oct4 and is a master regulator in murine ES cells. Since Lin28a and Sall4 are so important to Oct4, when NBMA targets Oct4, the expression of Lin28a and Sall4 genes should theoretically change accordingly. Therefore, we subsequently used quantitative real-time PCR to detect their expression levels in testicular tissues two weeks after administration. The results of qPCR experiments showed that the expression levels of Lin28a and Sall4 were significantly higher than those of the azoospermia group (Figure 4). This result supports our assumption.

Based on the results of the above-mentioned quantitative real-time PCR experiments of Lin28A and Sall4, we hope to continue to search for front-end genes that can regulate them. And a large number of research results show that Dazl (Deleted In Azoospermia Like) is an important regulator of Lin28A and Sall4.[23,24] Based on this, we suspect that the expression of Dazl may also change. In order to verify this conjecture, we subsequently performed transcriptome sequencing, enrichment analysis and qPCR verification experiments on the mouse testis after two weeks administration of NBMA. Sequencing and enrichment analysis showed that genes related to spermatogenesis, sperm differentiation, sperm development and immunity were significantly up-regulated (including Dazl, Ddx4, id4, Zbtb16, Taf4b, Sohlh1 and Sohlh2, and others (Figure 5), and subsequent qPCR verification experiments also proved seven genes including Dazl are significantly up-regulated (Figure 6).

The Dazl gene family encodes RNA binding proteins that are expressed in germ cells of males, which is essential for gametogenesis in males. It is a marker of germ cells and plays a central role during spermatogenesis. In addition, Dazl is the main translation regulator of many proteins in the process of mouse spermatogenesis (including Ddx4). Ddx4 (DEAD-Box helicase 4) is a protein coding gene and a key gene that affects spermatogenesis.[25] The gene is specifically expressed in the germ cell lineage in both sexes and functions in germ cell development. Given the importance of Dazl and Ddx4, we carried out both qPCR and western blot experiments to verify their expression. Quantitative real-time PCR and western blot experiments showed that the expression of Dazl and Ddx4 were up-regulated at the same time, indicating that these two genes are positively correlated (Figure 6).

Notably, Dazl also has an important regulatory effect on meiosis-related genes (Sycp1 and Sycp3).[24] Therefore, we believe that the increased expression of Dazl should promote the translation and expression of Sycp1 and Sycp3, and subsequent qPCR and western blot experiments proved this

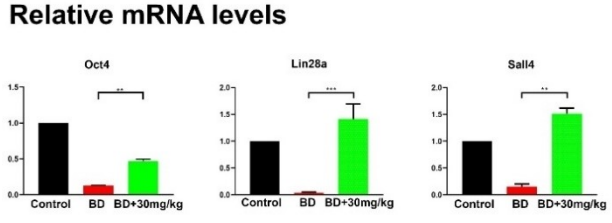

Figure 4. Expression levels of Oct4 and its important related genes Lin28a and Sall4 (The experiment was carried out six times).

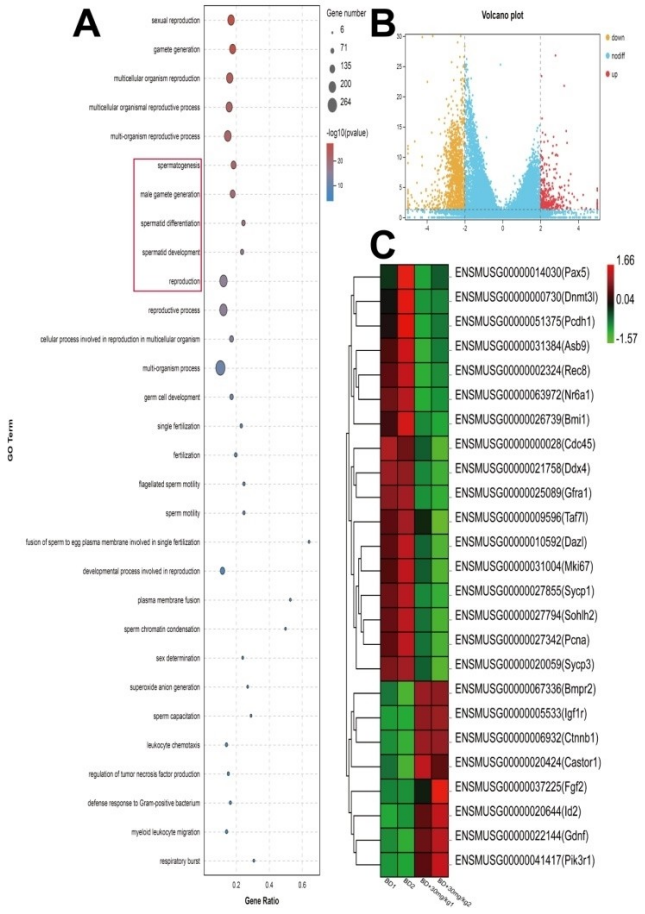

Figure 5. mRNA-seq data. (A) GO enrichment of up-regulated genes in the comparation of BD group vs. BD + 30 mg kg^{-1} group. (B) Volcano map of discrepant genes in the comparation. (C) Heat map of the differentially expressed genes, which are crucial for spermatogenesis in the comparation.

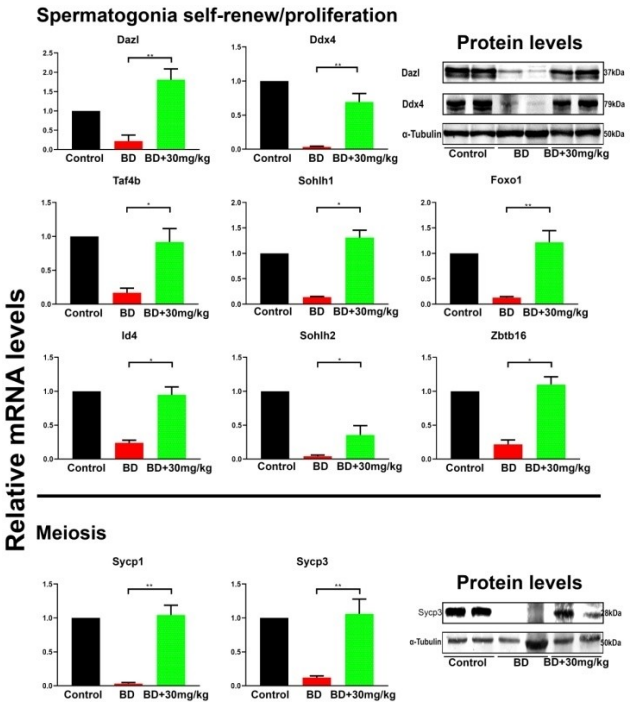

Figure 6. Dazl and its regulated gene expression levels. Expression levels of spermatogonia self-renew/proliferation related genes: Dazl, Ddx4, Taf4b, Sohlh1, Sohlh2. Foxo1, Id4, Zbtb16. Meiosis related genes: Sycp1, Sycp3. The results are presented as means ± SD, with n = 6 per group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ (compared to the BD group).

point (Figure 6). Finally, we conclude that the possible molecular regulation mechanism is that NBMA targets Oct4 to up-regulate the expression of Oct4, which subsequently causes the up-regulation of the expression of Dazl and other related genes, thereby promoting the differentiation of spermatogonial stem cells.

Conclusions

In summary, we used computer-aided drug molecular design and chemical synthesis strategies to obtain the Oct4-targeted drug molecule NBMA, and this molecule can effectively promote spermatogenesis. To further verify its molecular mechanism, we found differentially expressed genes closely related to spermatogenesis by transcriptome sequencing and enrichment analysis, and verified experimentally by qPCR and western blot experiments. Finally, it was found that the Oct4 pathway mediated by NBMA may be an important way to promote the recovery of spermatogenesis in azoospermic mice. The results of this study preliminarily clarified the molecular mechanism of NBMA to promote spermatogenesis, which will lay the foundation for the next step of our development of more effective azoospermia treatment drugs.

Experimental Section

Animals and Treatment

Six-week-old male ICR mice weighing 25–30 g (Laboratory Animal Center of Nantong University) were maintained in a 12 h dark/light cycle under temperature at 23 ± 2 °C and relative humidity of 45–55 %. The animals were fed with ad libitum access to water and food. After one week of adjusting, the animals were randomly divided into five groups (8 mice per group). Firstly, busulfan was dissolved in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO, USA). Before injection, distilled water was added to obtain final concentrations of 8 mg mL^{-1}. Four groups were received, i. p. 40 mg kg^{-1} in a volume of approximately 120 μL while the rest were untreated. 4 weeks after treatment of busulfan, those untreated mice in groups 1 received normal saline, serving as the control, mice in group 2 were treated with DMSO while mouse in groups 3, 4, 5 received i.g. 1 mg kg^{-1}, 10 mg kg^{-1} or 30 mg kg^{-1} of NBMA, which was dissolved in a mixture of dimethyl sulfoxide and water, per day for five days, respectively. Mice were sacrificed on two weeks and four weeks. Blood was collected, placed in a gel glass tube, and centrifuged at 1500 g for 10 minutes to collect serum samples. Serum samples were labeled and stored at −80 °C for hormone [testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH)] analysis. Furthermore, all testes were separated and weighed. One testis from each animal was frozen in liquid nitrogen and stored at −80 °C for subsequent gene and protein expression level analysis.

Epididymal Sperm Analysis

We cut the cauda of epididymis into fragments in warm phosphate-buffered saline (PBS; 1 mL; 37 °C; pH 7.4) to release the sperm, and incubated the sperm suspension for 10 minutes at 37 °C. Then we put 10 μL diluted sperm suspension on a glass slide and covered with a coverslip, followed analysis using by a computer-assisted sperm analysis (CASA) system. At least 5 fields of each mouse were captured, and the index, including sperm concentration, viability, and other parameters, were examined for a comprehensive assessment.

Histological Analysis

Testes per group collected from the two-time point were selected and fixed in Bouin's fluid, embedded in paraffin wax, cut into sections of 5 μm, and then deparaffinized for hematoxylin and eosin (HE) staining. HE staining was carried out by standard protocol. Approximately sixty sections of the seminiferous tubules were randomly selected from each testis. About two hundred tubules were checked per group. The results are presented as the three stages of spermatogenesis: spermatogonia, spermatocyte, spermatids, respectively, and the percentage of tubules of each stage **for** about 200 tubular sections in each group.

Western Blot Analysis

Testes were homogenized in the ice-cold PBS, and then the homogenates were lysed with RIPA buffer to obtain proteins. The protein concentrations of samples were measured using BCA Protein Assay Kit in a plate reader. An aliquot of 30 μg protein from each sample was electrophoresed in 10 % polyacrylamide gels containing sodium dodecyl sulphate, and then the separated proteins were electrically transferred onto the nitrocellulose membranes. After blocking with 5 % non-fat milk in Tris-buffered saline Tween-20 buffer for an hour, the membranes were incubated with primary antibodies against Dazl (1 : 1000), Ddx4 (1 : 1000), Sycp3 (1 : 1000), and Tubulin (1 : 3000) (listed in Supporting Table S2)

overnight at 4 °C. Then the membranes were washed and incubated with HRP-conjugated anti-rabbit or anti-goat IgG secondary antibody (1 : 5000, Bioword, United States) at room temperature for an hour. The immunoreactive bands were visualized by chemiluminescence using an Amersham Typhoon Fluorescent protein imaging system (Cytiva, Sweden). The intensity of the band was analysed with Image Lab software. The housekeeper protein α-tubulin was used to standardize protein density across samples.

RNA Isolation and Real-Time PCR (qPCR)

Total RNAs were purified from the testes of three groups of mice after DMSO or Drug treatment using the Trizol reagent (Invitrogen, Carlsbad, CA, USA), and the concentration of RNA was measured by reading OD value at 260 nm. The first strand (cDNA) was reversely transcribed and used as the template for qPCR analysis. The genes playing a role in the development of spermatogonia (Pax5, Dazl, Ddx4, Lin28a, Sohlh1, Sohlh2, Sall4, Foxo1, ID4, Ztbt16, Taf4b) and spermatocyte (Sycp1, Sycp3) were measured by using TB Green™ Premix Ex Taq™ II (Takara Biotechnology, Kyoto, Japan). The qPCR reaction mixture had 10 μL TB Green mix, 1.6 μLl forward and reverse primer mix, 2 μg diluted cDNA sample, and 5–8 μL RNase-free water. The reaction was processed on a Light Cycler 96 Real-Time System (Roche, Switzerland) by the following program: 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. The relative expression of testicular genes was normalized to β-Actin. The primers were listed in Table S3 (Supporting Information).

Immunofluorescence Assay

The tissue-containing slices were washed with phosphate-buffered saline and incubated for an hour in a blocking solution containing normal serum. Subsequently, the slices were incubated with primary rabbit anti-GPX4 (1 : 200) antibodies at 4 °C overnight. On the following day, anti-rabbit IgG Alexa Fluor 594 antibody (1 : 300) was added at room temperature for an hour. The slices were then rinsed and stained with a Hoechst 33342 staining solution and observed under a fluorescent microscope (Axio Imager M2, Zeiss, German).

Statistical Analysis

All data are presented as the mean ± SD from at least 3 independent experiments. Statistical significance was analysed using one-way ANOVA followed by ad hoc Turkey's multiple comparisons to the control. Statistical analysis was performed using GraphPad Prism (version 6, GraphPad Software Inc., San Diego, CA, United States). A $p < 0.05$ was considered statistically significant.

Ethics Statement

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Nantong University and Experiments were approved by the Animal Ethics Committee of SYXK(SU) 2007-0021.

Acknowledgements

We thank the National Key Research and Development Program of China (2018YFC1003500 to F. S.) and the Six Talent Peaks Project in Jiangsu Province (131219631004 to J. Y.) for financial support.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: male infertility · NBMA · Oct4 · spermatogenesis · targeted drug design

[1] S. Jiao, Y. Yang, S. Chen, *Hum. Reprod. Update* **2021**, *27*, 154–189.
[2] N. A. Kurhanewicz, D. Dinwiddie, Z. D. Bush, D. E. Libuda, *Curr. Biol.* **2020**, *30*, 5007–5017.
[3] A. Agarwal, A. Mulgund, A. Hamada, M. R. Chyatte, *Reprod. Biol. Endocrinol.* **2015**, *13*, 37.
[4] Y. Sato, A. Tajima, K. Tsunematsu, S. Nozawa, M. Yoshiike, E. Koh, J. Kanaya, M. Namiki, K. Matsumiya, A. Tsujimura, K. Komatsu, N. Itoh, J. Eguchi, I. Imoto, A. Yamauchi, T. Iwamoto, *Hum. Reprod.* **2015**, *30*, 1510–1514.
[5] J. Boivin, L. Bunting, J. A. Collins, K. G. Nygren, *Hum. Reprod.* **2009**, *24*, 2380–2383.
[6] E. Caroppo, E. M. Colpi, G. Gazzano, L. Vaccalluzzo, F. I. Scroppo, G. D'Amato, G. M. Colpi, *J. Assist. Reprod. Genet.* **2017**, *34*, 149–154.
[7] W. Liu, X. Gao, G. Ma, L. Yan, T. Chen, T. Li, R. M. Yu, J. L. Ma, *Andrologia* **2017**, *49*, e12705.
[8] K. Chiba, N. Enatsu, M. Fujisawa, *Med. Biol.* **2016**, *15*, 165–173.
[9] S. Sunderam, D. M. Kissin, Y. Zhang, S. G. Folger, S. L. Boulet, L. Warner, W. M. Callaghan, W. D. Barfield, *Assisted reproductive technology surveillance*, United States, 2016. *MMWR Surveillance Summaries* **2019**, *68*, 1.
[10] S. Yilmaz, N. Sezer, I. M. Gönenç, S. E. Ilhan, E. Yilmaz, *Cytotechnology* **2018**, *70*, 489–495.
[11] J. Takeda, S. Seino, G. I. Bell, *Nucleic Acids Res.* **1992**, *20*, 4613–4620.
[12] L. A. Boyer, T. I. Lee, M. F. Cole, S. E. Johnstone, S. S. Levine, J. P. Zucker, M. G. Guenther, R. M. Kumar, H. L. Murray, R. G. Jenner, D. K. Gifford, D. A. Melton, R. Jaenisch, R. A. Young, *Cell* **2005**, *122*, 947–956.
[13] Y. Atlasi, S. J. Mowla, S. A. M. Ziaee, P. J. Gokhale, P. W. Andrews, *Stem Cells* **2008**, *26*, 3068–3074.
[14] X. Wang, J. Dai, *Stem Cells* **2010**, *28*, 885–893.
[15] J. Kehler, E. Tolkunova, B. Koschorz, M. Pesce, L. Gentile, M. Boiani, H. Lomelí, A. Nagy, K. J. McLaughlin, H. R. Schöler, A. Tomilin, *EMBO Rep.* **2004**, *5*, 1078–1083.
[16] H. Niwa, J. Miyazaki, A. G. Smith, *Nat. Genet.* **2000**, *24*, 372–376.
[17] H. R. Scholer, G. R. Dressler, R. Balling, H. Rohdewohld, P. Gruss, *EMBO J.* **1990**, *9*, 2185–2195.
[18] G. Cauffman, H. Van de Velde, I. Liebaers, A. Van Steirteghem, *Mol. Hum. Reprod.* **2004**, *11*, 173–181.
[19] M. Pardo, B. Lang, L. Yu, H. Prosser, A. Bradley, M. M. Babu, J. Choudhary, *Cell Stem Cell* **2010**, *6*, 382–395.
[20] F. Fang, B. Angulo, N. Xia, M. Sukhwani, Z. Wang, C. C. Carey, A. Mazurie, J. Cui, R. Wilkinson, B. Wiedenheft, N. Irie, M. A. Surani, K. E. Orwig, R. A. Reijo Pera, *Nat. Cell Biol.* **2018**, *20*, 655–665.
[21] a) J. Yang, X. Wu, B. Yang, Y. Liu, R. Cheng, Z. Gong, F. Sun, *RSC Adv.* **2021**, *11*, 164–167; b) C. Wang, J. Yang, X. Meng, Y. Sun, X. Man, J. Li, F. Sun, *Dalton Trans.* **2019**, *48*, 4474–4478; c) Z. Shen, H. Huang, C. Zhu, S. Warratz, L. Ackermann, *Org. Lett.* **2019**, *21*, 571–574; d) C. Zhu, J. C. A. Oliveira, Z. Shen, H. Huang, L. Ackermann, *ACS Catal.* **2018**, *8*, 4402–4407; e) W. Liu, G. Cera, J. C. A. Oliveira, Z. Shen, L. Ackermann, *Chem. Eur. J.* **2017**, *23*, 11524–11528.
[22] a) M. Shen, B. E. Leslie, T. G. Driver, *Angew. Chem. Int. Ed.* **2008**, *47*, 5056–5059; *Angew. Chem.* **2008**, *120*, 5134–5137; b) C. Chen, W. Sun, Y.

Yan, F. Yang, Y. Wang, Y.-P. Zhu, L. Liu, B. Zhu, *Adv. Synth. Catal.* **2020**, *362*, 2970–2975.
[23] a) F. Ma, Z. Zhou, N. Li, L. Zheng, C. Wu, B. Niu, F. Tang, X. He, G. Li, J. Hua, *Sci. Rep.* **2016**, *6*, 1–12; b) H. H. Chen, M. Welling, D. B. Bloch, J. Muñoz, E. Mientjes, X. Chen, C. Tramp, J. Wu, A. Yabuuchi, Y.-F. Chou, C. Buecker, A. Krainer, R. Willemsen, A. J. Heck, N. Geijsen, *Stem Cell Rev.* **2014**, *3*, 892–904; c) M. M. Mikedis, Y. Fan, P. K. Nicholls, T. Endo, E. K. Jackson, S. A. Cobb, D. G. de Rooij, D. C. Page, *eLife* **2020**, *9*, e56523.
[24] H. Li, Z. Liang, J. Yang, D. Wang, H. Wang, M. Zhu, B. Geng, E. Y. Xu, *Natl. Sci. Rev.* **2019**, *6*, 455–468.
[25] a) N. A. Fraunhoffer, A. M. Abuelafia, I. Stella, S. Galliano, M. Barrios, A. D. Vitullo, *J. Ovonic Res.* **2015**, *8*, 1–7; b) J. V. Medrano, C. Ramathal, H. N. Nguyen, C. Simon, R. A. Reijo Pera, *Stem Cells* **2012**, *30*, 441–451.

Manuscript received: September 22, 2021
Revised manuscript received: January 26, 2022