Med1 regulates meiotic progression during spermatogenesis in mice

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Abstract

Spermatogenesis is a highly coordinated process. Signaling from nuclear hormone receptors, like those for retinoic acid (RA), is important for normal spermatogenesis. However, the mechanisms regulating these signals are poorly understood. Mediator complex subunit 1 (MED1) is a transcriptional enhancer that directly modulates transcription from nuclear hormone receptors. MED1 is present in male germ cells throughout mammalian development, but its function during spermatogenesis is unknown. To determine its role, we generated mice lacking *Med1* specifically in their germ cells beginning just before birth. Conditional *Med1* knockout males are fertile, exhibiting normal testis weights and siring ordinary numbers of offspring. RA-responsive gene products stimulated by RA gene 8 (*Stra8*) and synaptonemal complex protein 3 (*Sycp3*) are first detected in knockout spermatogonia at the expected time points during the first wave of spermatogenesis, and persist with normal patterns of cellular distribution in adult knockout testes. Meiotic progression, however, is altered in the absence of *Med1*. At postnatal day 7 (P7), zygotene-stage knockout spermatocytes are already detected, unlike in control testes, with fewer pre-leptotene-stage cells and more leptotene spermatocytes observed in the knockouts. At P9, *Med1* knockout spermatocytes prematurely enter pachynema. Once formed, greater numbers of knockout spermatocytes remain in pachynema relative to the other stages of meiosis throughout testis development and its maintenance in the adult. Meiotic exit is not inhibited. We conclude that MED1 regulates the temporal progression of primary spermatocytes through meiosis, with its absence resulting in abbreviated pre-leptotene, leptotene, and zygotene stages, and a prolonged pachytene stage.

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Introduction

Spermatogenesis is a tightly regulated process driven by a complex network of germ cell-autonomous gene transcription and extrinsic signaling from supporting somatic cells. In adult mice, seminiferous tubules contain layers of germ cells at different developmental stages. On the basis of the composition of germ cells in a given tubule cross-section the mouse seminiferous epithelial cycle consists of 12 distinct stages (Oakberg 1956). The germ cells develop through these stages in a spatiotemporally coordinated manner, resulting in constant sperm production. Progression of a cohort of germ cells through seminiferous epithelial cycles until they are released into the epididymis is referred to as a spermatogenic wave (Perey *et al.* 1961).

Ligands of nuclear hormone receptors have long been known to play an important role in spermatogenesis. Mice with deficiencies in vitamin A and vitamin D have impaired fertility (Howell *et al.* 1963, Thompson *et al.*

1964, Jensen 2014). Vitamin A is metabolized to retinoic acid (RA), and thus RA signaling is a readout for vitamin A levels. RA is required for spermatogonial differentiation as well as for meiotic entry (Van Pelt & De Rooij 1990, Zhou *et al.* 2008, Barrios *et al.* 2010). The male germ cells express a number of nuclear hormone receptors in a development-specific manner. RA receptors γ (RARG) and α (RARA) function in undifferentiated spermatogonia (Vernet *et al.* 2006, Gely-Pernot *et al.* 2012). Mice with either a global or a germ cell-specific RARG and RARA ablation have an increased number of degenerative seminiferous tubules with no mature germ cells, due to a block in spermatogonial differentiation (Gely-Pernot *et al.* 2012). The vitamin D receptor (VDR) is highly expressed in male germ cells (Jensen 2014). VDR-null mice are infertile and have abnormal histology, although the precise mechanisms are unknown (Kinuta *et al.* 2000).

Mediator is a large multi-protein complex that functions as a transcriptional enhancer. Mediator complex subunit 1 (MED1) specifically interacts with

nuclear hormone receptors, including RARs and VDR, to activate target genes (Zhu *et al.* 1997, Yuan *et al.* 1998, Ren *et al.* 2000, Urahama *et al.* 2005). MED1 promotes nuclear hormone receptor-mediated transcription in a ligand-dependent manner through an interaction between the LxxLL motif of MED1 and the AF2 domain of the receptor (reviewed in Chen & Roeder (2011)). The global knockout of *Med1* in mice is embryonic lethal in mid-gestation (Ito *et al.* 2000, Zhu *et al.* 2000). Conditional knockouts have revealed numerous roles for *Med1* in the regulation of cell differentiation and lineage specification, including myelomonocytic differentiation, erythroid development, mammary luminal epithelial cell determination, and hair differentiation (Urahama *et al.* 2005, Jiang *et al.* 2010, Stumpf *et al.* 2010, Oda *et al.* 2012).

Previous work by us demonstrated that *Med1* is highly expressed throughout male germ cell development (Huszar & Payne 2013). However, the functional role of *Med1* in spermatogenesis is unknown. Due to the role of MED1 in the promotion of nuclear hormone receptor-mediated gene transcription, we hypothesized that MED1 may play a role in regulating the onset and duration of spermatogonial differentiation. To this end, we generated mice with a conditional germ cell-specific ablation of *Med1*. Unexpectedly, our results establish a novel role for *Med1* in regulating the dynamics of meiotic progression, modulating the amount of time spermatocytes exist in leptotema, zygonema and pachynema.

Materials and methods

Mice

All procedures and care of animals were carried out in accordance with the Stanley Manne Children's Research Institute Animal Care and Use Committee (Manne IACUC). The Manne IACUC specifically approved this study, protocol #13-002.0. Generation of floxed *Med1* mice has been described previously (Jia *et al.* 2004). Germ cell-specific *Med1* knockout mice were generated by crossing hemizygous FVB-Tg(*Ddx4-cre*)1Dcas/ mice (referred to here as *Vasa-cre*) with floxed C57BL/6 *Med1^{fl/fl}* mice to generate *Vasa-cre⁺;Med1^{fl/+}* F₁ mice. These mice were then crossed with additional *Med1^{fl/fl}* to generate *Vasa-cre⁺;Med1^{Δ/fl}* F₂ mice. Control mice used were *Vasa-cre⁻;Med1^{Δ/fl}* or *Vasa-cre⁺;Med1^{fl/+}*. Genotyping was performed using PCR on genomic DNA. PCR primer sequences are available upon request. *Vasa-cre* mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Testis weights and body weights were measured when mice were killed. To assess the fertility of knockout mice, 10-week-old male mice were backcrossed with WT female mice for a period of 5 months, and the number of pups generated through such matings was evaluated.

Histology and immunohistochemistry

The testes were fixed with Bouin's solution at 4 °C, and dehydrated for paraffin embedding. The sections were cut to

a thickness of 5 μM, deparaffinized, and rehydrated. For histology, sections were stained with hematoxylin and eosin (H+E). For immunohistochemistry (IHC), slides were prepared by performing antigen retrieval. The slides were boiled in 0.01 M sodium citrate, pH 6.0, for 10 min. The sections were then blocked for 1 h at room temperature with 3% goat serum in PBS. The sections were incubated overnight at 4 °C with primary antibodies diluted in 3% goat serum in PBS. The primary antibodies used for IHC were anti-SYCP3 (#ab15093, Abcam, Cambridge, MA, USA), anti-γH2AX (#05-636, Millipore, Billerica, MA, USA), anti-STRA8 (#ab49602, Abcam), anti-MED1 (#ab64965, Abcam), anti-TRA98 (#ab82527, Abcam), anti-BOULE (gift of Eugene Xu, Northwestern University), and anti-SOHLH1 (gift of Aleksandar Rajkovic, University of Pittsburgh). The sections were then labeled with the appropriate fluorescence-conjugated secondary antibody for 1 h at room temperature. The slides were incubated for 10 min with 4',6-diamidino-2-phenylindole (DAPI). The coverslips were then affixed with Vectashield anti-fade mounting medium (Vector Laboratories, Burlingame, CA, USA) and samples were imaged using a Leica DMR-HC epifluorescence microscope with 10×, 20×, and 40× objectives and a QImaging Retiga 4000R camera (Surrey, BC, Canada).

Chromosome spreads

Chromosome spreads were prepared from testis following methods reported previously (Peters *et al.* 1997). Briefly, the tunica albuginea was removed from testes at postnatal days P7, P14, P28, and 6-weeks-old, and the seminiferous tubules were placed in a hypotonic extraction buffer containing 30 mM Tris, 50 mM sucrose, 17 mM trisodium citrate dehydrate, 5 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride at pH 8.2 for 60 min. A 20 μl drop of 100 mM sucrose at pH 8.2 was placed on a clean glass slide. A small section of a seminiferous tubule was placed in the sucrose and minced using fine forceps. An additional 20 μl of 100 mM sucrose was added and the cells were pipetted to create a suspension. The suspension was spread across two new slides that had been cleaned in 1% paraformaldehyde with 0.15% Triton X-100. The cells were allowed to dry for 4 h at room temperature with high humidity. The slides were then rinsed twice in 0.4% Photoflo (Kodak). To prepare slides for imaging, slides were blocked with 5% goat serum in PBS for 1 h at room temperature. They were then incubated overnight at 4 °C with anti-SYCP3 and anti-γH2AX. Finally, the slides were incubated with secondary antibodies and imaged. The cells were incubated for 10 min with DAPI and then coverslips were attached with Vectashield (Vector Laboratories). Chromosome spreads were made from three mice of each genotype.

Statistical analyses

All experiments were performed at least three times. Data are presented as the mean ± S.E.M. Statistical analyses were carried out using Prism 5 GraphPad Software (La Jolla, CA, USA). Significance between the means was determined using Student's *t*-test.

Figure 1 Vasa-cre-mediated *Med1* knockout (VMKO) testes exhibit normal morphology and weight. (A) Cross-sections of 6-week-old VMKO (left) and control (right) testes immunostained for MED1 (green) and the pan-germ cell marker TRA98 (red), and exposed to DNA stain DAPI (blue). Arrows identify Sertoli cell nuclei positive for MED1 but negative for TRA98. Scale bars = 50 μ m. (B) Cross-sections of 6-week-old VMKO (left) and control (right) testes stained with hematoxylin and eosin. Scale bars = 50 μ m. (C) Assessment of testis weight:body weight measurements of 10-week-old VMKO (left, $n=9$) and control (right, $n=25$) male mice. Differences are not statistically significant (Student's *t*-test).

Results

Med1 ablation in fetal prospermatogonia does not impact fertility in adult male mice

To examine the role that *Med1* plays in the developing male germ line, we generated germ cell-specific *Med1* knockout mice by intercrossing floxed *Med1* mice with *Vasa*-cre mice. These *Vasa*-cre;*Med1*^{Δ/fl} mice are referred

to here as *Vasa*-cre mediated-*Med1* knock out (VMKO) animals. *Vasa*-cre is specifically expressed in germ cells beginning at embryonic day 15 (Gallardo *et al.* 2007). To validate that *Med1* was only ablated in germ cells, 6-week-old VMKO mice testes were examined for the co-distribution of MED1 and germ cell-specific antigen TRA98 (Tanaka *et al.* 1997). We did not detect any MED1 in VMKO germ cells (Fig. 1A). Only Sertoli cells and testicular interstitial cells contained MED1 in the conditional knockout samples. Histological examination of 6-week-old VMKO testes did not reveal any abnormalities. Hematoxylin and eosin staining revealed an appearance of VMKO cross-sections that was comparable with control sections (Fig. 1B). Testis weights and body weights were measured from VMKO and control animals. No differences were seen in the VMKO body weights (25.8 g mean average $\pm$ 0.92 g S.E.M. vs 24.1 g mean average $\pm$ 0.74 g S.E.M. in controls) or in the testis weights (95.6 mg mean average $\pm$ 4.94 mg S.E.M. vs 95.2 mg mean average $\pm$ 4.07 mg S.E.M. in controls), resulting in statistically similar testis weight:body weight ratios (Fig. 1C). We next examined the fertility of male mice lacking *Med1* in their germ cells. Ten-week-old VMKO males were mated with WT females continuously for 5 months. VMKO males were fertile with normal litter sizes (Fig. 2A) and a normal accumulation of successive

Figure 2 VMKO male mice exhibit normal fertility. (A) VMKO and control males were mated with WT female mice for a period of 5 months. Average litter size for each group is shown. Graph exhibits mean values $\pm$ S.E.M. Difference is not statistically significant (NS, Student's *t*-test). (B) The cumulative number of pups sired by each animal over the entire breeding period.

litters (Fig. 2B). From these data, we conclude that VMKO animals produce sperm and exhibit normal fertility.

Male germ cells lacking Med1 exhibit normal STRA8 and SYCP3 localization but display altered meiotic progression

Because MED1 interacts with nuclear hormone receptors in many different cell types, we evaluated the cross-sections of adult VMKO testes for distribution patterns of RA-responsive gene products. The levels of two proteins, stimulated by RA gene 8 (STRA8) and synaptonemal complex protein 3 (SYCP3), increase in male germ cells upon exposure to RA (Snyder *et al.* 2011, Busada *et al.* 2014). Upregulation of *Stras8* occurs through the direct engagement of response elements near the gene by activated RARs (Raverdeau *et al.* 2012, Kumar *et al.* 2013), while the increase in *Sycp3* expression happens in a RAR-independent manner (Tedesco *et al.* 2013). Nonetheless, SYCP3 is an excellent marker of RA-induced differentiation. In 6-week-old VMKO testes, both STRA8 and SYCP3 appropriately localized to differentiating spermatogonia and meiotic spermatocytes (Fig. 3A and B). Equivalent numbers of STRA8⁺ and SYCP3⁺ cells were detected in the cross-sections of VMKO seminiferous tubule when compared with controls (Fig. 3A, B, and C). Since adult testes reflect steady-state spermatogenesis, we next wondered whether the first-wave spermatogenic cells in developing VMKO testes exhibited distribution patterns of STRA8 and SYCP3 comparable with control testes. Endogenous RA signaling usually commences in male germ cells beginning at postnatal day 3 (P3) or P4 (Zhou *et al.* 2008, Snyder *et al.* 2010). When P2 testes were examined for STRA8, indeed both VMKO and control samples completely lacked STRA8⁺ cells in their seminiferous tubules (Fig. 4A). By contrast, P4 testes from VMKO and control animals exhibited STRA8⁺ cells in equivalent numbers (Fig. 4A and B). This finding suggests that there is no premature RA signaling response occurring in male germ cells lacking *Med1*.

Spermatogonia committing to differentiation upregulate spermatogenesis- and oogenesis-specific basic helix-loop-helix-containing protein 1 (SOHLH1; Suzuki *et al.* 2012). This protein is essential for mitotic germ cell differentiation before entering meiosis (Ballow *et al.* 2006). P7 testes from VMKO males exhibited SOHLH1⁺ cells at numbers comparable with those in controls (Fig. 4B and C), indicating that the initial wave of spermatogonial differentiation is not disrupted in the absence of MED1. Subsequently, the first set of male germ cells enters the pre-leptotene and leptotene stages of meiosis in mice between P7 and P9, prompting our examination of VMKO testes for SYCP3 at this time window. At P9, cross-sections of VMKO testis reveal an abundant number of SYCP3⁺ cells that is indistinguishable in scope from control sections (Fig. 4C). This pattern

Figure 3 Adult VMKO testes contain normal numbers of retinoic-acid responsive germ cells. (A) Cross-sections of 6-week-old VMKO (left) and control (right) testes immunostained for STRA8 (green) and exposed to DAPI (blue). Arrows identify STRA8-positive cells. Scale bars = 50 μ m. (B) Cross-sections of 6-week-old VMKO (left) and control (right) testes immunostained for SYCP3 (green) and exposed to DAPI (blue). Arrows identify SYCP3-positive cells. Scale bars = 50 μ m. (C) Quantitation of STRA8⁺ and SYCP3⁺ cells per seminiferous tubule cross-section in 6-week-old VMKO and control testes. Graph exhibits mean values $\pm$ S.E.M. Differences are not statistically significant (NS, Student's *t*-test).

persists at P12, with both VMKO and control samples containing equivalent numbers of SYCP3⁺ cells (Fig. 4C).

We next prepared chromosome spreads from VMKO testes, beginning at P7, to more closely evaluate the meiotic stages represented in the samples. The spreads were co-stained for SYCP3 and H2A histone family, member X (H2AFX; γ H2AX), which allowed us to determine the specific stage of prophase I (Ashley 2004). At P7, we observed zygotene-stage spermatocytes in the testes of VMKO males but not in control males (Fig. 5A). At P9, we detected pachytene-stage spermatocytes in VMKO but not in control testes (Fig. 5B), reflecting a premature progression through prophase I of meiosis. When the overall percentage of SYCP3⁺ cells in

Figure 4 Neonatal and juvenile VMKO testes contain normal numbers of retinoic-acid-responsive germ cells. (A) Cross-sections of 2-day-old (P2, left) and P4 (right) VMKO and control testes immunostained for STRA8 (top) and exposed to DAPI (bottom). Arrows identify STRA8-positive cells. Scale bars = 50 μ m. (B) Quantitation of STRA8+ (P4 testis) and SOHLH1+ (P7 testis) cells per cross-section of seminiferous tubule in VMKO and control testes. Graph exhibits mean values $\pm$ S.E.M. Differences are not statistically significant (NS, Student's *t*-test). (C) Cross-sections of P7 VMKO and control testes immunostained for SOHLH1 (top) and exposed to DAPI (bottom). Scale bars = 50 μ m. (D) Cross-sections of P9 (left) and P12 (right) VMKO and control testes immunostained for SYCP3 (top) and exposed to DAPI (bottom). Scale bars = 50 μ m.

the P7 chromosome spreads was calculated, the values were statistically similar between VMKO and control samples (Fig. 5C). We determined the ratios of pre-leptotene, leptotene, and zygotene spermatocytes in P7 chromosome spreads and found that VMKO samples had fewer pre-leptotene and more zygotene spermatocytes when compared with controls (Fig. 5D). P14 chromosome spreads exhibited an even greater difference in the ratios. While significantly fewer VMKO spermatocytes were in pre-leptonema, leptotema, and zygonema at this time point, over twice as many VMKO cells were in

pachynema when compared with control samples (Fig. 5E). γ H2AX exhibits broad chromosomal distribution in leptotene and zygotene spermatocytes, but preferentially localizes to the sex chromosomes by the early-pachytene stage, appearing as a concentrated focus of immunostaining in nuclei (Fernandez-Capetillo *et al.* 2003). To assess whether pachytene spermatocytes were overrepresented in the meiotic cell populations of VMKO testes between P14 and adulthood, we examined the cross-sections of testis at P28 and observed that the majority of γ H2AX in VMKO samples appeared as concentrated foci (Fig. 6A). The control sections, by contrast, contained many germ cells in which γ H2AX

Figure 5 VMKO male germ cells prematurely enter zygonema and pachynema in the first meiotic prophase. (A and B) Meiotic chromosome spreads were prepared from P7 (A) and P9 (B) VMKO and control testes and immunostained for SYCP3 (green) and γ H2AX (red). Representative images are shown for all observed meiotic stages. Scale bars = 10 μ m. (C) Quantification of the percentage of P7 cells in the chromosome spreads that were SYCP3 positive. Difference is not statistically significant (NS, Student's *t*-test). (D and E) Quantification of the percentage of P7 (D) and P14 (E) cells in the chromosome spreads that were pre-leptotene, leptotene, zygotene, and pachytene. ****P* < 0.001; statistical significance calculated using Student's *t*-test. NO, not observed. All graphs exhibit mean values $\pm$ S.E.M.

Figure 6 VMKO testes exhibit a higher proportion of pachytene cells relative to other meiotic stages. (A and B) Cross-sections of P28 (A) and P30 (B) VMKO and control testes immunostained for γ H2AX (top) and exposed to DAPI (bottom). Asterisks demarcate seminiferous tubules enriched with pachytene cells (concentrated foci of γ H2AX). Arrows identify leptotene and zygotene spermatocytes. Scale bars = 50 μ m. (C and D) Quantification of the percentage of P28 (C) and 6-week-old (D) cells in the chromosome spreads that were pre-leptotene, leptotene, zygotene, and pachytene. ** $P < 0.01$ and *** $P < 0.001$; statistical significance calculated using Student's *t*-test. Graphs exhibit mean values $\pm$ S.E.M.

exhibited broad nuclear distribution, indicative of leptotene and zygotene spermatocytes. This pattern was also seen at P30 (Fig. 6B) and in 6-week-old testes (data not shown). Chromosome spreads from P28 testes revealed that, like in P14 testes, more VMKO spermatocytes were in pachynema when compared with control cells (Fig. 6C). This pattern persisted in 6-week-old testes, with greater numbers of pachytene spermatocytes and fewer numbers of pre-leptotene, leptotene, and zygotene spermatocytes in VMKO testes when compared with controls (Fig. 6D). Finally, to determine whether post-meiotic germ cell development exhibits normal initiation in VMKO testes, we evaluated the distribution of the RNA-binding protein BOULE, which is essential for round spermatids to begin the elongation process (VanGompel & Xu 2010). Adult VMKO germ cells exhibited normal BOULE distribution (Fig. 7), suggesting that the initial stages of spermiogenesis are not disrupted by an overrepresentation of pachytene spermatocytes in the meiotic cell population.

Discussion

In this study, we have described a role for MED1 in regulating the temporal progression of primary

spermatocytes through meiosis. *Med1*-depleted germ cells prematurely appear in zygonema and pachynema during the initial wave of spermatogenesis, with a reduced proportion of cells remaining in pre-leptonema and leptonema. Once formed, pachytene cells lacking *Med1* progress with temporal dynamics that result in a meiotic exit comparable with control cells. Subsequent spermatogenic waves retain this pattern of prolonged pachynema relative to other stages of the first meiotic prophase. Ultimately, fertility is normal in *Med1* conditional knockout males, resulting in normal numbers of offspring.

The role MED1 plays in enhancing transcription *in vivo* from nuclear hormone receptors, including VDR, estrogen receptors, and peroxisome proliferator-activated receptors, is well described (Oda *et al.* 2003, Jia *et al.* 2004, Zhang *et al.* 2005). While MED1 physically interacts with RARs, a functional role for a MED1–RAR interaction *in vivo* has not been shown. One study found that a human promyelocytic leukemia cell line was resistant to RA-induced differentiation *in vitro* following transient *Med1* knockdown (Urahama *et al.* 2005). RA is the main driver of meiotic entry in germ cells (Griswold *et al.* 2012). While loss of *Med1* does not inhibit spermatogonial differentiation or entry into meiosis, the *Med1* loss-of-function effect alters the timing of meiotic progression once cells have committed to this process. It is important to note that MED1 interacts with members from other classes of transcription factors, including the GATA family (Crawford *et al.* 2002). Thus, it is possible that MED1 regulates transcription in developing germ cells through a nonhormone receptor or another previously unidentified binding partner.

Several other factors have been shown to play a role in the temporal progression of meiosis. Insulin-like factor 6 is essential for late pachynema of prophase I, with few germ cells differentiating beyond this stage in its absence (Burnicka-Turek *et al.* 2009). Loss of Dicer 1 in the male

Figure 7 VMKO testes exhibit normal post-meiotic germ cell development. Cross-sections of 6-week-old VMKO (left) and control (right) testes immunostained for BOULE (green) and exposed to DAPI (blue). Scale bars = 25 μ m.

germline results in a higher proportion of leptotene and zygotene cells and a reduced proportion of pachytene, diplotene, and metaphase I cells (Romero *et al.* 2011, Greenlee *et al.* 2012). Claudin 3 knockdown in the testis, meanwhile, generates meiotic cells that exhibit a prolonged preleptonema with respect to other stages in meiosis (Chihara *et al.* 2013). In light of these previous findings, MED1 could mediate its activity in the spermatocytes downstream of growth factor secretion, tight junction formation, or noncoding RNA production. The mechanism of how MED1 regulates meiotic progression remains to be defined in further detail.

The spermatogenic process relies on a high level of coordination between many different cell types. While RA and other extrinsic signaling molecules play key roles influencing spermatogonial differentiation and meiosis, the effects of these molecules are ultimately regulated within the germ cells themselves. In this study, we describe a role for MED1 in the regulation of meiotic progression in male germ cells. Together with other transcriptional and epigenetic regulators, MED1 appears to modulate the influence of extrinsic signaling factors on germ cells to correctly time key events during spermatogenesis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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