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Deficient expression of JMJD1A histone demethylase in patients with round spermatid maturation arrest

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KEY MESSAGE

This study provides new insights into understanding the molecular basis of post-meiotic arrest in infertile men, which may have implications for future studies regarding diagnosis and treatment of male infertility.

A B S T R A C T

JMJD1A (jumonji domain-containing 1A), a known histone H3K9 demethylase, has been identified as a critical epigenetic regulator in male germ cells, activating the sperm chromatin-packaging genes encoding protamines (PRM) and transition proteins (TNP) required for spermatid elongation and condensation. This research investigated the expression pattern of JMJD1A protein in testicular biopsies of 79 infertile men who had undergone testicular sperm extraction. Samples were classified into obstructive azoospermia (OA, $n = 26$), round spermatid maturation arrest (SMA, $n = 29$) and Sertoli cell only syndrome (SCOS, $n = 24$). Experiments including the detection of mRNA and protein expressions of JMJD1A revealed a severe decrease of *JMJD1A*/JMJD1A in samples with SMA and SCOS compared with samples with OA ($P < 0.005$, Kruskal–Wallis test). Additional experiments, including incorporation of JMJD1A on the promoter regions of *TNP* and *PRM* genes, and the expression of these genes, showed a significant decrease in the SMA and SCOS versus the OA testes ($P < 0.005$, Kruskal-Wallis test). These findings show the low expression of *JMJD1A*/JMJD1A, as well as its low incorporation into chromatin in testes with round spermatid maturation arrest, suggesting that a deficient expression of *JMJD1A*/JMJD1A might be reflecting and/or contributing to round spermatid maturation arrest.

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Introduction

Spermatogenesis is a complex and unique process in male reproductive systems in which diploid spermatogonia sequentially undergo mitotic, meiotic and post-meiotic (spermiogenesis) phases to differentiate into haploid mature spermatozoa (D'Occhio et al., 2007). During mammalian spermiogenesis, an impressive degree of morphological changes and gene regulation occur that are associated with extensive remodelling in the structure of sperm chromatin (D'Occhio et al., 2007; Kimmins and Sassone-Corsi, 2005; Oliva and Castillo, 2011). The chromatin remodelling is an important epigenetic process during germ cell development that is essential for condensation and protection of paternal genome, sperm maturation and fertility (Carrell and Hammoud, 2010; Ward, 2010). This process is supported by the performance of specialized factors, in which most core histones are initially hyperacetylated and then replaced by transition proteins (TNP1 and TNP2), which are themselves later replaced by protamines (PRM1 and PRM2) (D'Occhio et al., 2007; Gaucher et al., 2010; Goudarzi et al., 2014).

Protamines and transition proteins are extremely basic sperm-specific nuclear proteins that bind to DNA and pack the genome into a highly compact form (D'Occhio et al., 2007; Meistrich et al., 2003; Oliva, 2006). Genetic ablation of these nucleoproteins disrupts the process of nuclear condensation, leading to defective spermatogenesis (Cho et al., 2001; Rathke et al., 2014; Shirley et al., 2004). Studies in male mice have indicated that the genes encoding these proteins are activated by JMJD1A histone demethylase function (Liu et al., 2010; Okada et al., 2007).

JMJD1A (jumonji domain-containing 1A, also known as TSGA/KDM3A/JHDM2A), a notable epigenetic modifier element, is dynamically expressed in the male germ cells (Pedersen and Helin, 2010). This protein participates in the transcriptional control of *TNP* and *PRM* genes by demethylating the repressive epigenetic mark of histone H3 lysine 9 in their promoters (Liu et al., 2010; Okada et al., 2007). A recent study has found that in addition to targeting histone substrates in the nucleus, JMJD1A can also act on non-histone substrates in the cytoplasm, and is necessary for cytoskeletal rearrangements during spermiogenesis (Kasioulis et al., 2014). JMJD1A is expressed in nucleus from late pachytene spermatocytes up to round spermatids, and in cytoplasm of elongating spermatids in the form of distinct foci (Okada et al., 2007). Histological analysis of *JMJD1A* knockout mice showed that spermatids failed to elongate due to several post-meiotic defects, such as impaired chromatin compaction, cytoskeletal defects, abnormal acrosome and defective heterochromatin distribution. The synergistic effect of these abnormalities led to blocked spermiogenesis and infertility in the knockout mice (Kasioulis et al., 2014; Liu et al., 2010; Okada et al., 2007).

Considering the proven role of JMJD1A in maturation of post-meiotic germ cells (Kasioulis et al., 2014; Liu et al., 2010; Okada et al., 2007), this study investigated the expression profile of this demethylating enzyme in testicular biopsies of infertile men to provide new insights into understanding the molecular basis of post-meiotic arrest of spermatogenesis.

Materials and methods

Patients

Testicular biopsy specimens were collected from 79 infertile men who referred to the Royan Institute and underwent testicular sperm extraction (TESE) procedures to obtain spermatozoa for intracytoplasmic sperm injection (ICSI). The Institutional Ethics Committee of the Royan Institute approved the study on 6 November 2012 (reference number EC91/1091), and written informed consent was gained from all patients before the collection of tissue samples. Patients with cystic fibrosis, Klinefelter syndrome and Y chromosome abnormalities were excluded from the study. Parameters, including LH, FSH, testosterone, age, karyotyping and Y chromosome microdeletion, were recorded. Measurement of the hormonal status was done using a competitive ELISA kit (Monobind, CA, USA) according to Zangeneh et al. (2015). Cytogenetic analysis was conducted based on standard methods on phytohemagglutinin (PHA)-stimulated peripheral lymphocyte cultured cells (Asia et al., 2014). The number of samples used in each experiment is described in **Table 1**.

Table 1 – Number of samples used in each experiment.

Experiment	OA	SMA	SCOS
qPCR	10	11	10
Chromatin-ELISA	1 + 4[a]	3 + 4[a]	1 + 5[a]
Immunohistochemistry	3	3	3
ChIP-qPCR	8 + 4[a]	8 + 4[a]	5 + 5[a]
All experiments	26	29	24

ChIP = chromatin immunoprecipitation; OA = obstructive azoospermia; qPCR = real-time quantitative PCR; SCOS = Sertoli cell only syndrome; SMA = round spermatid maturation arrest.
[a] Identical sample in each group.

Histological evaluation

After performing the TESE procedures, a small part of the testicular biopsies was immersed in Bouin's solution for the histological analysis and the remainder was used for this study. Histological evaluation was performed according to the study of McLachlan et al. (2007). On average, five sections and 40 seminiferous tubules per section were analysed for each sample. Considering the most advanced spermatogenetic cells identified in the combined histologic and cytologic findings, these samples were categorized into the following groups: the samples that revealed normal spermatogenesis served as positive control (obstructive azoospermia [OA], $n = 26$), the samples with spermatogenic failure consisted of round spermatid maturation arrest (SMA, $n = 29$) and Sertoli cell only syndrome (SCOS, $n = 24$). The clinical characteristics of these three groups are summarized in **Table 2**.

RNA isolation and cDNA synthesis

Total RNA was extracted from the tissue samples with Trizol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. After DNase-I treatment, complementary DNA was synthesized using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) as per manufacturer's protocol. All the samples were checked for the expression of germ cell markers (DAZL and VASA), by reverse transcription-PCR (RT-PCR) and designed primers (**Table 3**), to control the number of germ cells in these samples (**Supplementary Figure S1**).

Real-time quantitative PCR (qPCR)

qPCR was performed on a 7500 Real-Time PCR System (AB Applied Biosystems, Carlsbad, CA, USA) using SYBR Green master mix (AB

Table 2 – The clinical characteristics of patient groups.

Patient groups	No. of patients	Genetic analysis	Age (years)	LH (mIU/ml)	FSH (mIU/ml)	Testosterone (ng/ml)
OA	26	46XY/ normal AZF	34.05 ± 1.58 (26 – 48)	8.06 ± 1.28	9.15 ± 1.08	4.24 ± 0.42
SMA	29	46XY/ normal AZF	31.66 ± 1.27 (24 – 47)	7.95 ± 0.97	9.96 ± 1.44	4.93 ± 0.54
SCOS	24	46XY/ normal AZF	35.05 ± 2.14 (19 – 52)	8.60 ± 1.20	18.28 ± 1.90	3.88 ± 0.20
P-value	–	–	NS	NS	0.001*	NS

Values are mean ± SEM.
NS = not statistically significant; OA = obstructive azoospermia; SCOS = Sertoli cell only syndrome; SMA = round spermatid maturation arrest.
* Significant difference, Kruskal–Wallis test.

Applied Biosystems), with designed primers listed in **Table 3**. The PCR mixture for each reaction contained 10 µl SYBR Green (PCR Master Mix, 5 ml; Applied Biosystems), 1 µl of each primer (20 pmol), and 50 ng cDNA adjusted to a final volume of 20 µl with nuclease-free water. The reactions were carried out with initial denaturation at 95°C for 4 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 10 min. Three technical replicates were conducted per sample and gene. Data were analysed using a comparative Ct method for relative quantification ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001). The amount of target ($2^{-\Delta\Delta Ct}$) was obtained by normalization to an endogenous control (β-actin) and relative to a calibrator [$(Ct_{\text{target gene}} - Ct_{\text{endogenous control}})_{\text{sample}} - (Ct_{\text{target gene}} - Ct_{\text{endogenous control}})_{\text{calibrator}}$].

Table 3 – Primer pairs used in this study.

Gene	Primers (5′ 3′)	Product size (bp)	Annealing temperature (°C)
RT-PCR primers			
DAZL	F: CGTGGCTCCGCAAGATGGC	101	60
	R: TACAGGGACCAGGAGGGAACCA		
VASA	F: TATTGACAGATGCTCAACAGGA	124	60
	R: GCCCTTTCTGGTATCAACTG		
qPCR primers			
JMJD1A	F: CAGTTGCCTAAATGCCGA	111	60
	R: TGAATTGTAACCTCCTGAAGTG		
PRM1	F: AATAGCACATCCACCAAACTC	134	60
	R: CAACATTTATTGACAGGCGG		
PRM2	F: GCTGGAAGTTAAGAGAAAGTCAC	80	60
	R: GGCTTGAGCATTTGATGTAGG		
TNP1	F: GACCTGATGTTAGATCAAAGCC	75	60
	R: ATTCCTCATTTCGTCACAACTG		
TNP2	F: GGAAATCCAACTAATGAGACCG	127	60
	R: TAGTGTTGCGTAGAAATCACCA		
ACTB	F: AGCACAGAGCCTCGCCTT	163	60
	R: CACGATGGAGGGGAAGAC		
ChIP-qPCR primers			
PRM1	F: GGAGGAGTCATCTTGTATCG	147	60
	R: TCATTGTGAGGGCAAAGG		
PRM2	F: CTTCCAAATGACAATGTGCG	128	60
	R: TTGCCTTGCCTGTAAAGC		
TNP1	F: GTCTCTTGTACTCATCCAATGCC	187	60
	R: TACTGTGCTGTCACTCACCT		
TNP2	F: TTCTTCTAATGTCCGAATGAGG	76	60
	R: CTGAACAAGTCCCAGTTTCC		

bP = base pairs; ChIP = chromatin immunoprecipitation; F = forward; qPCR = real-time quantitative PCR; RT-PCR = reverse transcription-PCR; R = reverse.

Sheared chromatin preparation

The testicular biopsies were initially homogenized, and proteins were cross-linked to DNA by incubating the homogenates with formaldehyde (36.5%; Sigma, St Louis, MO, USA) for 10 min. Then, the formaldehyde was quenched by adding 1.25 mol/l glycine diluted to a final concentration of 125 mmol/l. The homogenates were subsequently washed with phosphate-buffered saline (PBS) and were lysed in 80 µl of lysis buffer (3.75 ml 10% SDS, 12.5 ml 20% Triton X-100, 7.5 ml 5 mol/l NaCl, 0.5 ml 0.5 mol/l EDTA, 0.5 ml 250 mmol/l EGTA, 10 ml 0.5 mol/l HEPES). Afterward, the samples were sonicated for 10 cycles (30 s ON and 30 s OFF) with a Diagenode Bioruptor sonicator (UCD-200). Finally, the sheared samples were centrifuged at 14,000*g* for 5 min at 4°C and the supernatants were collected in clean tubes.

Chromatin-ELISA

Chromatin-ELISA was performed according to Dai and Rasmussen (2007). Briefly, after spectrophotometery diluted sheared chromatin in coating solution (KPL, Gaithersburg, MD, USA) was coated onto each well of a Nunc-Maxisorp 96-well plate. Background of wells was obtained from control wells without sheared chromatin. After overnight incubation at 4°C, each well was washed four times with 200 µl wash solution (KPL) for 2.5 min. Afterward, they were blocked in 100 µl blocking solution (KPL) and incubated 1 h at room temperature. Blocking buffer was aspirated and 50 µl of anti-JMJD1A antibody (ab91252, Abcam, Cambridge, UK) were added to the wells. Some wells were probed with anti-histone H2A antibody (ab58550, Abcam) to serve as a loading control. After that the plate was incubated at room temperature for 1 – 2 h and subsequently washed as described above. Then 100 µl HRP-conjugated antibodies (ab6789 for the wells with anti-JMJD1A antibody and ab16284 for the wells with anti-histone H2A antibody) as secondary antibody (diluted in blocking solution) were added and incubated at room temperature for 1 h. After washing, 50 µl of 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate (KPL) was added to each well at room temperature for 10 min. Finally, 50 µl of 1 mol/l H_2SO_4 was added to each well to stop reaction. Optical absorption was read at 450 nm on an ELISA-reader, Thermo Scientific (Multiskan Spectrum). Relative assay level for incorporation of JMJD1A protein in chromatin fractions was calculated by normalizing JMJD1A ELISA signals (after background subtraction) to core histone H2A content.

Chromatin immunoprecipitation (ChIP)-qPCR

ChIP assay was performed using anti-JMJD1A antibody (ab91252, Abcam) and an Orange ChIP kit (Diagenode, Liège, Belgium) in accordance with its given instruction. Immunoprecipitated DNA and input control DNA were quantified by qPCR on a 7500 Real-Time PCR System (AB Applied Biosystems) using SYBR Green master mix and designed primers (**Table 3**). qPCR condition was as follows: initial denaturation at 95°C for 4 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, extension for 30 s at 72°C and a final polymerization at 72°C for 10 min. Results were normalized to input DNA and expressed as percentage of input DNA associated with immunoprecipitated chromatin.

Immunohistochemistry

Bouin's fixed paraffin-embedded biopsies were cut into 5 µm sections for JMJD1A localization. The sections were deparaffinized in xylol (10 min) and rehydrated in distilled water (3 min) at room temperature. Afterwards, they were treated in methanol containing 10% H_2O_2 for 30 min at room temperature to quench endogenous peroxidase. The sections were subsequently immersed in a 10 mmol/l sodium citrate buffer (pH 6.0) at 121°C for 20 min to retrieve antigens. After washing in PBS-tween 0.05%, tissue sections were incubated in PBS containing 0.5% Triton X-100 for 20 min at room temperature. Then, they were blocked for 1 h at 37°C with goat serum (Sigma-Aldrich, St Louis, MO, USA) and incubated with a primary antibody against JMJD1A (1:100; ab91252, Abcam) at 4°C overnight. The washed sections were incubated with HRP-conjugated secondary antibody (1:100; ab6789, Abcam) for 1 h at 37°C. Then the sections were stained with Liquid DAB+ Substrate Chromogen System (Dako, Glostrup, Denmark) at room temperature for 15 min. After staining, they were counterstained with haematoxylin (30 s at room temperature) and dehydrated with 70% and 100% ethanol (3 min each) at room temperature. Finally, the sections were immersed in xylol (5 min at room temperature) and mounted on Entellan (Merck Millipore, Darmstadt, Germany) for examination by light microscopy. Negative controls were prepared in the same manner as the positive control, except that the primary antibody was replaced with PBS solution, widely considered an adequate control for the monoclonal primary antibody, due to its high specificity.

Statistical analysis

Data were shown as mean ± SEM. Statistical comparisons between the groups were determined using a Kruskal-Wallis test followed by Dunn's multiple comparison post test. Differences were considered statistically significant at $P < 0.05$. Statistical analysis was performed by IBM SPSS statistics version 20 (IBM Corporation, Armonk, NY, USA).

Results

Clinical characteristics of patients

The results of the Kruskal–Wallis test showed no significant differences in age, LH and testosterone serum concentrations between the three groups (OA [$n = 26$], SMA [$n = 29$], and SCOS [$n = 24$]), whereas the statistical difference in serum FSH concentration between these groups was significant ($P = 0.001$, **Table 2**). Besides, Dunn's multiple comparison test revealed much higher concentrations of FSH in the SCOS group compared with that of the SMA and OA ($P < 0.005$). There was no considerable variation in the FSH concentration between the specimens with SMA and OA.

Expression pattern of the JMJD1A gene

The mRNA levels of the *JMJD1A* gene in different testicular biopsy specimens were evaluated by using qPCR. There was a significant difference in the expression of the *JMJD1A* gene among the three sample groups ($P < 0.005$, Kruskal–Wallis test). As shown in **Figure 1A**, the expression of *JMJD1A* was significantly lower in the SMA ($n = 11$) and the SCOS ($n = 10$) than that of the positive control (OA, $n = 10$) ($P < 0.005$, Dunn's post test). However, no substantial variation was seen between the two groups with defective spermatogenesis (SMA and SCOS).

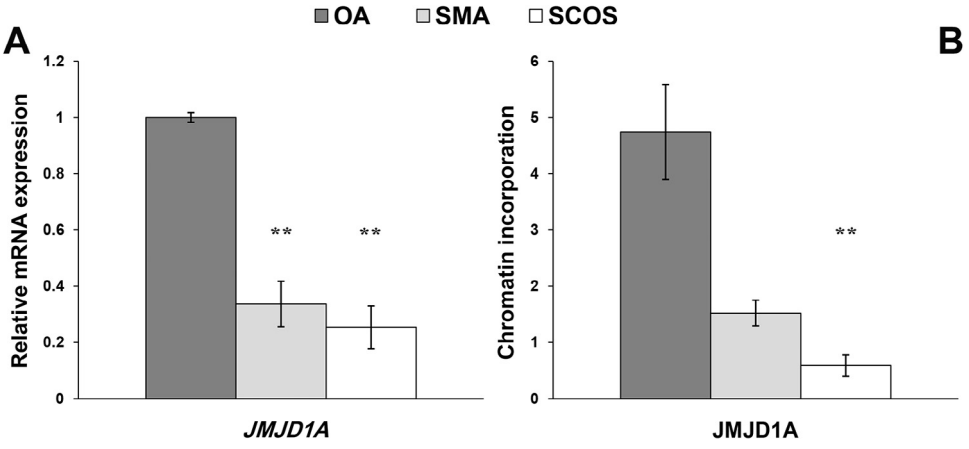

Figure 1 – (A) Relative mRNA expression of the *JMJD1A* gene normalized to β-actin in samples with OA ($n = 10$), SMA ($n = 11$) and SCOS ($n = 10$). (B) Total chromatin incorporation of JMJD1A protein normalized to core histone H2A content in OA ($n = 5$), SMA ($n = 7$) and SCOS ($n = 6$) groups. OA = obstructive azoospermia; SCOS = Sertoli cell only syndrome; SMA = round spermatid maturation arrest. Data are shown as mean ± SEM and were analysed using a Kruskal–Wallis test with Dunn's post-hoc test. Asterisks indicate significant differences compared with control (OA), $^{**}P < 0.005$.

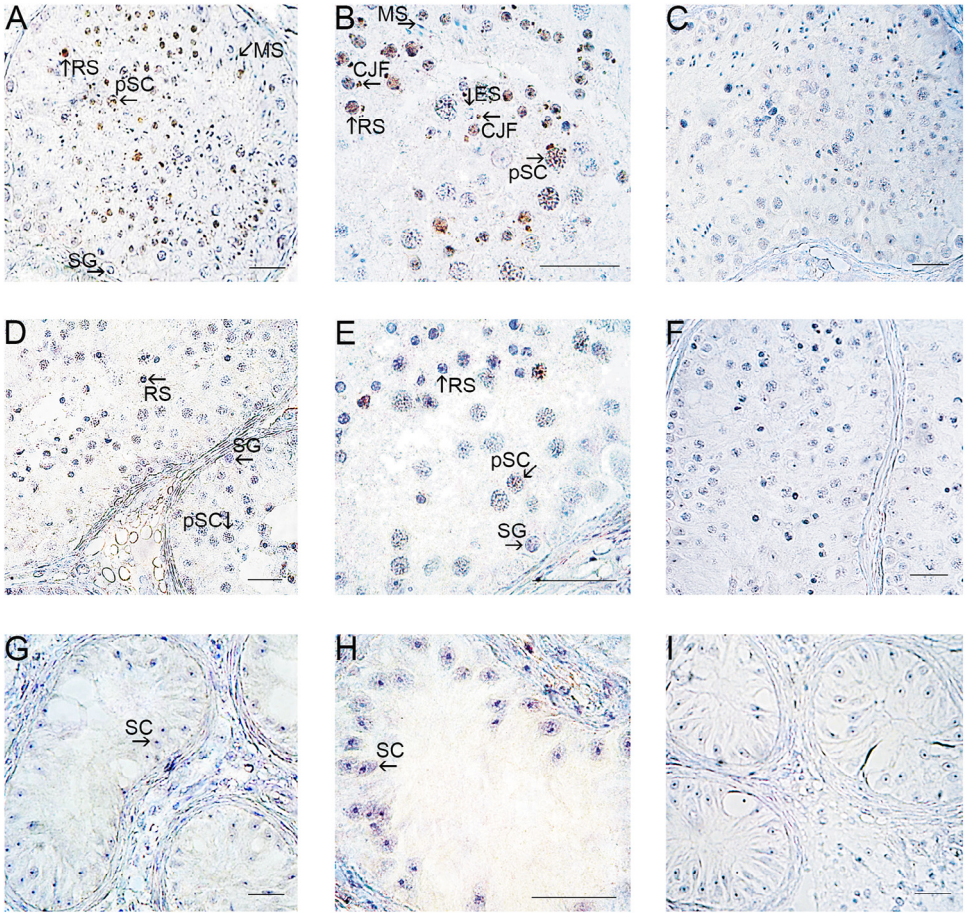

Figure 2 – Immunohistochemistry of JMJD1A in human testicular sections. (A, B) Testicular sections of samples with OA (obstructive azoospermia) stained with anti-JMJD1A antibody. (C) Testicular section of a sample with OA without anti-JMJD1A antibody. (D, E) Testicular sections of samples with SMA (round spermatid maturation arrest) stained with anti-JMJD1A antibody. (F) Testicular section of a sample with SMA without anti-JMJD1A antibody. (G, H) Testicular sections of samples with SCOS (Sertoli cell only syndrome) stained with anti-JMJD1A antibody. (I) Testicular section of a sample with SCOS without anti-JMJD1A antibody. CJF = cytoplasmic JMJD1A foci; ES = elongating spermatids; MS = mature spermatids; pSC = pachytene spermatocytes; RS = round spermatids; SC = Sertoli cells; SG = spermatogonia. Scale bar = 20 μm.

Total chromatin incorporation of JMJD1A protein

Total concentrations of JMJD1A incorporation into chromatin extracts of the three groups of testicular biopsy samples were assessed by chromatin-ELISA technique. A significant difference in the total chromatin incorporation of JMJD1A was noted between the SCOS, SMA and OA groups ($P < 0.005$, Kruskal–Wallis test). The incorporation of JMJD1A was significantly decreased in specimens with SCOS ($n = 6$) against OA group ($n = 5$) ($P < 0.005$, Dunn's post test). There was also a much lower incorporation of JMJD1A in the SMA group ($n = 7$) compared with OA, but this difference did not reach statistical significance (Dunn's post test). Moreover, there was no significant alteration between the groups with SMA and SCOS (**Figure 1B**).

Immunohistochemical analysis of JMJD1A protein

The expression of JMJD1A protein was analysed using immunohistochemistry. It was found that this protein expressed in the nuclei of spermatocytes from pachytene stage onwards, and also in round spermatids with higher intensity, while it was undetectable in mature spermatozoa and Sertoli cells. Cytoplasmic JMJD1A foci in round and elongating spermatids were also observed. Furthermore, the expression of JMJD1A was considered in the samples with SMA ($n = 3$), SCOS ($n = 3$) and OA ($n = 3$) groups, and was determined that the JMJD1A signal is identifiable in the OA testicular biopsies while it is absent or barely visible in SMA and SCOS samples (**Figure 2**).

JMJD1A incorporation on the promoter regions of TNP and PRM genes, in accordance with the expression profile of the genes

The presence of JMJD1A protein on the promoter regions of *TNP* and *PRM* genes was estimated quantitatively via ChIP-qPCR assay and a set of primers designed from the promoter regions of these genes (**Table 3**). Significant differences between the three groups were found in terms of JMJD1A incorporation on the promoter of *TNP1*, *TNP2*, *PRM1* and *PRM2* genes ($P < 0.005$, Kruskal–Wallis test). Dunn's test for multiple comparisons revealed that the incorporation of JMJD1A on the aforementioned promoters was considerably impaired in the samples with SMA ($n = 12$) group versus OA ($n = 12$) (*TNP1*, *TNP2*

[$P < 0.05$], *PRM1* and *PRM2* [$P < 0.005$]). Besides, the incorporation of JMJD1A in the SCOS ($n = 10$) group was significantly reduced in comparison with the OA group (*TNP1*, *PRM1*, *PRM2* [$P < 0.005$] and *TNP2* [$P < 0.05$], Dunn's post test). No significant changes were observed for the JMJD1A incorporation on the aforesaid promoters between the samples with SMA and SCOS, except for *TNP1* ($P < 0.05$, **Figure 3A**).

Expression of *TNP* and *PRM* genes were quantitatively checked in the three aforementioned testicular biopsy samples as well. The Kruskal–Wallis test among these groups indicated significant differences in the expression of *TNP1*, *TNP2*, *PRM1* and *PRM2* genes ($P < 0.005$). Data displayed much less expression of *TNP1*, *TNP2*, *PRM1* and *PRM2* genes in the SCOS ($n = 10$) groups compared with those of the OA group ($n = 10$) ($P < 0.005$, Dunn's post test). Furthermore, there was a sharp decline in the expression of *TNP1*, *PRM1*, *PRM2* ($P < 0.005$) and *TNP2* ($P < 0.05$) genes in the samples with SMA ($n = 11$) versus OA (Dunn's post test). However, there were no considerable changes between the specimens with defective spermatogenesis (**Figure 3B**).

Discussion

Epigenetic regulation of gene expression in germ cells is directed by highly specialized programmes, and deviation from these programmes can result in male infertility (Zamudio et al., 2008). One major event occurring in mammals during the differentiation of round spermatids into spermatozoa is the tight compaction of sperm nucleus, achieved through the replacement of most histones first by TNP and then with PRM (Miller et al., 2010; Rathke et al., 2014). The sequential expression of *TNP* and *PRM* genes is regulated by JMJD1A, a histone demethylase specific for H3K9me2/me1 modifications and indispensable for spermiogenesis (Liu et al., 2010; Okada et al., 2007).

In the current study, the expression profile of JMJD1A histone demethylase in testicular tissues of infertile men with OA, SMA and SCOS was evaluated. The incorporation of JMJD1A protein on the promoter regions of *TNP* and *PRM* genes and the expression of these genes were considered in the three aforementioned samples as well.

The measurement of clinical parameters, including age and serum concentrations of LH, FSH and testosterone, in the SCOS, SMA and OA groups showed that there are no relationships between these parameters and this study.

The results revealed that the *JMJD1A* gene expression and the total incorporation of JMJD1A into chromatin in the samples with SCOS were significantly lower than those of specimens with OA (positive control). On the other hand, immunohistochemistry analysis of JMJD1A expression in the testis tissues showed that this protein is not expressed in Sertoli cells and is restricted only to germ cells. Due to the absence of germ cells in the SCOS samples, no expression of JMJD1A was expected in this group. But the detection of JMJD1A at low levels in the specimens with SCOS by qPCR technique may indicate the presence of occult foci of spermatogenesis in these samples. There are several cases of patients with SCOS histology who have undergone successful sperm retrieval (Lin et al., 2005). The sperm retrieval rate in this category has been reported to be 24 – 48% (Stahl et al., 2011).

The mRNA expression of the *JMJD1A* gene in the SMA group was significantly reduced compared with that of the OA group. In addition, chromatin-ELISA technique showed a very poor incorporation of JMJD1A protein in the aforementioned group relative to the control, although this difference was not significant. This might be because of the smaller sample size and also the lower sensitivity of this test compared with qPCR. To ensure the low expression of this protein in the samples with SMA, immunohistochemistry technique was performed in the next step. Immunohistochemical staining in the control samples exhibited that JMJD1A was expressed in stages from pachytene spermatocytes up to elongating spermatids, consistent with the previous report (Okada et al., 2007). By contrast, this protein was absent or barely visible in spermatocytes and round spermatids of the SMA samples. These results reinforce the possibility of actual reduction of JMJD1A expression in the SMA patients, and suggest that the low expression of this protein in the SMA is not merely because of missing cells and may reflect a true pathogenic factor. Interestingly, a previous study demonstrated a single nucleotide polymorphism (SNP) in the *JMJD1A* gene (rs34605051) which was significantly associated with spermatogenic failure (Aston et al., 2010).

In order to infer a possible pathogenic mechanism of JMJD1A in the specimens with SMA, the incorporation of this protein on the promoter regions of *TNP* and *PRM* genes were assessed as well as the expression of these genes in the testicular biopsies. The data indicated that the amounts of JMJD1A on the promoter regions of *TNP* and *PRM* genes and the expression of these genes were significantly decreased in the SMA group versus OA group. Regarding the importance of the regulatory role of JMJD1A on the expression of *TNP* and *PRM* genes, and also the observation of impaired chromatin condensation and blocked spermiogenesis in JMJD1A knockout mice (Liu et al., 2010; Okada et al., 2007), it is likely that JMJD1A deficiency triggers a pathogenic mechanism in the samples with SMA by reducing the expression of *TNP* and *PRM* genes. Furthermore, the study of Kasioulis and his colleagues showed that JMJD1A also plays an important role on cytoplasmic components during spermatogenesis, so that its deficiency leads to acrosomal and cytoskeletal disorders in JMJD1A mutant mice (Kasioulis et al., 2014). In this regard, the down-regulation of the *JMJD1A* gene may have the potential to contribute to post-meiotic arrest via cytoplasmic mechanisms in addition to the nuclear mechanisms involved in chromatin remodelling.

Altogether, this study's findings reveal a defect in the expression of JMJD1A histone demethylase in human testes with SMA, suggesting that its known role in spermatid maturation seemingly extends to humans, and its deficiency might be contributing to the SMA phenotype. However, additional studies with larger sample size are needed to confirm the validity of these findings. Besides, further work is required to distinguish the cases where JMJD1A would be a causal factor for spermatids maturation arrest from those where its lower expression would be a symptom associated with this phenotype.

Acknowledgement

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2016.09.005.

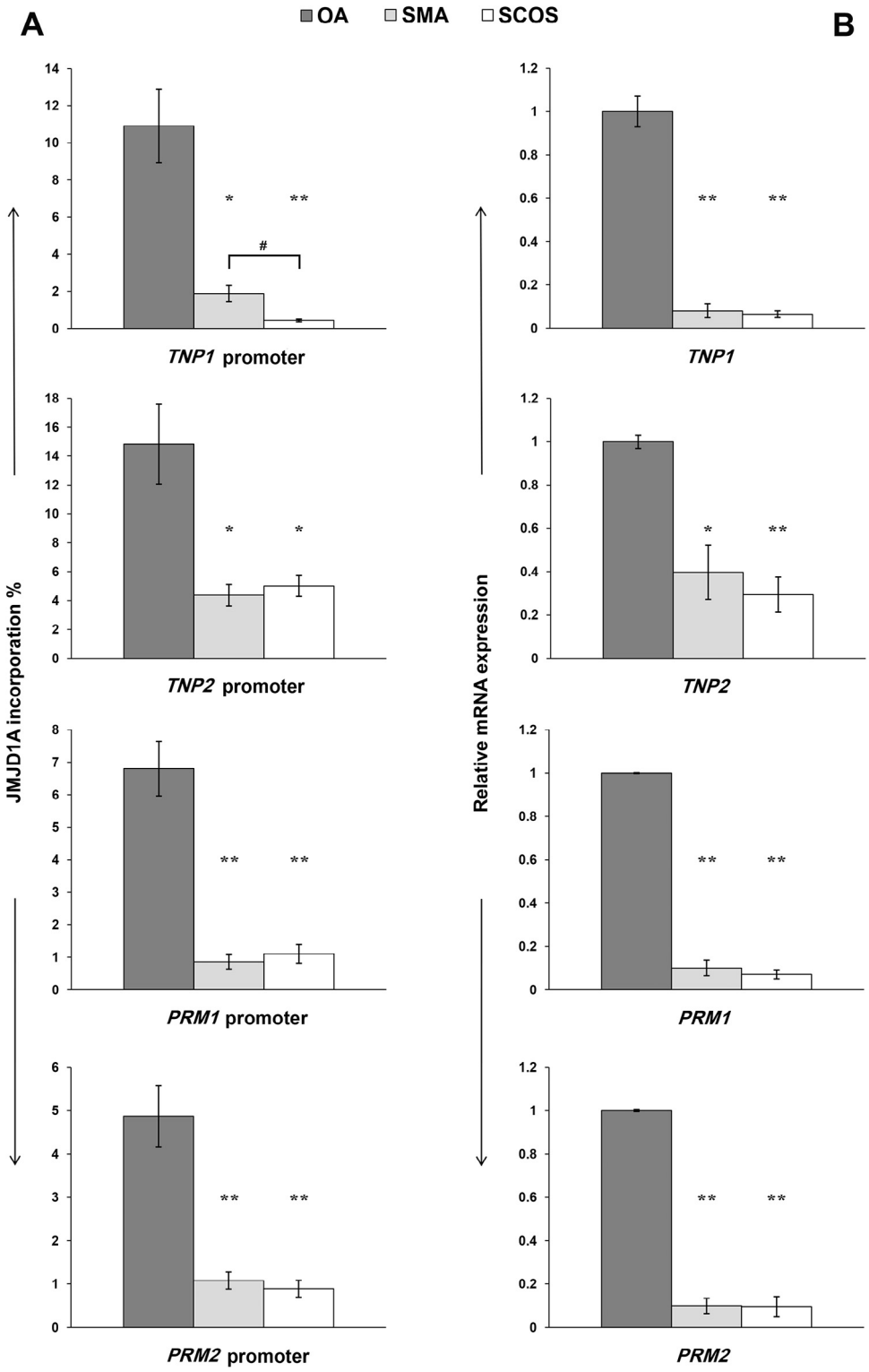

Figure 3 – (A) Chromatin immunoprecipitation of JMJD1A protein on the promoter regions of *TNP1*, *TNP2*, *PRM1* and *PRM2* genes in patients with OA (n = 12), SMA (n = 12) and SCOS (n = 10). (B) Relative mRNA expression normalized to β-actin of *TNP1*, *TNP2*, *PRM1* and *PRM2* genes in OA (n = 10), SMA (n = 11) and SCOS (n = 10) groups. OA = obstructive azoospermia; SMA = round spermatid maturation arrest; SCOS = Sertoli cell only syndrome. Statistical significance for multiple comparisons was determined by a Kruskal–Wallis test with Dunn's post-hoc test (mean ± SEM). Asterisks indicate significant differences compared with control (OA), $^{**}P < 0.005$, $^{*}P < 0.05$. Hash shows significant difference between SMA and SCOS groups, $\#P < 0.05$.

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REFERENCES

Asia, S., Vaziri Nasab, H., Sabbaghian, M., Kalantari, H., Zari Moradi, S., Gourabi, H., Mohseni Meybodi, A., 2014. A rare de novo Complex Chromosomal Rearrangement (CCR) involving four chromosomes in an oligoasthenosperm infertile man. Cell J. 16, 377–382.

Aston, K.I., Krausz, C., Laface, I., Ruiz-Castané, E., Carrell, D.T., 2010. Evaluation of 172 candidate polymorphisms for association with oligozoospermia or azoospermia in a large cohort of men of European descent. Hum. Reprod. 25, 1383–1397.

Carrell, D.T., Hammoud, S.S., 2010. The human sperm epigenome and its potential role in embryonic development. Mol. Hum. Reprod. 16, 37–47.

Cho, C., Willis, W.D., Goulding, E.H., Jung-Ha, H., Choi, Y.C., Hecht, N.B., Eddy, E.M., 2001. Haploinsufficiency of protamine-1 or-2 causes infertility in mice. Nat. Genet. 28, 82–86.

Dai, B., Rasmussen, T.P., 2007. Global epiproteomic signatures distinguish embryonic stem cells from differentiated cells. Stem Cells 25, 2567–2574.

D'Occhio, M.J., Hengstberger, K.J., Johnston, S.D., 2007. Biology of sperm chromatin structure and relationship to male fertility and embryonic survival. Anim. Reprod. Sci. 101, 1–17.

Gaucher, J., Reynoird, N., Montellier, E., Boussouar, F., Rousseaux, S., Khochbin, S., 2010. From meiosis to postmeiotic events: the secrets of histone disappearance. FEBS J. 277, 599–604.

Goudarzi, A., Shiota, H., Rousseaux, S., Khochbin, S., 2014. Genome-scale acetylation-dependent histone eviction during spermatogenesis. J. Mol. Biol. 426, 3342–3349.

Kasioulis, I., Syred, H.M., Tate, P., Finch, A., Shaw, J., Seawright, A., Fuszard, M., Botting, C.H., Shirran, S., Adams, I.R., Jackson, I.J., van Heyningen, V., Yeyati, P.L., 2014. Kdm3a lysine demethylase is an Hsp90 client required for cytoskeletal rearrangements during spermatogenesis. Mol. Biol. Cell 25, 1216–1233.

Kimmins, S., Sassone-Corsi, P., 2005. Chromatin remodelling and epigenetic features of germ cells. Nature 434, 583–589.

Lin, Y.M., Kuo, P.L., Lin, Y.H., Teng, Y.N., Nan Lin, J.S., 2005. Messenger RNA transcripts of the meiotic regulator BOULE in the testis of azoospermic men and their application in predicting the success of sperm retrieval. Hum. Reprod. 20, 782–788.

Liu, Z., Zhou, S., Liao, L., Chen, X., Meistrich, M., Xu, J., 2010. Jmjd1a demethylase-regulated histone modification is essential for cAMP-response element modulator-regulated gene expression and spermatogenesis. J. Biol. Chem. 285, 2758–2770.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2– ΔΔCT method. Methods 25, 402–408.

McLachlan, R.I., Rajpert-De Meyts, E., Hoei-Hansen, C.E., de Kretser, D.M., Skakkebaek, N.E., 2007. Histological evaluation of the human testis–approaches to optimizing the clinical value of the assessment: mini review. Hum. Reprod. 22, 2–16.

Meistrich, M.L., Mohapatra, B., Shirley, C.R., Zhao, M., 2003. Roles of transition nuclear proteins in spermiogenesis. Chromosoma 111, 483–488.

Miller, D., Brinkworth, M., Iles, D., 2010. Paternal DNA packaging in spermatozoa: more than the sumof its parts? DNA, histones, protamines and epigenetics. Reproduction 139, 287–301.

Okada, Y., Scott, G., Ray, M.K., Mishina, Y., Zhang, Y., 2007. Histone demethylase JHDM2A is critical for Tnp1 and Prm1 transcription and spermatogenesis. Nature 450, 119–123.

Oliva, R., 2006. Protamines and male infertility. Hum. Reprod. Update 12, 417–435.

Oliva, R., Castillo, J., 2011. Proteomics and the genetics of sperm chromatin condensation. Asian J. Androl. 13, 24–30.

Pedersen, M.T., Helin, K., 2010. Histone demethylases in development and disease. Trends Cell Biol. 20, 662–671.

Rathke, C., Baarends, W.M., Awe, S., Renkawitz-Pohl, R., 2014. Chromatin dynamics during spermiogenesis. Biochim. Biophys. Acta 1839, 155–168.

Shirley, C.R., Hayashi, S., Mounsey, S., Yanagimachi, R., Meistrich, M.L., 2004. Abnormalities and reduced reproductive potential of sperm from Tnp1-and Tnp2-null double mutant mice. Biol. Reprod. 71, 1220–1229.

Stahl, P.J., Mielnik, A., Schlegel, P.N., Paduch, D.A., 2011. Heat shock factor Y chromosome (HSFY) mRNA level predicts the presence of retrievable testicular sperm in men with nonobstructive azoospermia. Fertil. Steril. 96, 303–308.

Ward, W.S., 2010. Function of sperm chromatin structural elements in fertilization and development. Mol. Hum. Reprod. 16, 30–36.

Zamudio, N.M., Chong, S., O'Bryan, M.K., 2008. Epigenetic regulation in male germ cells. Reproduction 136, 131–146.

Zangeneh, F., Salman Yazdi, R., Naghizadeh, M.M., Abedinia, N., 2015. Effect of Ramadan fasting on stress neurohormones in women with polycystic ovary syndrome. J. Family Reprod. Health 9, 51–57.