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ORIGINAL ARTICLE

Paternal vitamin D deficiency is associated with offspring testicular developmental abnormalities, fibrosis, and altered sperm miRNA profiles

Yu-Ming Chen[1] · Zhi-Hong Tian[1,2] · Shi-Yuan Li[1] · Juan Dai[3] · Xing-Ru Wang[1] · Guo-Ying Sun[1,4,5] · Min Liu[1,4,5] · Berthold Hocher[2,6,7,8] · Mei Tian[1,4,5]

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Abstract

Background Paternal environmental factors can influence offspring development and health through sperm-carried epigenetic information, but the underlying mechanisms are still not fully understood. This study aims to investigate whether paternal vitamin D deficiency can modify the sperm-derived miRNAs and whether it affects the testicular development of the offspring, and to explore the potential molecular mechanisms.

Methods Four-week-old male C57BL/6J mice were fed with vitamin D-sufficient or -deficient diet for 16 weeks, then mated with healthy female mice to obtain F1 offspring. High-throughput sequencing of paternal sperm miRNAs and bioinformatics analysis were performed. All offspring were raised to adulthood under standard feeding conditions, and their body weight, reproductive organ development, testicular histomorphology and molecular characteristics were systematically evaluated.

Results Paternal vitamin D deficiency induced significant alterations in the sperm miRNA expression profile, with a total of 16 differentially expressed miRNAs being identified. The target genes of these miRNAs were found to be enriched in pathways related to oxidative stress and fibrosis. Compared to the control group, the male offspring of VDD father group exhibited decreased body weight and testicular weight, accompanied by abnormal testicular tissue structure. At the molecular level, the expression of antioxidant-related genes in the offspring testes was down-regulated, while the TGF-β/SMAD2 signaling pathway and fibrosis markers were significantly up-regulated, suggesting enhanced oxidative stress and activation of the fibrotic remodeling.

Conclusions This study suggests that paternal vitamin D deficiency may reshape the oxidative stress and fibrosis-related pathways in offspring's testes accompanied by altering sperm miRNA-mediated epigenetic information.

Keywords Paternal vitamin D deficiency · Sperm miRNA · Epigenetics · Offspring testicular development · Inflammation and oxidative stress

Introduction

Developmental origins of health and disease (DOHaD) proposes that environmental conditions during critical windows of early life, including the preconception, intrauterine, and early postnatal periods, can shape disease susceptibility and health outcomes later in life [1]. Although early DOHaD research has largely emphasized maternal and intrauterine influences, paternal exposures are increasingly recognized as important contributors to offspring development [2]. Experimental studies have shown that adverse paternal conditions, including high-fat diet, obesity, sleep deprivation, and combined exposure to nicotine, ethanol, and caffeine, are associated with impaired sperm function, altered offspring metabolism or fertility, and adverse pregnancy outcomes [3–6]. These findings indicate that paternal

Yu-Ming Chen and Zhi-Hong Tian contributed equally to this work and share first author.

Extended author information available on the last page of the article

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environmental factors should be considered an integral component of developmental programming.

The mechanisms by which paternal exposures exert long-term effects on offspring phenotypes remain incompletely understood. Sperm-borne molecular information, particularly epigenetic signals, has been proposed as a key mediator of this process [7, 8]. In mice, a paternal high-fat diet alters testicular gene expression [9], increases sperm DNA damage and reactive oxygen species (ROS) levels [10, 11], and modifies histone acetylation [12]. These molecular alterations are associated with reduced sperm function and disrupted embryonic development [10, 13]. It should be noted that sperm DNA damage primarily reflects genomic integrity rather than a canonical epigenetic mark, although it often co-occurs with epigenetic alterations. Consistently, human studies have linked obesity with decreased sperm concentration and motility, as well as increased sperm DNA damage [14]. In animal models, reproductive phenotypes induced by paternal high-fat diet or genotoxic exposure can persist into subsequent generations [11, 15], suggesting that sperm-derived molecular changes may contribute to intergenerational or, in some cases, transgenerational effects. Candidate epigenetic carriers include chromatin organization, DNA methylation, histone modifications, and coding or non-coding RNA cargo [16, 17].

Among these candidates, sperm small non-coding RNAs (sncRNAs), particularly microRNAs (miRNAs), have emerged as sensitive mediators of paternal environmental information [18]. Sperm miRNA profiles dynamically respond to paternal metabolic status, diet, stress, and lifestyle factors. For example, RNA sequencing studies have reported increased expression of inflammation-related miRNAs, including miR-155 and miR-122, in the plasma and sperm of obese men compared with controls [19]. Animal studies further support this concept: paternal obesity alters sperm miRNA profiles, and these changes have been linked to metabolic and reproductive phenotypes in offspring [20, 21]. Exercise intervention can remodel sperm miRNA signatures, including miRNAs involved in adipocyte differentiation and insulin signaling, such as miR-193b and miR-204 [22]. Beyond metabolic exposures, paternal glucocorticoid or stress exposure also changes sperm sncRNA or miRNA profiles and affects neuroendocrine and behavioral phenotypes in offspring [23, 24]. Together, these findings suggest that sperm sncRNAs are highly responsive to paternal environmental perturbations and may participate in the transmission of acquired phenotypic information across generations.

Vitamin D deficiency (VDD) is highly prevalent worldwide and remains an important public health concern [25, 26]. Beyond its classical role in calcium and phosphate homeostasis, vitamin D acts through endocrine, paracrine, and autocrine pathways and influences multiple organs and physiological systems. Accumulating evidence suggests that vitamin D signaling is involved in male reproductive function, including steroidogenesis, spermatogenesis, and sperm physiology [27]. Animal studies indicate that vitamin D is required for normal sex hormone signaling and sperm motility [28]. In men, serum 25-hydroxyvitamin D [25(OH)D] and free 25(OH)D levels have been positively associated with sperm motility, morphology, and fertility-related parameters [29, 30]. Consistent with these observations, vitamin D receptor-deficient mice exhibit reduced sperm motility, viability, and sperm count [31], whereas dietary vitamin D deficiency in male rodents impairs sperm motility and morphology and reduces mating success and pregnancy rates [32].

Although VDD has been linked to impaired sperm quality, whether paternal VDD affects offspring reproductive development through sperm epigenetic alterations remains unclear. In particular, evidence connecting paternal VDD, sperm sncRNA or miRNA remodeling, and testicular development in male offspring is still limited. Therefore, this study aimed to investigate whether paternal vitamin D deficiency alters sperm epigenetic information and whether these changes are associated with impaired testicular development in offspring.

Materials and methods

Reagents

Ethanol was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). TRIzol reagent (AG21102) was obtained from Accurate Biochnology Co., Ltd. (Changsha, China), and chloroform substitute (G3014-01) was purchased from Servicebio Biotechnology Co., Ltd. (Wuhan, China). Reverse transcription kit (AU341-02) and qPCR kit (11201ES08) were obtained from TransGen Biotech Co., Ltd. (Beijing, China) and Yeasen Biotechnology Co., Ltd. (Shanghai, China), respectively. Serum testosterone levels were measured using a mouse testosterone ELISA kit (SEKSM-0073, Solarbio Biotech Co., Ltd.)

Animals and ethics

Male C57/BL6J mice (4 weeks old) and female C57BL/6J mice (8–9 weeks old) used in this study were purchased from Jiangsu Jicui Yao Kang Co., Ltd. After a one-week acclimatization period in the quarantine room of Hunan Normal University Health Science Center, they were housed five per cage in SPF-grade animal facilities. Housing conditions: Maintained under a 12 h/12 h light/dark cycle at

20–25 °C with free access to food and water. All animal experiments were reviewed and approved by the Ethics Committee of Hunan Normal University (Ethics Approval No. D2023009), and procedures strictly adhered to the Regulations on the Management of Laboratory Animals. Purified feed was purchased from Shenzhen Ruidi Biotechnology Co., Ltd.

Dietary interventions and mating study

Prior to mating, male C57BL/6 mice were randomly assigned to receive either a control diet (CD, $n = 20$; 1000 IU/kg vitamin D) or a vitamin D-deficient diet (VDD, $n = 20$; 0 IU/kg vitamin D) [33]. After the dietary intervention, F0 males were mated with untreated vitamin D-replete female mice. The parental and offspring generations were designated as F0 and F1, respectively. All F0 female mice were maintained on the same standard purified diet containing sufficient vitamin D throughout the study, thereby minimizing potential maternal dietary confounding factors. After birth, litter size was standardized to four pups per litter on postnatal day 3, with sex balance maintained as far as possible. F1 offspring from both paternal groups were fed a standard vitamin D-replete purified diet after weaning.

Serum testing

At 20 weeks of age, the blood drawn from the orbital venous plexus of anesthetized F0 generation male mice was injected into a prepared 1.5 ml EP tube. Serum was separated by centrifugation at 3000 g for 10 min at room temperature and stored at -80 °C. Ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was employed to determine the concentration levels of total 25-hydroxyvitamin D [25(OH)D] and its two metabolites, $25(OH)D_2$ and $25(OH)D_3$ in F0 paternal mice [34]. The Mouse Testosterone ELISA Kit was used to detect serum testosterone levels.

Sperm sample collection

The paternal mice were sacrificed, and cauda epididymides with ~ 1 cm vas deferens were dissected, minced, and incubated at 37 °C for 1 h. The suspension was filtered through a 40 μm strainer, and 10 μL was used for sperm concentration, motility, and morphology assessment according to the WHO guidelines [35]. All counts were performed double-blindly, and averages were used for analysis. Filtered sperm were centrifuged at 2,000 × g for 2 min, the supernatant discarded, and somatic cells lysed with 250 μL Triton X-100/0.05 g SDS in 50 mL DEPC water on ice for 40 min. Absence of somatic cells was confirmed microscopically. Sperm were washed twice with PBS, centrifuged at 600 × g for 5 min, resuspended in 1 mL TRIzol, and stored at − 80 °C. Total sperm RNA was extracted and qualified samples sent for miRNA sequencing.

Small RNA sequencing and bioinformatic analysis

Small RNA libraries were prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina according to the manufacturer's instructions and sequenced on an Illumina NovaSeq 6000 platform. Raw sequencing reads were subjected to quality control using FastQC, followed by adapter trimming and length filtering using Cutadapt. Clean reads ranging from 18 to 30 nucleotides in length were retained for subsequent analysis. The processed reads were mapped to the mouse reference genome GRCm39 using Bowtie. Known miRNAs were identified by alignment to the miRBase database (version 22.1). The expression levels of miRNAs were quantified and normalized as counts per million. Differentially expressed miRNAs between the control diet (CD) and vitamin D deficiency (VDD) groups were identified using DESeq2. miRNAs with a false discovery rate (FDR) less than 0.05 and an absolute $\log_2$ fold change greater than 1 were considered significantly differentially expressed. Potential target genes of differentially expressed miRNAs were predicted using TargetScan and miRanda. Functional enrichment analyses, including Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway analyses, were performed using the clusterProfiler package in R.

Analysis of offspring development and glucose metabolism

Body weight of F1 mice was recorded weekly from 4 to 24 weeks of age [20]. Intraperitoneal glucose tolerance tests (IPGTTs) were performed at 12 and 24 weeks of age. Mice were fasted for 6 h before testing. Glucose was administered by intraperitoneal injection at a dose of 2 g/kg body weight. Blood glucose concentrations were measured at 0, 30, 60, 90, and 120 min after glucose injection. Fasting blood glucose levels, glucose tolerance curves, and the area under the curve (AUC) over 120 min were calculated and used for analysis.

Mouse tissue collection

At 24 weeks, F1 mice were fasted 12 h and weighed. Blood was collected via orbital vein under 3% isoflurane. Serum stored at − 80 °C. Testes were weighed (wet weight). Organ index = 100 × wet weight / body weight. The left testis was

snap-frozen at −80 °C, and the right testis was fixed in 4% paraformaldehyde.

Histological analysis

Right testicles from offspring mice fixed in 4% paraformaldehyde were harvested, embedded in paraffin, and sectioned for hematoxylin and eosin (HE) staining. Imaging was performed using an Olympus AH-2 light microscope (Olympus, Tokyo, Japan). Two researchers independently examined five randomly selected areas per section under a light microscope to evaluate relevant indicators ($n = 12$ mice per group). Masson staining of mouse testicular tissue was used to assess the degree of testicular fibrosis.

Total RNA extraction, reverse transcription, and qPCR

Total RNA was isolated from testicular tissue using TRIzol reagent and reverse transcribed into cDNA using a cDNA synthesis kit. qPCR was performed using the SYBR Green qPCR Premix Reagent Kit and the LightCycler 480 real-time fluorescent quantitative PCR cycler. The primer sequences for mouse genes used in this study are shown in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for quantitative normalization.

Western blotting

Frozen mouse testicular tissues were homogenized on ice in RIPA lysis buffer supplemented with phenylmethylsulfonyl fluoride (PMSF, 100:1) using a BeadRuptor 12 homogenizer. The homogenates were incubated on ice for 20 min and then centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatants were collected, and protein concentrations were determined using a BCA protein assay kit according to the manufacturer's instructions. Equal amounts of protein (50 µg per sample) were mixed with 2× Laemmli sample buffer, denatured by boiling, and separated by 12% SDS-PAGE. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membranes using a semi-dry transfer system. The membranes were blocked with 5% bovine serum albumin (BSA) in TBST at room temperature for 1 h and incubated overnight at 4 °C with primary antibodies against TNF-α (346654, Zen-bioscience, 1:5000), IL-1β (516288, Zen-bioscience, 1:5000), IL-10 (502171, Zen-bioscience), 1:5000, TGF-β1 (346599, Zen-bioscience, 1:5000), SMAD2 (R25742, Zen-bioscience, 1:5000), phosphorylated SMAD2 (p-SMAD2) (R26361, Zen-bioscience, 1:5000), α-smooth muscle actin (α-SMA) (R23450, Zen-bioscience, 1:5000), and GAPDH (GB15004-100, Servicebio, 1:10000). After washing with TBST, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (AS014, ABclonal, 1:10000) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection system and captured using a GenoSens2000 imaging system (CLINX, China). Band intensities were quantified using ImageJ software and normalized to GAPDH.

Measurement of oxidative stress in testicular tissue

Prepare 5% or 10% testis homogenates and centrifuge. Using the supernatant, GSH, MDA (TBA), and T-SOD (hydroxylamine) levels were measured as per Nanjing Jiancheng Bioengineering Institute kit instructions.

Statistical analysis

The Shapiro–Wilk test is used to test the normality of each dataset. Outliers are eliminated in accordance with the Dixon criterion. Results are mean ± SEM. Analyses were performed in GraphPad Prism 9.5.0 ($P < 0.05$).

Table 1 Sequences of primers used in RT-qPCR

Genes	Forward primer	Reverse primer
Cat	5'-ATTGCCGTCCGATTCTCC-3'	5'-CCAGTTACCATCTTCAGTGTAG-3'
Gst	5'-CCCAGGCTAGGAGTGGTCAT-3'	5'-TATCCTCTGGAATGCGGTCG-3'
Sod	5'-ACTTCGAGCAGAAGGCAAGC-3'	5'-GTCTCCAACATGCCTCTCTTCAT-3'
Nf-kb	5'-ACCTGAGTCTTCTGGACCGCTG-3'	5'-CCAGCCTTCTCCCAAGAGTCGT-3'
Tnf-α	5'-ACGGCATGGATCTCAAAGAC-3'	5'-GTGGGTGAGGAGCACGTAGT-3'
IL-1β	5'-GCTGCTTCCAAACCTTTGAC-3'	5'-AGCTTCTCCACAGCCACACAAT-3'
IL-10	5'-CTGTCATCGATTTCTCCCCTGTG-3'	5'-TGGTCTTGGAGCTTATTAAATCAC-3'
Star	5'-TGCCGAAGACAATCATCAAC-3'	5'-CAGGTCAATGTGGTGGACAG-3'
Cyp11a1	5'-CCTTTATGAGATGGCACACAA-3'	5'-GATGCTGGCTTTGAGGAGTG-3'
α-SMA	5'-GTCCCAGACATCAGGGAGTAA-3'	5'-TCGGATACTTCAGCGTCAGGA-3'
Col3a1	5'-TCTGCCACCCCGAACTCA-3'	5'-TGCTTACGTGGGACAGTCATG-3'
Tgf-β1	5'-GAGCCCTGGATACCAACTATTG-3'	5'-GCAGGGTCCCAGACAGAAG-3'
Smad2	5'-AATACGGTAGATCAGTGGGACA-3'	5'-CAGTTTTCGATTGCCTTGAGC-3'
GAPDH	5'-GACAACTTTGGCATTGTGGA-3′	5'-ATGCAGGGATGATGTTCTGG-3'

Results

Paternal VDD induces sperm miRNA remodeling

To establish a vitamin D deficiency mouse model, the control group was given a purified diet with normal vitamin D content (1000 IU/kg), while the vitamin D deficiency group was given a purified diet without vitamin D (0 IU/kg). After 16 weeks of dietary intake, serum tests showed that the levels of 25(OH)D and 25(OH)D3 in male mice of the vitamin D deficiency group were significantly lower than those in the control group (Fig. 1A, B), indicating that the vitamin D deficiency model was successfully established.

We performed small RNA sequencing on sperm from two groups of father mice to determine whether VDD treatment alters sperm miRNA expression. By comparing the differences in sperm miRNA expression between the VDD group and the CD group of fathers, we evaluated the regulatory effect of paternal VDD exposure on sperm miRNAs. Hierarchical clustering analysis using Pearson correlation as an indicator successfully distinguished the samples of the VDD group and the CD group, and the results are shown in a heatmap (Fig. 1C). Differential expression analysis identified 98 miRNAs that were upregulated by ≥2-fold (counts per million, CPM) and 142 miRNAs that were downregulated by ≥2-fold in the sperm of the VDD group (Fig. 1D). The full list of differentially expressed miRNAs is provided in Supplementary Table S1. To further reveal the potential mechanism of the transgenerational effects, we focused on miRNAs with a fold change≥2 and identified a total of 16 significantly differentially expressed miRNAs. These miRNAs predicted a total of 1472 downstream target genes. Further functional enrichment analysis using the R package clusterProfiler found that signaling pathways related to redox and fibrosis were significantly enriched (Fig. 1E). For these pathways, we identified miRNAs that may be involved in their regulation and confirmed that their target genes all have corresponding miRNA binding sites (Fig. 1F).

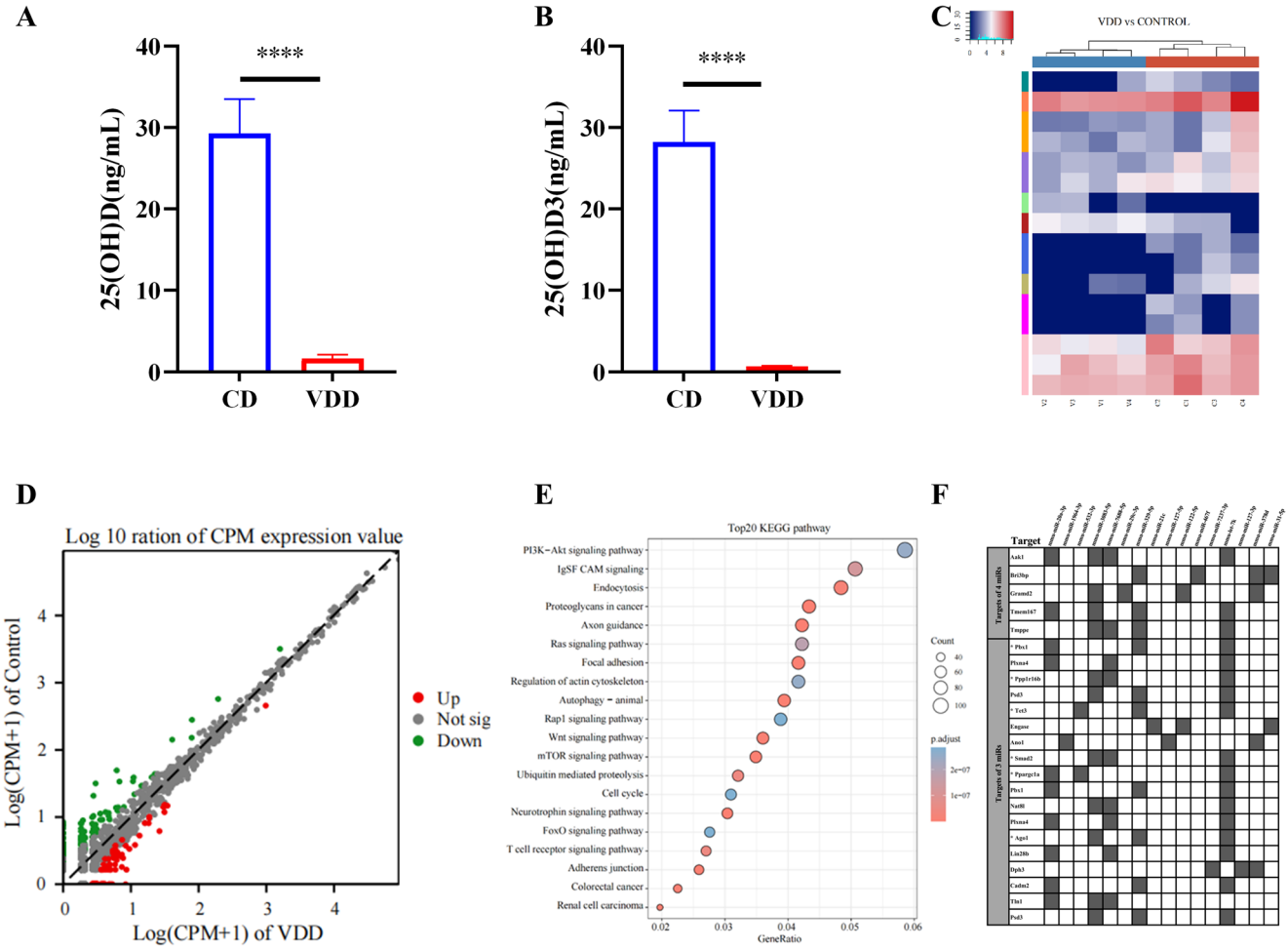

Fig. 1 Paternal VDD induces sperm miRNA remodeling. (**A**) 25(OH)D level; (**B**) 25(OH)D3 level; (**C**) Sample heatmap; (**D**) Expression values of genes with expression changes vs. the average value of the control group; (**E**) KEGG enrichment analysis of differentially expressed genes; (**F**) Genes with predicted miRNA binding sites were found to have altered expression in sperm of VDD-treated animals. ($n=4$, ns indicates $P>0.05$, ****indicates $P<0.0001$)

Paternal VDD affects offspring growth and metabolic trajectories

To evaluate the effects of paternal VDD on offspring growth and metabolism, body weight and organ indices were monitored in male F1 offspring from 4 to 24 weeks of age. From week 7 onward, male offspring of VDD fathers showed significantly lower body weight gain than offspring of CD fathers ($P<0.05$) (Fig. 2A). Analysis of organ weights showed that testis weight was significantly reduced in VDD-F1 offspring compared with CD-F1 offspring ($P<0.01$) (Fig. 2B). The liver weight ($P<0.05$) and testis organ weight ratio ($P<0.05$) of offspring mice in the VDD group were significantly lower than those in the CD group (Fig. 2C, D). These data suggest that paternal VDD affects the body weight of offspring mice, and the organ indices of the testis and liver are abnormal.

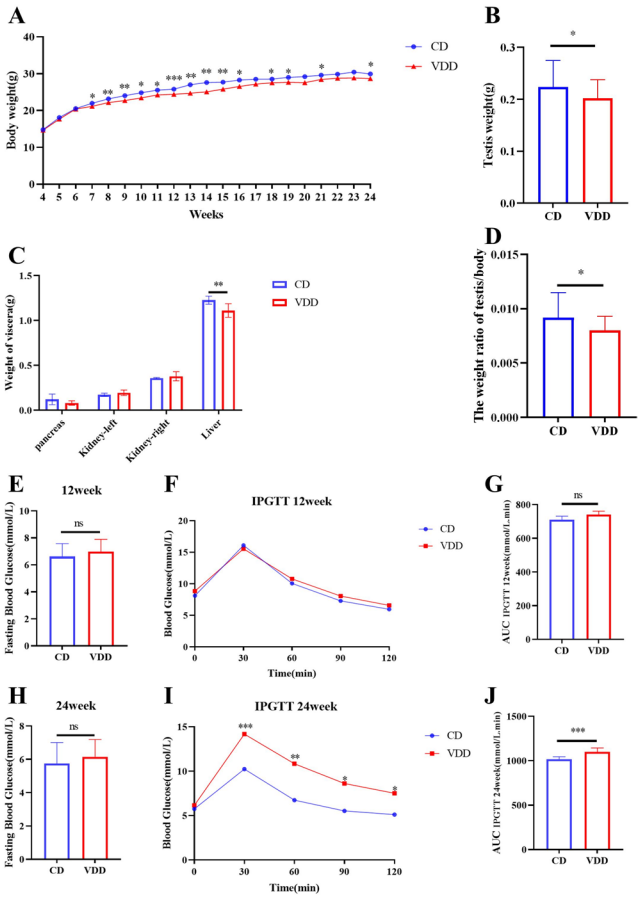

Fig. 2 Paternal VDD affects offspring growth and metabolic trajectories. (**A**) Body weight changes of F1 male offspring from 4 to 24 weeks ($n=12$); (**B**) Left testis weight of male mice ($n=12$); (**C**) Weights of major organs in F1 male offspring ($n=12$); (**D**) Testis organ weight ratio of male mice ($n=12$); (**E**) Fasting blood glucose of 12-week-old male offspring; (**F-G**) Glucose tolerance test and its AUC of 12-week-old male offspring; (**H**) Fasting blood glucose of 24-week-old male offspring; (**I-J**) Glucose tolerance test and its AUC of 24-week-old male offspring; CD ($n=12$) VDD ($n=12$). Data are presented as mean ± SEM. Statistical analyses were performed using unpaired t-test for **A–E**, and two-way repeated measures ANOVA with Bonferroni correction for F and I. (ns indicates $P>0.05$; *, ** and *** represent $P<0.05$, $P<0.01$ and $P<0.001$, respectively. CD: normal diet; VDD: vitamin D deficiency diet; AUC: area under curve)

In addition, we measured the glucose metabolism indices of the offspring mice. We detected the fasting blood glucose of F1 offspring mice at 12 weeks and 24 weeks of age and performed glucose tolerance tests. As shown in Fig. 2E, there was no significant difference in fasting blood glucose between the two groups of 12-week-old mice ($P>0.05$). As shown in Fig. 2F-G, after intraperitoneal glucose injection, the GTT curves of the two groups of 12-week-old mice rose rapidly and reached a peak at 30 min, followed by a slow decline. There were no significant differences in blood glucose levels between the two groups at 30 min, 60 min, 90 min, and 120 min ($P>0.05$), and there was also no significant difference in the area under the GTT curve (GTT-AUC) between the two groups ($P>0.05$). As shown in Fig. 2H, there was no significant difference in fasting blood glucose between the two groups of 24-week-old offspring mice ($P>0.05$). As shown in Fig. 2I-J, the GTT curve of the 24-week-old VDD group offspring mice rose rapidly after intraperitoneal glucose injection and reached a peak at 30 min, followed by a slow decline. The blood glucose levels of the VDD group offspring mice at 30 min, 60 min, 90 min, and 120 min were significantly higher than those of the CD group offspring mice ($P<0.001$), and the area under the GTT curve (GTT-AUC) of the VDD group offspring mice was also significantly higher than that of the CD group offspring mice ($P<0.001$).These results indicate that paternal VDD impairs glucose regulation and insulin sensitivity in male offspring, leading to glucose intolerance later in life.

Paternal VDD affects offspring testicular development and spermatogenic cells counts

The morphological and spermatogenic differences between the two groups of testicular tissues were compared through testicular gross observation and HE staining.

Gross examination of testes revealed that the testicular volume of the male offspring in the experimental group was significantly reduced (Fig. 3A). HE staining showed no significant difference in the number of seminiferous tubule lumens ($n=12$; $P>0.05$; Fig. 3B, F). The diameter of the seminiferous tubules in the offspring of the VDD group was significantly reduced ($n=12$; $P<0.05$; Fig. 3C, G). Within the seminiferous tubules, Sertoli cells and all major stages of spermatogenic cells (spermatogonia, primary spermatocytes, spermatids, and spermatozoa) were observed. Counting revealed that compared with the control group, the numbers of primary spermatocytes ($n=12$; $P<0.01$) and spermatids ($n=12$; $P<0.001$) in the testes of offspring in

the VDD group were significantly reduced (Fig. 3C, H). The above results indicate that paternal VDD exposure affects testicular development and spermatogenesis in offspring. Analyzing the sperm malformation rate in the cauda epididymis of male offspring (Fig. 3E), compared with the control group, the sperm malformation rate of male offspring in the VDD group did not show significant decrease ($P>0.05$, Fig. 3I). A sperm count was performed, and no difference in sperm count was found between the two groups. ($n=8$; $P>0.05$; Fig. 3D, J).

To explore whether paternal VDD affects the serum testosterone level of male offspring, the testosterone level was measured using an ELISA kit. The results are shown in the figure (Fig. 3K), and the serum testosterone level of male offspring in the VDD group was significantly lower than that in the CD group ($n=12$; $P<0.05$). While, the mRNA expression levels of key steroidogenic genes Star and Cyp11a1 in testicular tissue showed no significant differences between VDD and CD offspring ($n=8$; $P>0.05$; Fig. 3L, M).

Paternal VDD induces oxidative stress and inflammatory environment imbalance in offspring testes

We examined the changes in oxidative stress markers in the offspring's testicular tissue. Compared with the CD group, the activities of antioxidant indicators SOD ($P<0.05$) and GSH ($P<0.05$) in the testes of offspring mice in the VDD group were significantly decreased (Fig. 4A, B), while the level of oxidative stress product MDA ($P<0.05$) was significantly increased (Fig. 4C). RT-qPCR analysis showed that the mRNA expressions of antioxidant-related genes Sod ($P<0.05$), Cat ($P<0.05$), and Gst ($P<0.05$) in the testicular tissue of offspring mice in the VDD group were significantly down-regulated (Fig. 4D, E, F). These results indicate that paternal VDD exposure causes oxidative stress damage in the testicular tissue of offspring. The expression of key inflammatory cytokines was assessed in testicular tissue, focusing on the pro-inflammatory factors TNF-α and IL-1β, and the anti-inflammatory factor IL-10. In the testicular tissues of offspring mice in the VDD group, the mRNA and protein expressions of TNF-α ($P<0.05$) and IL-1 ($P<0.05$) were significantly increased, while the mRNA and protein expression of the anti-inflammatory cytokine IL-10 ($P<0.05$) was significantly decreased (Fig. 4E, F, G).

Paternal VDD is associated with testicular fibrosis and activation of the TGF-β/SMAD2 pathway in offspring

Next, we performed Masson staining on the testicular tissue of the offspring. The Masson staining method was used to label the collagen fiber tissue in the testes. Compared with the control group (Fig. 5A), the content of collagen fibers (blue part) around the walls of some seminiferous tubules and in the interstitium of the experimental group

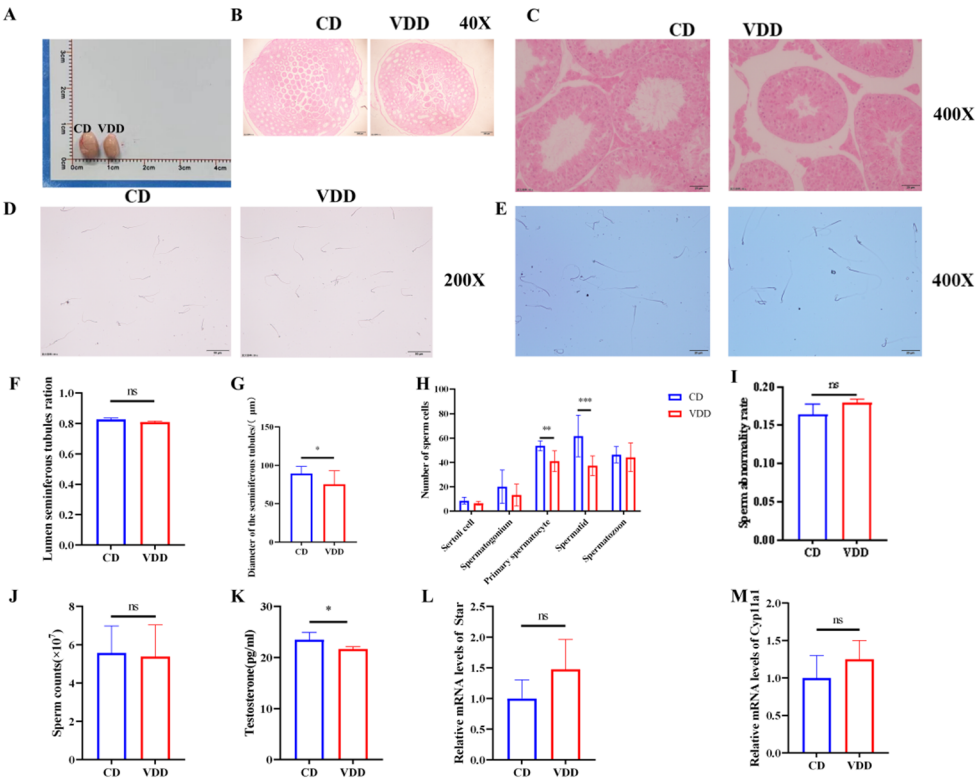

Fig. 3 Paternal VDD affects offspring testicular development and spermatogenic cells counts. (**A**) Gross view of testis; (**B**) HE staining of mouse testis (40×); (**C**) HE staining of mouse testis (400×); (**D**) Sperm suspension (200×); (**E**) Sperm Sect. (400×); (**F**) Lumen seminiferous tubules section; (**G**) The diameter of seminiferous tube; (**H**) Cell count during spermatogenesis observed in seminiferous tubules; (**I**) Sperm abnormal rate; (**J**) Sperm counts ($\times 10^7$); (**K**) Serum testosterone ($n=12$); (**L**) Relative mRNA levels of Star ($n=8$); (**M**) relative mRNA levels of Cyp11a1 ($n=8$); (scale bars: 200 μm, 50 μm, 20 μm, ns indicates $P>0.05$, * indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$)

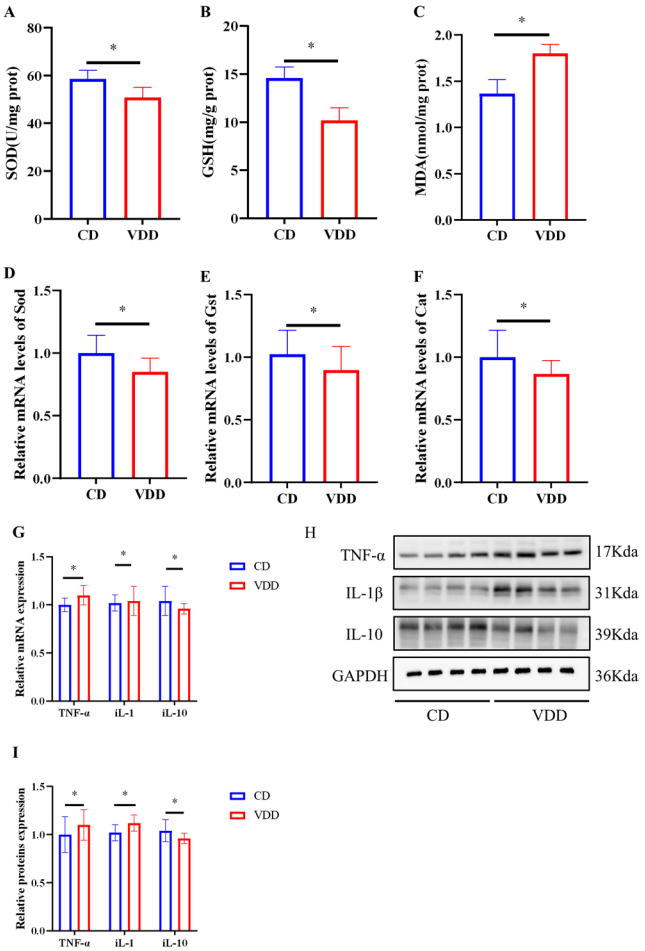

Fig. 4 Paternal VDD induces testicular oxidative stress and imbalance of inflammatory environment in offspring. (**A-C**) Micro-method detection of testicular SOD, GSH and MDA levels ($n=12$); (**D-F**) RT-qPCR detection of Sod, Gst and Cat expression ($n=8$); (**G**) Effect of paternal VDD on the levels of testicular inflammatory factor-related genes in offspring; (**H**) Western blot analysis of the expression of testicular inflammatory factor-related proteins in offspring affected by paternal VDD; (**I**) Calculation of relative protein levels with GAPDH as the internal control; Data are expressed as mean±standard deviation. ($n=4$, * indicates $P<0.05$)

increased significantly (Fig. 5B); the blue collagen fibers were evaluated (Fig. 5C). The results in the figures show that the content of testicular collagen fibers in the male offspring of the VDD group was significantly higher than that in the CD group. To further explore the internal reasons why paternal VDD leads to testicular fibrosis in offspring, we detected fibrosis-related indicators. First, the TGF-β/SMAD2 signaling pathway is involved in regulating tissue fibrosis, so we detected the expression of key molecules in this signaling pathway in the testicular tissue of offspring mice. RT-qPCR showed that paternal VDD significantly upregulated the mRNA levels of fibrosis-related indicators α-SMA, Col3A1, and pathway-related genes Tgf-β and Smad2 ($P<0.05$, Fig. 5D). Western blot analysis confirmed that the protein expressions of α-SMA, COL3A1, TGF-β, and P-SMAD2 were increased in the offspring mice of the VDD group ($P<0.05$, Fig. 5E, F). These data indicate that compared with the CD group, paternal VDD may activate the TGF-β/SMAD2 signaling pathway, thereby leading to fibrosis in the testicular tissue of offspring.

Discussion

In this study, we found that paternal VDD remodeled sperm miRNA profiles and was associated with impaired reproductive development in male offspring. Male F1 offspring derived from VDD fathers exhibited reduced body weight gain, impaired glucose tolerance in adulthood, decreased testis weight, reduced seminiferous tubule diameter, disrupted spermatogenic cell composition, and lower serum testosterone levels. At the tissue level, offspring testes showed increased oxidative stress, inflammatory imbalance, collagen deposition, and activation of the TGF-β/SMAD2 signaling pathway. Collectively, these findings support an association between paternal VDD, sperm miRNA alterations, and impaired testicular development in offspring.

Paternal nutritional status has increasingly been recognized as an important determinant of offspring health [36]. Previous studies have shown that adverse paternal dietary exposures, including high-fat diet, low-protein diet, calorie restriction, and abnormal methyl-donor intake, can alter sperm epigenetic features and influence offspring metabolic and reproductive phenotypes [37–40]. Among these, paternal high-fat diet has been most extensively studied and has been shown to affect sperm DNA methylation, histone modifications, and small non-coding RNA profiles, thereby influencing offspring metabolic tissues and reproductive function [41–43]. In contrast, the paternal effects of vitamin D deficiency remain insufficiently characterized. Our findings extend current knowledge by suggesting that paternal VDD is associated with both altered sperm molecular signatures and impaired reproductive outcomes in offspring.

Oxidative stress may represent an important mechanism linking paternal VDD-associated sperm alterations to offspring testicular dysfunction. Mature spermatozoa have limited cytoplasmic content and low transcriptional activity, making them particularly vulnerable to oxidative damage and dependent on tightly regulated antioxidant systems [44]. In the present study, male offspring of VDD fathers showed reduced testicular SOD activity and GSH levels, increased MDA accumulation, and decreased expression of antioxidant-related genes, including Sod, Cat, and Gst. These findings indicate that paternal VDD is associated with a disrupted redox balance in offspring testes. Given that oxidative stress can impair germ cell integrity and

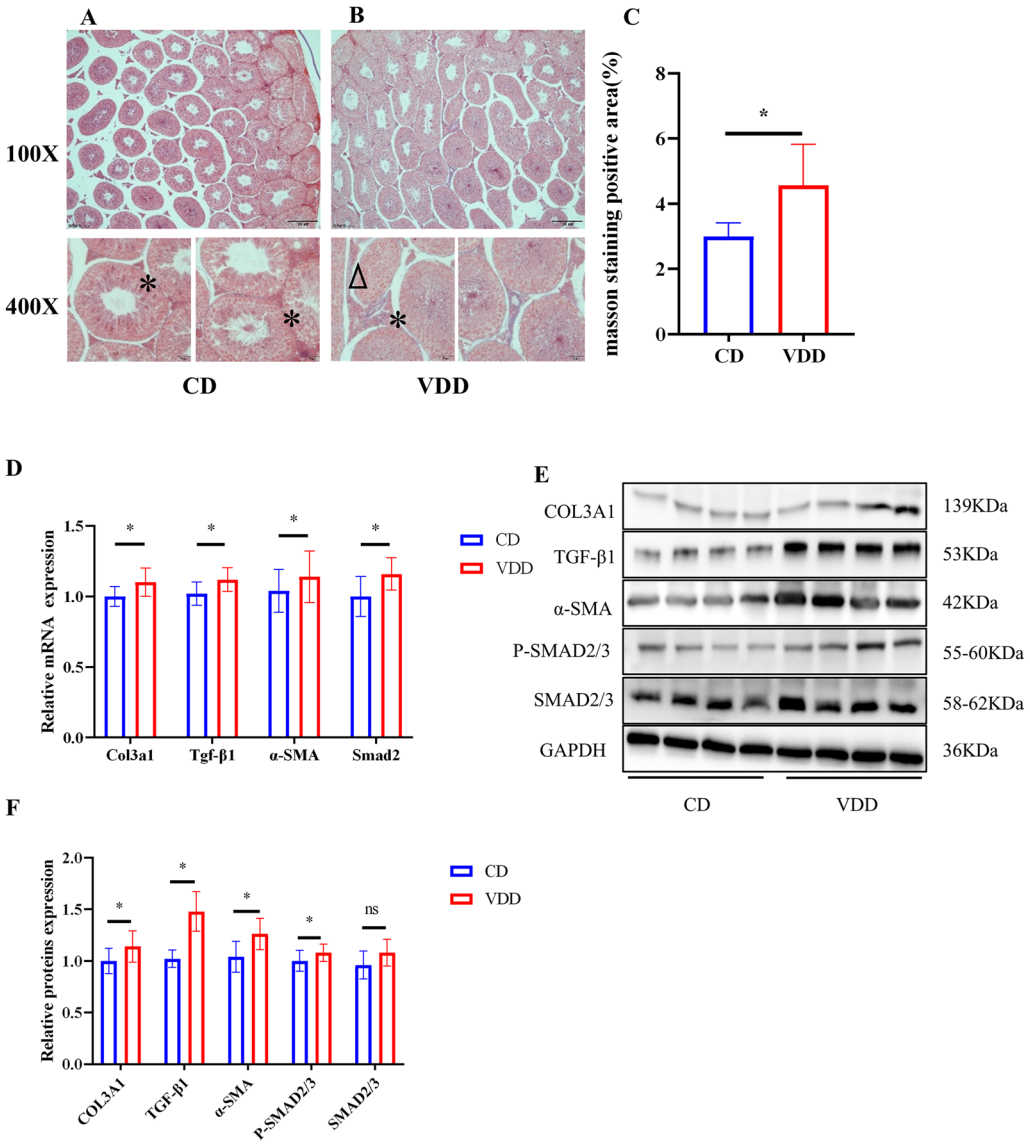

Fig. 5 Paternal VDD drives offspring testicular fibrosis by activating the TGF-β/SMAD2 pathway. (**A**) Masson staining of testicular tissue in offspring mice from the normal group; (**B**) Masson staining of testicular tissue in offspring mice from the VDD group; (**C**) Collagen fiber content; (**D**) Effect of paternal VDD on the mRNA levels of genes related to the TGF-β/SMAD2 pathway in offspring testes; (**E**) Western blot analysis of the expression of the TGF-β/SMAD2 pathway in offspring testes affected by paternal VDD; (**F**) Calculation of relative protein levels with GAPDH as the internal control ($n=4$); Arrows indicate collagen deposition in the peritubular region of the seminiferous tubules; Asterisks indicate collagen deposition in the testicular interstitial region; Images represent results from at least 3 independent experiments, scale bars: 100 μm, 20 μm. Data are presented as mean ± standard deviation. (ns indicates $P>0.05$, * indicates $P<0.05$)

spermatogenesis, it may contribute to the reduced seminiferous tubule diameter and decreased germ cell populations observed in VDD-F1 males.

Inflammatory imbalance and fibrotic remodeling may further exacerbate testicular injury [45]. We observed increased expression of the pro-inflammatory cytokines TNF-α and IL-1β and decreased expression of the anti-inflammatory cytokine IL-10 in offspring testes. In parallel, Masson staining revealed increased collagen deposition in the peritubular and interstitial regions. The upregulation of α-SMA, COL3A1, TGF-β, and phosphorylated SMAD2 suggests activation of a profibrotic signaling program. Given that TGF-β/SMAD signaling is a canonical pathway in tissue fibrosis [46], these results support the presence of a fibrotic microenvironment in VDD-F1 testes. However, causal involvement of this pathway requires further validation using pathway inhibition or genetic approaches.

An additional finding of this study was the reduction in serum testosterone levels in male offspring of VDD fathers. Testosterone biosynthesis depends on cholesterol transport, mitochondrial function, Leydig cell activity, and multiple steroidogenic enzymes [47]. Although genes such as Star

and Cyp11a1 are commonly used as markers of steroidogenic capacity [48, 49], their mRNA levels were not significantly altered in our study despite reduced circulating testosterone. This discrepancy suggests that transcriptional regulation of these genes alone may not fully explain the observed phenotype. Other mechanisms, including altered Leydig cell number, cholesterol availability, luteinizing hormone signaling, mitochondrial function, or enzymatic activity (e.g., Hsd3b, Cyp17a1, Hsd17b), may contribute and warrant further investigation [50].

This study has several limitations. First, although paternal VDD was associated with sperm miRNA remodeling and offspring testicular phenotypes, direct causality was not established. Future studies should test whether microinjection of VDD-associated sperm miRNAs into normal zygotes can reproduce the offspring testicular phenotype and whether inhibition or restoration of candidate miRNAs can rescue testicular abnormalities. Second, the predicted miRNA target genes require experimental validation using approaches such as luciferase reporter assays, miRNA gain- and loss-of-function experiments, and target gene expression analysis in offspring testes. Morover, investigating miRNA expression in offspring testis would be valuable. Third, this study focused mainly on F1 offspring; therefore, the findings support an intergenerational paternal effect but do not establish transgenerational inheritance. Studies involving F2 or later generations will be needed to determine whether the effects persist beyond the F1 generation.

Overall, our findings suggest that paternal vitamin D status before conception may influence offspring reproductive development. The data support a model in which paternal VDD remodels sperm miRNA profiles and is associated with oxidative stress, inflammatory imbalance, and fibrotic signaling in offspring testes. These findings provide evidence linking paternal micronutrient status to offspring reproductive health and identify sperm miRNAs as candidate mediators that require further mechanistic validation.

Conclusion

This study shows that paternal vitamin D deficiency can induce epigenetic reprogramming of sperm by altering the sperm miRNA expression profile, and the male offspring showed symptoms such as inflammation of the testicular tissue, enhanced oxidative stress, and fibrosis. From the perspective of epigenetics, this study expands the biological connotation of vitamin D in reproductive and metabolic health, provides a new theoretical basis for understanding the long-term impact of paternal nutritional factors on offspring health, and also offers scientific references for nutritional intervention in men of childbearing age and early prevention of paternal diseases.

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Data availability Data will be made available on request.

Declarations

Competing interests The authors declare no competing interests.

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Authors and Affiliations

Yu-Ming Chen[1] · Zhi-Hong Tian[1,2] · Shi-Yuan Li[1] · Juan Dai[3] · Xing-Ru Wang[1] · Guo-Ying Sun[1,4,5] · Min Liu[1,4,5] · Berthold Hocher[2,6,7,8] · Mei Tian[1,4,5]

✉ Min Liu
38431897@qq.com

✉ Berthold Hocher
berthold.hocher@medma.uni-heidelberg.de

✉ Mei Tian
tianmei@hunnu.edu.cn

1 School of Basic Medical Sciences, Hunan Normal University Health Science Center, Changsha 410005, Hunan, China

2 Fifth Department of Medicine (Nephrology/Endocrinology/ Rheumatology/ Pneumology), University Medical Center Mannheim, University of Heidelberg, 69120 Mannheim, Germany

3 Maternal and Child Health Family Planning Service Centre, Liuyang, Hunan, China

4 Key Laboratory of Study and Discovery of Small Targeted Molecules of Hunan Province, School of Medicine, Hunan Normal University, Changsha, Hunan, China

5 Key Laboratory of Model Animals and Stem Cell Biology in Hunan Province, School of Medicine, Hunan Normal University, Changsha, Hunan, China

6 Institute of Medical Diagnostics (IMD), 12247 Berlin, Germany

7 Reproductive and Genetic Hospital of CITIC-Xiangya, Changsha 410008, China

8 School of Medicine, Central South University, Changsha 410078, China