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Nutrient restriction, inducer of yeast meiosis, induces mammalian meiotic initiation

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ABSTRACT

From yeasts to mammals, the molecular machinery and chromosome structures carrying out meiosis are frequently conserved. However, the signal to initiate meiosis appears divergent: while nutrient restriction induces meiosis in the yeast system, retinoic acid (RA), a chordate morphogen, and its target, *Stra8*, are necessary but not sufficient to induce meiotic initiation in mammalian germ cells. Here, by using a combination of genetic, transcriptomic, cytologic approaches in mouse primary spermatogonial culture without the support of gonadal somatic cells, we show that nutrient restriction is both necessary and sufficient to robustly induce *Spo11*-dependent meiotic DNA double strand breaks (DSBs) and *Stra8*-dependent meiotic gene programs with RA, recapitulating those of early meiosis *in vivo*. Moreover, distinct network of 11 nutrient restriction-upregulated transcription factor genes was identified, whose expression does not require RA and is associated with early meiosis *in vivo*. Thus, our study proposes a conserved model, in which nutrient restriction induces meiotic initiation by upregulating key transcriptional factors for meiotic gene programs, and provides an *in vitro* platform to recapitulate meiotic initiation that will facilitate research and haploid gamete production.

TEXT

Sexual reproduction depends on the formation of haploid gametes through meiosis, which exchanges replicated parental chromosomes through homologous recombination during meiotic prophase, followed by two rounds of successive divisions to generate haploid gametes carrying novel genetic constitution¹. Meiotic initiation requires the activation of a specialized meiotic gene program that implements the chromosomal events during meiosis prophase, including homologous pairing, synapsis, and recombination². From yeasts to mammals, meiosis-specific chromosome structures exhibit remarkable evolution conservation, including synapsis formation mediated by the assembly of the synaptonemal complex (SC), homologous recombination through the formation and the subsequent repair of meiotic DNA double-strand breaks (DSBs). Many genes underlying these events are often evolutionarily-related or -conserved. For instances, *Spo11* encodes a DNA topoisomerase-like enzyme that catalyzes meiotic DSB formation^{3,4}. *Dmc1* encodes a meiotic recombinase that repairs DSBs by searching for allelic DNA sequences on the homologous chromatids^{5,6}. *Hormad* genes encode meiosis-specific chromosome factors (Hop1 in yeasts and Hormad1/2 in mammals) that are critical for synapsis and DSB formation and repair^{7,8}.

Despite these evolutionary conservations in meiotic genes and structures, the overarching signal to trigger meiosis appears to be divergent. In yeasts, transition from mitotic to meiotic cell cycles is induced simply by nutrient restriction. Subsequently, nutrient-sensing pathway induces the expression of *inducer of meiosis 1 (IME1)*, which encodes a single master transcriptional activator for meiotic genes, including *Spo11*, *Dmc1*, and *Hop1* (ref. 9, 10). In mammalian germ cells, meiotic initiation requires retinoic acid (RA), an activate metabolite of vitamin A. RA is a morphogen essential for growth and development in chordate animals¹¹. In female oogenesis, RA is synthesized primarily in the mesonephric ducts in embryonic ovaries¹². In male spermatogenesis, RA is produced by meiotic and somatic cells in testes¹³. RA induces meiotic gene program primarily by activating *stimulated by retinoic acid gene 8 (Stra8)* (ref. 14, 15). Although STRA8 by itself or with its cofactor MEIOSIN is necessary for meiotic initiation^{16,17}, neither RA nor STRA8 is sufficient, suggesting that other signal works with RA to induce meiotic initiation in mammals.

Our recent work in *Stra8*-deficient mice reveals that an autophagy-inducing factor is engaged on meiosis-initiating germ cells¹⁸. Interestingly, nutrient restriction, the aforementioned inducer of yeast meiosis, is perhaps the most potent inducer of autophagy¹⁹. Thus, we asked whether nutrient restriction might have a conserved role in inducing meiotic initiation in mammalian germ cells. To test this, we established primary spermatogonial culture by using neonatal mouse testicular cells in C57BL/6 X DBA/2 F1 hybrid background²⁰ (**fig. S1A**; see below for transcriptomic analysis for cultured primary spermatogonia). Nutrient restriction was applied to cultured cells by adding 90% Earle's Balanced Salt Solution (EBSS) to the complete culture medium.

Co-treatment of nutrient restriction and RA (referred to as “NRRA”) for 2 days triggered a distinct morphology of cellular enlargement in culture cells, suggesting cell differentiation (**Fig. 1A**; **fig. S1B**). NRRA induced a significant activation of essential meiotic genes, including *Spo11*, *Dmc1*, and *Sycp3* (**Fig. 1B**). *Sycp3* encodes a lateral element of synaptonemal complex that forms between two meiotic homologous chromosomes²¹. Consistently, phosphorylated histone H2AX (γ H2AX) shows that DNA DSBs were most profoundly formed in cells from NRRA-treated culture (**Fig. 1C**; **fig. S1C**). These effects were not observed in cultured cells treated with RA or NR alone (**Fig. 1A-C**). Similar effects of NRRA in meiotic gene activation were observed in F9 premeiotic cells (**fig. S2**). Importantly, the meiotic origin of these DSBs was confirmed by DMC1 staining (**Fig. 1D**). In addition, cells from primary spermatogonial culture generated from *Spo11*-deficient mice lack DMC1 foci upon NRRA treatment, constituting genetic evidence that NRRA-induced meiotic DSB formation *in vitro* requires *Spo11* (**Fig. 1D**; see below for the transcriptomic analysis of *Spo11*-deficient culture, which shows meiotic gene activation). Consistently, cultured cells exhibited rapid loss of Gfra1 and E-cadherin expression, markers for undifferentiated spermatogonia (**fig. S1D**).

Transcriptomic analysis shows that NRRA treatment for 2 days induced a gene expression pattern distinct from the treatment of RA or NR alone in primary spermatogonial culture (**Fig. 1E**; **fig. S3**; **table S1**). Four clusters of gene sets were identified by unsupervised hierarchical clustering (UHC) (**table S2**). Notably, genes in Cluster 2, which appear to be genes upregulated by RA and NRRA, are enriched with genes bearing gene ontology (GO) functional terms related

to meiosis (**Fig. 1E and fig. S4**). In contrast, Cluster 1, 3, or 4 is enriched with genes for “mitochondrion organization” (Cluster 1, mostly nutrient restriction-downregulated genes), “regulation of innate immune response” (Cluster 3, mostly nutrient restriction-upregulated genes), “ncRNA metabolic process” (Cluster 4, mostly RA-downregulated genes) (**fig. S4**). Consistently, nonparametric and unsupervised gene set variation analysis (GSVA) and hallmark gene set analysis revealed stronger positive correlations of meiosis-related pathways (male meiotic nuclear division, chromosome organization in meiotic cell cycle, synaptonemal structure) and gametogenesis-related (spermatogenesis) in genes upregulated by NRRA than those upregulated by RA (**Fig. 1F and G, fig. S5**) (**table S3**). These data together suggest that nutrient restriction in combination with RA is sufficient to induce meiotic gene and chromosome programs *in vitro*.

To examine whether the effect of NRRA in inducing meiotic gene program depends on genetic backgrounds, we established primary spermatogonial culture using testes from neonatal CD1 inbred mice. RNA-seq show that cultured cells in CD1 background exhibited comparable transcriptomic changes including activation of meiotic genes, including *Dmc1*, *Sycp3*, in response to NRRA treatment (**fig. S6**), suggesting that the sufficient role of NRRA in inducing meiotic gene program is independent of genetic backgrounds.

To delineate the role of nutrient restriction in meiotic gene expression, we assembled a set of 193 meiosis genes by combining two collections of genes that were previously found to be associated with mammalian early meiosis during mouse² and human²² spermatogenesis (**fig. S7**).

Unsupervised UHC divided 165 differentially expressed genes (DEGs) among these 193 genes into five major clusters (**Fig. 1H; table S4**). Nutrient restriction appears to play four roles on the expression of key meiotic genes by: 1) inducing a subset of meiotic gene expression that was not regulated by RA, such as *Rad21l*; 2) initiating the activation of a subset of meiotic genes, which was further enhanced by RA, such as *Zfp541*; 3) synergizing with RA to induce the activation of a subset of meiosis gene expressions, such as *Sycp1*, *Sycp3*, *Mei1*, *Msh5*, and *Stag3*; and 4) augmenting the expression of a subset of meiotic genes induced by RA, including *Dmc1*, *Smc1b*, *Ugt8a*, *Stag3*, *Hormad1*, as well as *Gm4969*, which encodes MEIOSIN, a transcriptional cofactor for STRA8 required for meiotic prophase program¹⁷. Notably, many meiotic genes

whose expression is STRA8-dependent require nutrient restriction (**Fig. 1H**)². Together, these data suggest crucial roles of nutrient restriction by itself or by synergizing with RA in inducing meiotic gene expression; in the absence of nutrient restriction, RA alone is not sufficient (see the effect of *in vivo* RA treatment on meiotic gene expression despite *Stra8* activation in **fig. S8**)²³.

To examine NRRA-induced meiotic initiation and its potential to support meiotic progression at the single cell resolution, we isolated single cells from primary spermatogonia culture treated with NRRA for 2 days to induce meiotic initiation, followed by a medium that is supposedly to promote meiotic progression for an additional 1 and 2 days. Culture treated with normal medium for 4 days were used as a control. Single cell RNA-sequencing (scRNA-seq) was then performed using the 10x Genomics platform (**Fig. 2A**). A total of 25,607 cells were sequenced from these samples. Using stringent quality control, 18,088 cells were selected for further analysis (**fig. S9**). All of the cells were pooled to perform clustering analysis, which revealed four major cell clusters based on their distinct gene expression patterns (**Fig. 2B**). These four clusters were subsequently annotated by using known marker genes (**Fig. 2C; fig. S10**). Cluster 0 appears to be undifferentiated spermatogonia (e.g., *Gfra1*, *Etv5*). Clusters 1, 2, and 3 appear to be differentiating spermatogonia/pre- and meiotic spermatocytes at progressively advanced meiotic stages with upregulated expression of spermatogonial differentiation (e.g., *Sohlhl1*) and meiotic genes (e.g., *Dmc1*, *Sycp3*). Cluster 4 appears to be mostly feeder cells (mouse embryonic fibroblast or MEF) due to the expression of fibroblast genes (*s100a4*). Progressive upregulation of meiotic genes and downregulation of undifferentiated spermatogonia genes on each time points were confirmed by qRT-PCR analysis (**fig. S11**).

Analysis of the distribution of the different cell clusters at each time point demonstrate a dramatic transition of cell populations (**Fig. 2D**). Analysis of GO functional terms for DEGs revealed rapid changes in their cellular processes (**Fig. 2E; table S5**). Cluster 0 is enriched in genes involved in regulation of cellular amide metabolic process (e.g., *Sox4*), stem cell division (e.g., *Zbtb16*, *Etv5*), etc (**fig. S12A**). Cluster 1 is enriched in genes involved in the regulation of gene silencing (e.g., *Hist1hle*), mitochondrial electron transport, NADH-ubiquinone (e.g., *Park7*), retinoic acid and metabolic process/response (e.g., *Stra8*), etc (**fig. S12B**). Cluster 2 is enriched in genes involved in reproduction in multicellular organisms (e.g., *Sycp3*), sperm-egg

recognition (e.g., *Ly6k*), etc (**fig. S12C**). Cluster 3 is enriched in genes for meiotic cell cycle (e.g., *Prdm9*), cytoplasmic translation (e.g., *Rpl18a*), translation initiation (e.g., *Eif1*), chromosome condensation (e.g., *Dnajc19*), etc (**fig. S12D**).

Importantly, cells from Cluster 1 to Cluster 3 exhibit a progressive and coordinated upregulation of meiotic genes bearing mouse meiosis-related GO terms, including DNA double-strand break formation, histone variants, meiotic nuclear division, synapsis (**fig. S13**). Notably, meiotic genes that are fully (*Dmc1*, *Hormad1*, *Mei1*, *Mlap*) or partially (*Smc1b*, *Stag3*, *Sycp1*, *Sycp2*, *Sycp3*, *Ugt8a*, *Meioc*) (**Fig. 2F**; **fig. S14**) dependent on STRA8 expression displayed progressive upregulation from Cluster 1 to Cluster 3. Since activation of RA signaling with *Stras8* expression is not sufficient to induce their expression *in vitro* and *in vivo* (**Fig. 1E**; **fig. S8**)²³, this data suggests that nutrient restriction is a critical signal to work with RA in activating meiotic genes.

To assess the relationship between NRRA-induced meiotic initiation and progression *in vitro* with those during *in vivo* meiosis during spermatogenesis, we performed principal component analysis (PCA) analysis with a published scRNA-seq database²⁴ (**fig. S15**), which shows that the transcriptional profiles of Clusters 0 to 3 correlate with meiotic initiation (leptotene, the first stage of meiotic prophase) and progression (zygotene and early pachytene) during *in vivo* spermatogenesis²⁴ (**Fig. 2G**). Subsequently, hclust and differential gene correlation analysis (DGCA) demonstrate independently that cell population in Cluster 0 from *in vitro* meiosis correlates with undifferentiated spermatogonia during *in vivo* spermatogenesis, cell population in Cluster 1 with differentiating spermatogonia/preleptotene spermatocytes, cell population in Cluster 2 with leptotene/zygotene spermatocytes, and cell population in Cluster 3 with early pachytene spermatocytes (**Fig. 2H and I**). A hallmark event that occurs in male germ cells during pachytene stage is meiotic sex chromosome inactivation (MSCI)²⁵. Similarly, we observed a progressive decline in sex chromosome gene transcription from Cluster 0 to Cluster 3, supporting that cells in Cluster 3 have reached to a pachytene-like stage (**Fig. 2J**).

Moreover, past study indicates that early meiosis during *in vivo* spermatogenesis follows a step-wise pattern without lineage branching²⁴ (**Fig. 2K**; **fig. S15**). To examine the transcriptional dynamics of *in vitro* meiotic initiation and progression, we performed pseudo-time analysis,

which shows that the projected timeline recapitulated early meiosis during *in vivo* spermatogenesis (**Fig. 2K, fig. S16-18**). The pseudo-time indicates that Cluster 0 is mainly at the start of the projected timeline trajectory, that Clusters 1, followed by Cluster 2, is positioned in the middle, and that Cluster 3 is at the end. Consistently, expression of genes specific to undifferentiated spermatogonia was located preferentially at the beginning of the trajectory, while expression of genes functionally involved in many essential meiotic programs (e.g., cohesion, DNA DSB formation, chromosome segregation) was located preferentially towards to the end of the trajectory, which further supports the validity of the analysis (**fig. S17 and S18**).

Stra8 is a best characterized gatekeeper of meiotic initiation in vertebrates ¹⁴. To examine whether NRRA-induced meiotic initiation meets this genetic requirement, we generated primary spermatogonia culture using *Stra8*-deficient mice. Consistent with the *in vivo* role of STRA8 in meiotic DSB formation ^{14, 26} (**fig. S19**), *Stra8*-deficient culture did not exhibit DMC1 foci upon NRRA treatment (**Fig. 3A**), suggesting that NRRA-induced *in vitro* meiotic DSB formation requires *Stra8* (**Fig. 3A**). Moreover, RNA-seq analysis shows that WT, *Stra8*-deficient, and *Spo11*-deficient cultures exhibited similar transcriptome profiles in normal medium (**Fig. 3B**). However, upon parallel NRRA treatment for 2 days, *Stra8*-deficient culture failed to upregulate a cluster of genes, which is enriched for meiosis-related GO terms, including meiotic cell cycle, meiosis I, synapsis (**Fig. 3B; Table S6**) ². This is consistent with the essential role of STRA8 in activating the gene programs of meiotic prophase ², and confirms that lack of meiotic DSBs in *Stra8*-deficient and *Spo11*-deficient cultures resulted from discrete mechanisms: SPO11 is an enzyme that directly responsible for meiotic DSB formation ^{3, 4}. GSVA analysis shows that indeed STRA8 sits at the foundation of NRRA-induced meiotic initiation *in vitro*: in contrast to WT and *Spo11*-deficient cultures, despite activation of retinoid acid receptor signaling pathway, gametogenesis- and meiosis-related pathways were not activated in *Stra8*-deficient culture upon NRRA treatment (**Fig. 3C**). Moreover, STRA8-dependent meiotic genes (*Sycp3*, *Mei1*, *Dmc1*, *Stag3*, *Sycp2*) were only upregulated in WT, but not *Stra8*-deficient, culture (**Fig. 3D; fig. S20; Table S7**). And the genes only upregulated in *Stra8*-deficient culture bears no meiosis-related GO terms, which is consistent with the robust meiotic initiation arrest phenotype of *Stra8*-deficient germ cells *in vivo* ^{14, 26}. Additionally, our system reveals that many genes associated with undifferentiated spermatogonia were not downregulated in *Stra8*-deficient culture (e.g.,

Pou5f1, *Etv5*), which is in line with the role for STRA8 in promoting spermatogonial differentiation¹³ (**Fig. 3E; fig. S21; Table S7**).

Our results show for the first time that *Stra8*-dependent and *Spo11*-dependent meiotic DSBs are induced *in vitro* (**Figs. 1D and 3A**). To further examine the processing of these meiotic DSBs, our spread analysis shows that, similar to *in vivo* meiosis, sing-strand DNA (ssDNA)-binding proteins, SPATA22 and MEIOB^{27,28}, were progressively recruited onto the meiotic chromosomes from a leptotene-like to an early pachytene-like stage in culture, suggesting that meiotic DSBs formed *in vitro* were resected into ssDNA before recruiting meiotic recombinases RAD51 and DMC1 for repair (**fig. S22A**). Quantification show that similar numbers of SPATA22, MEIOB, RAD51, and DMC1 foci were detected on meiotic chromosomes per germ cell between *in vivo* meiosis and *in vitro* meiosis (**Fig. 4A and B**). In addition, histone methyltransferase PRDM9 directs meiotic DSBs to be distributed to recombination hotspots by^{29,30}. PRDM9 expression is induced by NRRA in cultures at both mRNA (**Figs. 1H and 2C**) and protein levels (**Fig. 4C**). Consistently, chromatin immunoprecipitation (ChIP) assay for DMC1-associated ssDNA fragments, a direct method to detect recombination hotspots³¹, revealed that the DSBs formed *in vitro* upon NRRA treatment (**Fig. 4D**) were mapped to the strong hotspots of meiotic recombination *in vivo* (**fig. S22B**)³².

To examine the mechanism of nutrient restriction-induced meiotic initiation, we identified that nutrient restriction upregulated 120 transcription factor (TF) genes, whose expression does not require RA (**Fig. 5A and B**). To identify those potentially involved in regulating the meiotic gene programs, we examined the expression of these genes in the transcriptomic database for mouse spermatogenesis and found that 30 of them are expressed before meiotic prophase (**Fig. 5B and C; fig. S23**)²⁴. Then, we further investigated their correlation with meiotic gene expression in the Genotype-Tissue Expression database (GTEx)³³. Using *Dmcl*, *Sycp3*, *Hormad1* as three representatives, we found that 11 of them shows strong (Pearson correlation > 0.6) or moderate correlation (Pearson correlation > 0.4) with meiotic gene expression (**Fig. 5B and D, fig. S23**). We further confirmed that the expression of these 11 TFs *in vivo* does not require RA (**fig. S24**)²³. Notably, these 11 TFs include *Sohlh1* and *Sox3*, two characterized TFs implicated in early meiosis and spermatogenesis. *Sohlh1* is basic helix-loop-helix (bHLH)

transcription factor, whose deletion results in many tubules lacking meiotic spermatocytes³⁴. Recently, *Sohlh1* is shown to regulate meiotic gene expression (*Sycp1*, *Sycp3*) by directly binding to their proximal promoters³⁵. *Sox3* is expressed exclusively in spermatogonia committed to differentiation³⁶, and loss of *Sox3* impairs early meiosis, including a downregulation of *Sycp3* expression in young adult testes³⁷. Thus, these data suggest that nutrient restriction induces a distinct network of TFs to complement RA signaling in the meiotic gene activation (**Fig. 5E**).

Nutrient restriction is in essence a metabolic cue. Our scRNA-seq revealed that, while genes involved in the glycolytic pathways were downregulated, genes involved in mitochondrial function pathways were upregulated by NRRA-induced *in vitro* meiotic initiation and subsequent progression (Cluster 0 to Cluster 3) (**fig. S25**), which mirrors the transition from glycolysis to mitochondrial biogenesis and oxidative phosphorylation during *in vivo* spermatogonial differentiation³⁸. Thus, our data at the transcriptomic, cytologic, mechanistic, and metabolic levels establish that nutrient restriction in combination with RA is sufficient to induce meiotic initiation that faithfully recapitulates this process during *in vivo* meiosis and spermatogenesis. For first the time to our knowledge, this is achieved without the support of gonadal somatic cells. How do male germ cells receive nutrient restriction signal and achieve this metabolic switch *in vivo*? A model has been proposed for the differential microenvironments resided by undifferentiated and differentiating spermatogonia in the testis, which are influenced by the distances to blood supply and oxygen³⁸. Moreover, unlike the nutrient-rich basal compartment where mitotic spermatogonia reside, the apical compartment of the mammalian seminiferous tubule, where meiosis takes place, is a nutrient-restricted microenvironment (i.e., low in glucose and most amino acids) created by the blood-testis barrier (BTB)³⁹. Thus, nutrient restriction as a physiological stimulus for meiotic initiation warrants further investigation. Our study provides an *in vitro* platform to study meiotic initiation and to facilitate production of haploid gametes via meiosis in culture.

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AUTHOR CONTRIBUTIONS

X.Z. performed all the experiments. S.G. assisted the analyses of RNA-seq and scRNA-seq data. X.Z. and N.W. designed the experiments, analyzed and curated the data. N.W. and X.Z. wrote the manuscript.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

DATA AND MATERIALS AVAILABILITY

All data are available in the manuscript or the supplementary material.

SUPPLEMENTARY MATERIALS

This manuscript contains supplementary materials, including Materials and Methods, 26 Supplementary Figures, and 10 Supplementary Tables.

FIGURE LEGEND

Fig. 1. NR is required to induce meiotic initiation with RA in primary spermatogonial culture. **(A)** Bright-field images of cultured spermatogonia with indicated treatments for 2 days. Scale bars, 50 μ m. **(B)** Relative expression of *Spo11*, *Dmc1*, and *Sycp3* against *Gapdh* analyzed by qRT-PCR in cultured spermatogonia with indicated treatments for 2 days. Results shown represent mean $\pm$ SEM. * $P < 0.05$. n = 3 independent cultures. n.s., no significant. **(C)** Immunostaining for γ H2AX (red) and DAPI (blue) with indicated treatment for 2 days. Scale bars, 10 μ m. **(D)** Immunostaining for DMC1 (red), DDX4 (green), and DAPI (blue) in *Spo11*^{+/+} and *Spo11*^{-/-} primary spermatogonial culture. Scale bars, 10 μ m. **(E)** (Left) UHC and heatmap of gene expression in cultured primary spermatogonia with indicated treatments for 2 days. (Right) Top GO enrichments with representative genes in each cluster. **(F)** GSVA analysis for indicated treatments. In the heatmap, rows are defined by the selected gene sets, and columns by consensus scores for each treatment. Group enriched gene sets are highlighted by different color. **(G)** Bar plot shows p -value of GO functions between RA and NRRA treatment. **(H)** UHC and heatmap for the expression of 165 early meiosis-associated genes. STRA8-dependent genes characterized in a previous study in ref. ² are shown in red.

Figure 2

Fig. 2. scRNA-seq analysis of primary spermatogonial culture during NRRA-induced meiotic initiation and progression. **(A)** Workflow of scRNA-seq experiment. Number of cells collected: 6,884 (0 day), 6,936 (2 day), 6,200 (3 day), and 5,587 (4 day). **(B)** A t-distributed Stochastic Neighbor Embedding (tSNE) plot for analyzed cells. Cluster 0 – 3 were germ cells and Cluster 4 was somatic cells. Number of cells selected for analysis: Cluster 0 (6,487 cells), Cluster 1 (5,286 cells), Cluster 2 (3,860 cells), Cluster 3 (2,170 cells), Cluster 4 (285 cells). **(C)** Violin plots showing the expression level of representative genes in each cluster. **(D)** A bar plot showing the proportion of the different cell clusters at different time points. **(E)** A heatmap showing the expression of marker genes and GO functions in each cluster. **(F)** Gene expression patterns of indicated genes on tSNE plots and violin plots. **(G)** PCA analysis showing the relationship between single cell clusters from NRRA-treated culture *in vitro* (n = 4 clusters) and early mouse spermatogenesis *in vivo* (n = 6 clusters) based on the transcriptional profiles of commonly expressed genes (n = 7,803 genes). **(H)** Hierarchical clustering showing the relationship between single cell clusters from NRRA-treated culture *in vitro* and early mouse spermatogenesis *in vivo*. **(I)** Scatter plots comparing marker genes expression profile between *in vitro* clusters and *in vivo* clusters. **(J)** (Left) Heatmap of sex chromosome genes in Clusters 0 – 3. (Right). Violin plots showing percentage of X and Y chromosome genes profiles in Clusters 0 – 3. The median is shown by a red line. **(K)** Scatter plots showing cells along the projected pseudo-time in Cluster 0 – 3 (upper) and in indicated clusters from early mouse spermatogenesis *in vivo* (lower).

A

C

D

B

E

Figure 3

Fig. 3. *Stra8* is required for NRRA-induced meiotic initiation. **(A)** Immunostaining for DMC1 (red), DDX4 (green), and DAPI (blue) in *Stra8*^{+/+} and *Stra8*^{-/-} primary spermatogonial culture. Scale bars, 10 μm. **(B)** (Left) UHC and heatmap of gene expression in cultured primary spermatogonia with indicated genotypes for 2 days. (Right) Top GO enrichments with representative genes in each cluster. **(C)** GSVA analysis for indicated genotypes. In the heatmap, rows are defined by the selected gene sets, and columns by consensus scores for each genotype. Group enriched gene sets are highlighted by different color. **(D and E)** Venn plots with top GO enrichments and representative genes for NRRA-upregulated (**D**) and -downregulated (**E**) genes in *Stra8*^{+/+} and *Stra8*^{-/-} cultures.

Figure 4

Fig. 4. Number and distribution of meiotic DSB formation *in vitro*.

(A) Representative chromosome spreads stained by RAD51, DMC1, MEIOB, SPATA22, and SYCP3 from juvenile mice (day 14 of age) or primary spermatogonial culture following NRRA-induced meiotic initiation and progression are shown. (B) Quantification of foci for zygonema-early pachynema stage. Error bars, mean $\pm$ SD. Each solid circle indicates the total number of foci from a single nucleus. Total number of cells quantified from three independent cultures are shown on each graph. n.s., not significant. (C) Immunofluorescence staining for SYCP3 and PRDM9 on chromosome spreads in testicular germ cells from juvenile mice (day 14 of age) or in cells from primary spermatogonial culture following NRRA-induced meiotic initiation and progression. Scale bars, 10 μ m. (D) Enrichment of the DNA corresponding to several published hotspots by anti-DMC1 ChIP estimated by qPCR. ChIP was performed from primary spermatogonial culture following NRRA-induced meiotic initiation and progression. Enrichment of the DNA corresponding to genomic regions on Chr17, Chr5, Chr8, Chr11, Chr13, Chr14, and the *β -actin* gene was estimated by qPCR. Standard deviation from duplicate reactions are shown.

Figure 5

Fig. 5. Nutrient restriction induces a network of TF genes involved in early meiosis. **(A)** A workflow showing filters to identify genes of interest. **(B)** Heatmap of 120 TF genes upregulated by nutrient restriction. 11 identified TF genes involved in early meiosis are shown on the right. **(C)** Gene expression patterns of 3 representative TF genes during early spermatogenesis from a published scRNA-seq database ²⁴. **(D)** GTEx database showing correlation of representative TF genes (*Sohlh1*, *Pou2f2*, *Bcl11a*) with selected meiotic genes in 165 testis samples. **(E)** Schematics of nutrient restriction in combination with retinoic acid in inducing meiotic gene programs. Nutrient restriction alone is sufficient to activate a class of meiotic genes that do not require retinoic acid (e.g., *Rad21l*). Retinoic acid alone is sufficient to activate *Rec8*, an essential component of cohesion, and *Stra8*. Nutrient restriction and retinoic acid synergistically induce key meiotic genes for synapsis and recombination.

SUPPLEMENTARY MATERIALS FOR

Nutrient restriction, inducer of yeast meiosis, induces meiotic initiation in mammals

- **Materials and Methods**
- **Supplementary tables 8 – 10**
- **Supplementary figure legends**

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Materials and Methods

Animal

All genetically modified mice were obtained from Jackson Laboratory: *Stra8*-deficient mice (Jax stock number: 023805), *Spo11*-deficient mice (Jax stock number: 019117). CD1 and DBA/2 mice were raised under specific-pathogen-free (SPF) condition. All animal work is approved according to the guidelines of the Animal Care and Use Committee of the University of Kansas Medical Center.

Mouse primary spermatogonial culture

We generated primary spermatogonial culture in both CD1 inbred and C57BL/6XDBA/2 F1 hybrid backgrounds as previously described (1). For the primary spermatogonial culture, testes from 6-day-old mouse pups was used by dissociating via a two-step enzymatic digestion procedure. Primary spermatogonial cells were cultured on 0.1% gelatin-coated plates with germline stem cell culture medium overnight. After stem cell enrichment, primary spermatogonial cells were transfer onto murine embryonic fibroblast (MEF) feeder cells (from E13.5 embryos) for long-term culture. For indicated treatments to induce meiotic initiation, 1.0×10^6 primary spermatogonial cells were plated on 60-mm dishes coated with MEF feeder cells and treated for 48 hours. At meiotic initiation, meiotic progression was induced by a medium supplemented with melatonin, FSH, TGF β , BPE, and DHT (2) for another 48 to 72 hours. Medium compositions used in this study were summarized in Supplemental Table 8.

RNA isolation, qPCR

For RNA isolation, Total RNA was isolated from collected cells using TRIzol™ (Invitrogen,15596018). The RT reaction was carried out with SuperScript II First-Strand Synthesis Kit (Invitrogen,18080-051). qPCR was performed with gene specific primers that were listed in Supplemental Table 9. qPCR with amplified cDNAs was performed using the Power SYBR Green Master Mix (Applied Biosystems) on a real-time qPCR system.

Immunofluorescence (IF) analysis

For the IF staining, cultured cells were fixed with 4% paraformaldehyde contained 0.1% Triton X-100. Then cells were washed with PBS. Blocking was performed using 5 % BSA for 1 hour at room temperature. The primary antibodies were added and incubated for overnight at 4°C. After washed in PBS, the secondary antibodies were added and incubated for 1 hour at room temperature. Antibodies used were listed in Supplementary Table 9. Images were captured using a confocal laser microscope (Nikon A1R).

Chromosome spreads

Testis were dissociated after removing the tunica albuginea in 15 mL tubes with 5 mL TIM buffer (104 mM NaCl, 45 mM KCl, 1.2 mM MgSO₄, 0.6 mM KH₂PO₄, 6.0 mM sodium lactate, 1.0 mM sodium pyruvate, 0.1% glucose) contained 1mg/ml collagenase IV. Tissue was left shaking for 1 hour at room temperature. After incubation, TIM buffer was removed after centrifugation for 5 min at 300 g at room temperature. A 1 ml tip was used to disperse the tissue further by pipetting up and down for 5 min. Single cell suspension was resuspended in 500 µl TIM. 75 mM sucrose solution was added to tube. 200 µl cell mix was transfer to Superfrost glass slides (Fisher Scientific) coated with a thin layer of 1% paraformaldehyde (PFA), and half-dried in a closed slide box for 1 hour, then dried with half-open lid for 2 hours at room temperature. Slides were washed with 0.4% PhotoFlow (Nikon), air-dried and stored in -80°C.

Western blot

Total protein was isolated in RIPA buffer contained with 1 mM PMSF (Sigma) and protease inhibitor cocktail (Sigma P8340). Lysates were removed by centrifugation at 12,000 X g for 15 min at 4°C, and protein concentrations in supernatants were determined (DC protein assay; BioRad). Equal amount of protein from each sample was mixed with LDS sample buffer (Invitrogen) plus sample reducing agent (Invitrogen), and denatured for 10 min at 70°C. Proteins were resolved in 4-12% Bis-Tris gels (Thermo Fisher), and transferred to PVDF membranes. Blots were probed with antibodies overnight at 4°C, washed and reacted with secondary antibody. Detection was performed with the Clarity ECL Western Blotting Substrate (BioRad).

Chromatin immunoprecipitation (ChIP)

Testicular cells or collected cells were dissociated after remove the tunica albuginea and fixed for 10 min in 1% formaldehyde. Followed by quench the tissue with homogenized and washing in the PBS twice. Cells were lysed in 1ml of the lysis buffer (from EZ-ChIP kit, Millipore, 17-371RF) and the chromatin was sheared to ~1000 bp by sonication using a Diagenode pico bioruptor. The sample was pre-cleared with Protein G beads and 10 µl of pre-cleared chromatin was saved and referred to as input. The rest of the chromatin was incubated with DMC1 antibody or preimmune IgG overnight at 4°C, followed by a 2-hour incubation with Protein Agarose G beads. Following washing, the chromatin was eluted from the agarose beads by using 1% SDS, 0.1M NaHCO₃ (pH 9.0) at 65°C and crosslinking was reversed at 65°C overnight. DNA was deproteinized for 2 hours at 45°C and DNA was purified. Enrichment of the characterized hotspots was evaluated by qPCR analysis using previously reported primers (3).

RNA-sequencing (RNA-seq)

The stranded mRNA-Seq was performed using the Illumina NovaSeq 6000 Sequencing System at the University of Kansas Medical Center – Genomics Core (Kansas City, KS). Quality control on RNA submissions was completed using the Agilent TapeStation 4200 using the RNA ScreenTape Assay kit (Agilent Technologies 5067-5576). Total RNA (1ug) was used to initiate the library preparation protocol. The total RNA fraction was processed by oligo dT bead capture of mRNA, fragmentation, reverse transcription into cDNA, end repair of cDNA, ligation with the appropriate Unique Dual Index (UDI) adaptors, strand selection and library amplification by PCR using the Universal Plus mRNA-seq with NuQuant library preparation kit (Tecan Genomics 0520-A01).

Library validation was performed using the D1000 ScreenTape Assay kit (Agilent Technologies 5067-5582) on the Agilent TapeStation 4200. Concentration of each library was determined with the NuQuant module of the library prep kit using a Qubit 4 Fluorometer (ThermoFisher/Invitrogen), libraries were pooled based on equal molar amounts and the multiplexed pool was quantitated, in triplicate, using the Roche Lightcycler96 with FastStart Essential DNA Green Master (Roche 06402712001) and KAPA Library Quant (Illumina) DNA Standards 1-6 (KAPA Biosystems KK4903). Using the qPCR results, the RNA-Seq library pool was adjusted to 2.125nM for multiplexed sequencing.

Pooled libraries were denatured with 0.2N NaOH (0.04N final concentration) and neutralized with 400mM Tris-HCl pH 8.0. A dilution of the pooled libraries to 425 pM is performed in the sample tube, on instrument, followed by onboard clonal clustering of the patterned flow cell using the NovaSeq 6000 S1 Reagent Kit (200 cycle) (Illumina 20012864). A 2x101 cycle sequencing profile with dual index reads is completed using the following sequence profile: Read 1 – 101 cycles x Index Read 1 – 8 cycles x Index Read 2 – 8 cycles x Read 2 – 101 cycles. Following collection, sequence data is converted from .bcl file format to fastq file format using bcl2fastq software and de-multiplexed into individual sequences for data distribution using a secure FTP site or Illumina BaseSpace for further downstream analysis.

Data analysis of RNA-seq

Bulk RNA-seq data analysis was performed using the R software (version 3.6). Differentially expressed genes (DEGs) were calculated by DEseq2. The heatmap was generated using the pheatmap. Gene Ontology (GO) analysis of genes were analyzed by clusterProfiler package. GSVA heatmap was generated by R package, GSVA.

Single-Cell RNA-Seq (scRNA-seq)

Treated cells were digested by collagenase IV for 10 min at room temperature. The digestion was then stopped by media. The cells were pelleted by centrifugation at 300 g for 5 min. And then, supernatant was removed, and the cell pellet was washed with PBS twice. Single cells were obtained by filtering through 40 µm strainers. Cell number was counted using Countess II FL automated cell counter (Invitrogen).

The 10X Genomics Single Cell 3' Expression library preparation is performed using the 10X Genomics Chromium Controller. The cells prepared from disassociated tissue or tissue culture are validated for viability and cell concentration using the Countess II FL Automated Cell Counter (Life Technologies) targeting $\geq 75\%$ cell viability. If debris or cell clumping is present in the cell suspension, the preparation is filtered through a FLOWMI Cell Strainer, 40µm (ThermoFisher 50-136-7555) to yield a homogenous single cell suspension. Cell counts are redetermined by using the Countess II FL and adjusted to ~1000 cells/ul by low speed centrifugation at 4°C and resuspended in 1xPBS without calcium or magnesium (ThermoFisher

MT21040CV) supplemented with 0.04% BSA to prepare cells for emulsification. The cell emulsification is performed with the 10X Chromium Controller using the Chromium Next GEM Single Cell 3' GEM Library & Gel Bead Kit v3.1 (10X Genomics 1000120) and Chromium Next GEM Chip G Single Cell Kit (10X Genomics 1000127). Well 1 of Chip G is loaded with the reverse transcription (RT) Master Mix + cell suspension containing ~16,000 cells to target 10,000 emulsified cells at ~65% efficiency of emulsification. Well 2 of Chip G is filled with 50ul of the Next GEM GEL Beads. Well 3 of Chip G is filled with 45ul Partitioning Oil. Any unused wells are filled with 50% glycerol at a volume designated for the well number. A gasket is applied to the Chip G and the loading cassette and inserted into the Chromium Controller for GEM creation Using the Chromium Single Cell G run program. Emulsified GEMs are recovered from each well and transferred to 200ul strip tubes. The RT reaction to generate 10X barcoded single stranded cDNA, in the single cell containing GEMs, is performed using an Eppendorf MasterCycler Pro thermal cycler. Post GEM-RT Cleanup is conducted by breaking the GEMS with 10X Recovery Agent to separate the aqueous phase from the Recovery Agent and Partitioning oil. A cleanup of the sscDNA containing aqueous phase is completed using Dynabeads MyOne Silane beads (Life Technologies 37002D). The second strand cDNA amplification is performed using the cDNA Amplification Mix on the Eppendorf MasterCycler Pro. The 3' gene expression library construction is initiated with fragmentation, end repair and A-tailing of the dscDNA followed by adapter ligation and a sample index PCR using the Illumina compatible indexed adapters in the Chromium i7 Multiplex kit (10X Genomics 120262). Validation of the single cell library is conducted using the Agilent TapeStation 4200 ScreenTape assay (Agilent 5067-5576). Single cell library quantification is completed using a Roche LightCycle96 using FastStart Essential Green Master (Roche 06402712001) and KAPA Library Quant (Illumina) DNA Standards (KAPA KK4903). Library concentrations are adjusted to 3nM and pooled. The library pool is diluted to 1.00nM for a final clustering concentration of 200pM on a NovaSeq6000. The sequencing was performed using a NovaSeq6000 100 cycle Reagent Kit (Illumina 20012865) with an asymmetrical sequencing profile (read 1 - 28 cycle: i7 index read - 8 cycle : i5 index read – 0 cycle : read 2 – 94 cycles). Bcl2fastq conversion and demultiplexing is performed using the 10X Genomics Cell Ranger and Loupe Browser software suite.

Analysis of single-cell RNA-seq data

Cell clustering was performed by Seurat (<https://satijalab.org/seurat/>, R package, v3.1). Genes expressed in less than 3 cells were filtered out and cells with expressed genes less than 200 were excluded. Seurat object was built after filtered and normalized with default settings. Specifically, cells were retained only if they contained > 2000 ufeature, and had < 10% reads mapped to mitochondrial genome. Then graph-based clustering analyses and t-distributed stochastic neighbor embedding (t-SNE) were performed by using the top 6,000 highly variable genes and 30 PCs, which showed the most significant *p* values. The edges of two cells were measured by Jaccard distance to construct SNN graph. Cluster define was by specific marker genes using “FeaturePlot” function. Differential gene expression analysis for different cell types was by using the Monocle (<http://cole-trapnell-lab.github.io/monocle-release/>, R package, v2.12) following the default settings. Pseudotime lineage reconstruction was performed using diffusion map by Destiny R package on the set of 6000 highly variable genes from Seurat analysis. Trajectories was calculated with Slingshot R package by using the diffusion map results as an input data. Pheatmap package were used for heatmap plotting and clustering. Gene Ontology (GO) analysis of genes were analyzed by clusterProfiler package.

Statistical analysis

All experiments were replicated at least three times independently. Different mice, tissues or cells were used during each experimental replicate. Quantitative data from the experimental replicates were pooled and are presented as the mean $\pm$ SEM or mean $\pm$ SD as indicated in the figure legend. Compiled data were analyzed by Student's *t*-test.

Code availability

Source code of the analysis is publicly available on GitHub at this address:
<https://github.com/iamzhangxiaoyu/scRNA-seq>

Data availability

FastQ files of single-cell RNA-seq and bulk RNA-seq are available on GEO GSE.

REFERENCES:

1. M. Kanatsu-Shinohara *et al.*, Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol Reprod* **69**, 612-616 (2003).
2. Q. Zhou *et al.*, Complete Meiosis from Embryonic Stem Cell-Derived Germ Cells In Vitro. *Cell Stem Cell* **18**, 330-340 (2016).
3. F. Smagulova *et al.*, Genome-wide analysis reveals novel molecular features of mouse recombination hotspots. *Nature* **472**, 375-378 (2011).

Supplementary Figure Legends

fig. S1. Mouse primary spermatogonial cell culture.

(A) Confocal immunofluorescence detection of spermatogonia marker by staining of the cultured spermatogonia *in vitro*. (B) Morphology of cells in primary spermatogonial culture when exposed to NRRA. Appearance of A_{pr}, A₄, A₈ and A_{al} spermatogonia, reminiscent of *in vivo* spermatogonial differentiation, were detected after 2 days. Scale bar, 20 μ m. (C) Cell lysates from primary spermatogonial culture with indicated treatments for 2 days were subjected to Western-blot analysis using γ H2AX antibody. (D) Immunostaining of primary spermatogonial culture with indicated antibodies as markers for undifferentiated spermatogonia combined with DAPI (blue). Scale bar, 10 μ m.

fig. S2. NRRA induces meiotic gene expression in F9 premeiotic cells.

qRT-PCR analysis of meiotic genes in F9 cells with indicated treatment normalized to β -actin. Data represent mean $\pm$ SEM; n = 3. $P < 0.05$ (Student's *t*-test). F9 cells were cultured in DMEM medium with 10% FBS (complete medium). Nutrient restriction was applied to F9 cells by adding 90% of EBSS to the complete medium.

fig. S3. Differentially expressed genes (DEGs) in primary spermatogonial culture upon normal medium, RA, NR, and NRRA treatment.

Scatter plot representations of DEGs between the indicated groups (left panel). The up- or down-regulated genes (fold change > 2 or < -2) in each culture conditions are plotted in red and blue, respectively. The GO function analyses of the upregulated (middle panel) and downregulated (right panel) DEGs between two treatment groups are presented. Representative genes in each GO category are indicated.

fig. S4. GO function analysis for differentially expressed genes upon normal medium, RA, NR, NRRA treatment.

(A-D) The bubble plots show GO enrichment analysis for four gene clusters in Fig. 1E.

fig. S5. GSVA and hallmark analyses for RA and NRRA treatments in primary spermatogonial culture.

GSEA (upper panels) and hallmark (lower panels) analyses reveal distinct enriched gene sets between different groups. In the heatmap, rows are defined by the selected gene sets, and columns by consensus scores for each group. Groups enriched gene sets are highlighted by different color.

fig. S6. RNA-seq analysis of primary spermatogonial culture in CD1 background.

(A) UHC and heatmap of globally changed genes in primary spermatogonial culture with indicated treatments and genetic backgrounds. (B) Scatter plot representations of DEGs between normal medium and NRRA treatment groups in CD1 background. The up or down-regulated genes (fold change > 2 or < -2) in each cell culture conditions are plotted in red and blue, respectively. (C) The bar plots show GO enrichment analysis data for up and down genes (fold change > 2 or < -2) when NRRA treatment in CD1 background.

fig. S7. Changes in the expression of early meiosis-related genes upon NRRA treatment in primary spermatogonial culture.

(A) Heatmap showing the expression levels of 104 mouse early meiosis-related genes (Soh *et al.*, 2015) with indicated treatments. (B) Heatmap showing the expression levels of 107 human early meiosis-related genes (Guo *et al.*, 2017) with indicated treatments.

fig. S8. Changes of meiosis-related gene expression upon RA treatment in a previously reported scRNA-seq database.

(A) Heatmap showing the expression levels of 165 meiosis-related genes in DMSO and RA treatment groups in testes of postnatal day 5. (B) Violin plots showing representative meiotic genes profiles with DMSO and RA treatments. Please note that *Stra8* is a direct target gene of RA, and *Stra8* expression serves as “a positive control” for the activation of RA signaling upon RA treatment.

fig. S9. 10X genomics quality control in scRNA-seq.

(A) QC metrics. (B) A subset of features that exhibit high cell-to-cell variation in the dataset. (C) An alternative heuristic method generates an ‘Elbow plot’: a ranking of principle components based on the percentage of variance explained by each one (ElbowPlot function).

fig. S10. Expression pattern of marker genes for Cluster 0 – 4 in scRNA-seq.

Violin plot and scatter plot showing the expression of marker genes for undifferentiated spermatogonia (A), differentiating spermatogonia (B), meiocytes (C), and fibroblast (D) in each cluster.

fig. S11. Expression of undifferentiated spermatogonia and meiosis genes in primary spermatogonial culture.

qRT-PCR analysis of undifferentiated spermatogonia marker genes (A) and meiosis genes (B) in primary spermatogonial culture with indicated treatment durations normalized to *β-actin*. Standard deviation from replicate reactions are shown.

fig. S12. GO function and KEGG analysis for Cluster 0 to Cluster 3 in scRNA-seq.

(A) The bubble plots show GO enrichment and KEGG pathway analysis for cluster 0 DEGs. (B) The bubble plots show GO enrichment and KEGG pathway analysis for cluster 1 DEGs. (C) The bubble plots show GO enrichment and KEGG pathway analysis for cluster 2 DEGs. (D) The bubble plots show GO enrichment and KEGG pathway analysis for cluster 3 DEGs.

fig. S13. Analysis of Clusters 0 – 3 and meiotic pathways enriched in GO function terms.

(A-B) Heatmap of “DNA double strand break formation” and “meiotic prophase-specific histone variants” pathway-enriched genes. (C-D) Heatmap (left) and violin plot (right) of the enriched “meiotic nuclear division” and “synapsis” pathway-enriched genes. Violin plots showing the relative percentage expression levels of “meiotic nuclear division” and “synapsis” pathway-enriched genes versus all the genes in each cluster.

fig. S14. Expression of meiotic genes in scRNA-seq.

(A) Violin plot and scatter plot showing the expression of meiotic genes whose expression is fully dependent on STRA8 in each cluster. (B) Violin plot and scatter plot showing the

expression of meiotic genes whose expression is partially dependent on STRA8 in each cluster. (C) Heatmap describing the expression levels of 165 early meiosis-related genes in Cluster 0 – 4 from single cell RNA-seq. Please compare to fig. S8, which shows that RA alone is not sufficient to induce meiosis-related gene expression (except *Stra8* and *Rec8*, which are direct targets of RA).

fig. S15. Analysis of early spermatogenesis to meiotic prophase in a published scRNA-seq database on mouse spermatogenesis.

(A-C) Identification of different stages of early spermatogenesis to meiotic prophase. (D) Pseudo-time analysis of different stages early spermatogenesis to meiotic prophase.

fig. S16. Single-cell trajectories reveal dynamic biological transitions in Cluster 0 – 3 in pseudo-time.

(A) Clusters of genes that were differentially expressed across pseudotime and top GOs. (B) KEGG analysis for Clusters 0 – 3 in pseudotime DEGs.

fig. S17 – S18. Single-cell trajectories reveal dynamic meiotic function changes in Cluster 0 – 3 (C1-C4) in pseudotime.

Expression levels (vertical axis) of key genes for “meiotic cell cycle”, “cohesion”, “chromosome segregation” and “DNA double-strand break formation” are ordered in pseudotime.

fig. S18. Single-cell trajectories reveal dynamic meiotic function changes in Cluster 0 – 3 (C1-C4) in pseudotime.

Expression levels (vertical axis) of key genes for “DNA repair”, “homologous recombination” and “synapsis” are ordered in pseudotime.

fig. S19. *Stra8*-deficient germ cells (C57BL/6XDBA/2 F1 hybrid background) lack meiotic DSB formation *in vivo*.

Immunofluorescent staining for DMC1, MEIOB, and SPATA22 is showed in testicular cross-sections from WT, *Stra8*^{-/-}, *Spo11*^{-/-}, *Stra8*^{-/-};*Spo11*^{-/-} mice (28 days of age). This data indicates

that the essential role for meiotic DSB formation is not genetic background-dependent. Scale bars, 10 μ m.

fig. S20. GO function analysis for upregulated genes in WT and *Stra8*^{-/-} primary spermatogonial cultures.

(A) The bubble plots show GO enrichment analysis for genes upregulated in WT only. (B) The bubble plots show GO enrichment analysis for genes upregulated in both WT and *Stra8*KO. (C) The bubble plots show GO enrichment analysis for genes upregulated in *Stra8*KO only.

fig. S21. GO function analysis for downregulated genes in WT and *Stra8*^{-/-} primary spermatogonial culture

(A) The bubble plots show GO enrichment analysis for genes downregulated in WT only. (B) The bubble plots show GO enrichment analysis for genes downregulated in both WT and *Stra8*KO. (C) The bubble plots show GO enrichment analysis for genes downregulated in *Stra8*KO only.

fig. S22. Cytological analysis of meiotic DSB formation *in vitro*.

(A) RAD51, DMC1, MEIOB, and SPATA22 foci on meiotic chromosomes (SYCP3) were used to identify different stages of meiosis (leptotene-like to zygo-/early-pachytene-like stage). Percentages of leptotene-like to zygo-/early-pachytene-like stage in culture on each day following treatment is shown on the right. (B) Enrichment of the DNA corresponding to several published hotspots by anti-DMC1 ChIP estimated by qPCR. ChIP was performed from juvenile testis extracts (*in vivo*; upper panel) and primary spermatogonial culture following NRRA treatment (*in vitro*; lower panel). Enrichment of the DNA corresponding to genomic regions on Chr17, Chr5, Chr8, Chr11, Chr13, Chr14, and the β -actin gene was estimated by qPCR. Standard deviation from replicate reactions are shown.

fig. S23. Correlation of NR-induced TF genes with representative meiotic genes.

Scatter plots showing correlation of representative TF genes with selected meiotic genes in 165 testis tissues based on the data from Genotype Tissue Expression (GTEx). Note that every dot represents one tissue type. Correlation coefficient (r) and *P* values were calculated by Pearson's

correlation analysis. The expression pattern of each TF genes in scRNA-seq dataset during *in vivo* spermatogenesis is shown on the right.

fig. S24. NR-induced TF genes do not respond to RA treatment *in vivo*.

(A) Heatmap describing the expression levels of RA-responsive genes and NR-induced TF genes in DMSO and RA treatment in testes at day 5 of age (Velte *et al*, 2019). (B) Violin plots showing NR-induced TF genes profiles in DMSO and RA treatment in *in vivo* (Velte *et al*, 2019).

fig. S25. Analysis of metabolic pathways enriched in GO function terms in Clusters 0 – 3.

(A-F) Heatmap (left) and violin plot (right) of the enriched glycolytic process, glycolysis and oxidative phosphorylation related genes. Violin plot showing the relative percentage expression levels of glycolytic process, glucose metabolic process, and oxidative phosphorylation related genes versus all the genes in each cluster. (G-J) Heatmap (upper) and violin plot (lower) of the enriched mitochondrial respiratory chain complex I & IV assembly and mitochondrial organization related genes. Violin plot showing the relative percentage expression levels of mitochondrial respiratory chain complex I & IV assembly and mitochondrial electron transport NADH to ubiquinone related genes versus all the genes in each cluster.

Supplementary Table S8. Medium composition

	SSC medium	Nutrient restriction medium	Meiotic process medium
N2	1 X	1 X	1 X
Pyruvic Acid	200 µg/ml	200 µg/ml	200 µg/ml
D-(+)-glucose	6 mg/ml	6 mg/ml	6 mg/ml
DL-Lactic Acid	1 µl/ml	1 µl/ml	1 µl/ml
BSA	5 mg/ml	5 mg/ml	5 mg/ml
L-Glutamine	2 mM	2 mM	2 mM
2-Mercaptoethanol	5X10 ⁻⁵ M	5X10 ⁻⁵ M	5X10 ⁻⁵ M
MEM Vitamin	1 X	1 X	1 X
NEAA	1 X	1 X	1 X
Ascorbic Acid	10 ⁻⁴ M	10 ⁻⁴ M	10 ⁻⁴ M
d-Biotin	10 µg/ml	10 µg/ml	10 µg/ml
Beta-Estradiol	30 ng/ml	30 ng/ml	30 ng/ml
Human FGF2	10 ng/ml	10 ng/ml	-
Rat GDNF	15 ng/ml	15 ng/ml	-
FBS	10 µl/ml	10 µl/ml	10 µl/ml
KSR	50 µl/ml	50 µl/ml	100 µl/ml
EBSS	-	90% +10%SSC medium	-
RA	-	10 nM	-
TGF beta	-	-	10 ng/ml
Melatonin	-	-	10 ⁻⁷ M
DHT	-	-	10 ⁻⁶ M
BPE	-	-	50 ng/ml
FSH	-	-	200 ng/ml

Supplementary Table S9. Antibodies used in this study

	Company	Catalog number
DMC1	Sigma	HPA001232
SYCP3	Santa	Sc-74569
SPATA22	Proteintech	16989-1-AP
MEIOB	Gift from Jeremy Wang's lab	
RAD51	Millipore	PC-130
γ H2AX	Millipore	05-636
DDX4	Abcam	Ab13840
GFRA1	R&D	AF560
CDH1	BD	610181
AlexaFluor 488-conjugated donkey anti-mouse IgG	ThermoFisher	A-21202
AlexaFluor 546-conjugated donkey anti-rabbit IgG	ThermoFisher	A-10040
AlexaFluor 546-conjugated donkey anti-mouse IgG	ThermoFisher	A-10036
AlexaFluor 647-conjugated donkey anti-rabbit IgG	ThermoFisher	A31573

Supplementary Table S10. Primers used in this study.

	Forward: 5'→3'	Reverse: 5'→3'
<i>Dmc1</i>	ATCGGGATTCCAAGATGATG	TGCAGAGGGCTCTTCTTGTT
<i>Sycp3</i>	ACATGGAAAGAAAAGATCTGCTG	CTGGCTTTGAAAGAAGCTTTG
<i>Spo11</i>	TTGATTGCTGGCAACTTGAG	GAAATGTTGCATCCTTCTCG
<i>Actin</i>	CTGCCGCATCCTCTTCCTC	GCCACAGGATTCCATACCCA
<i>Gfra1</i>	CACTCCTGGATTTGCTGATGT	AGTGTGCGGTACTTGGTGC
<i>Zbtb16</i>	ATAAGTAGATCTGCTTTTCTCCCA	ATGTCGACGTTCTTTCCGCCTCAGAACG
<i>Id4</i>	CAGTGCGATATGAACGACTGC	GACTTTCTTGTTGGGCGGGAT
<i>Pou5f1</i>	CACGAGTGGAAAGCAACTCA	AGATGGTGGTCTGGCTGAAC
<i>Nanos2</i>	TTCTTGGCAGTGACGATCAG	TGAGGTACCTGTCACCCACA
<i>Meiob</i>	GGCCCGGAAGTATCCGATTT	GCCTTTGACATCTGTTTTCCCA
<i>Spata22</i>	GCTGAAGGAGGCGAGGTTTAG	CAATGGCACAGGCAAACAGC

fig. S1

fig. S2

fig. S3

fig. S4

fig. S5

fig. S6

fig. S7

fig. S8

fig. S9

fig. S10

fig. S11

A

B

fig S12A

Cluster 0

fig S12B

fig. S12C

fig. S12D

Cluster 3

Fig. S13

fig. S14

fig. S15

fig. S16

fig. S17

fig. S18

fig. S19

fig. S20

GO_terms_BP (Genes upregulated in WT only)

GO_terms_BP (Genes upregulated in both WT and Stra8KO)

GO_terms_BP (Genes upregulated in Stra8KO)

fig. S21

fig. S22

fig. S23

fig. S24

fig. S25

Figures

Figure 1

NR is required to induce meiotic initiation with RA in primary spermatogonial culture. (A) Bright-field images of cultured spermatogonia with indicated treatments for 2 days. Scale bars, 50 μ m. (B) Relative expression of *Spo11*, *Dmc1*, and *Sycp3* against *Gapdh* analyzed by qRT-PCR in cultured spermatogonia with indicated treatments for 2 days. Results shown represent mean $\pm$ SEM. * $P < 0.05$. $n = 3$ independent cultures. n.s., no significant. (C) Immunostaining for γ H2AX (red) and DAPI (blue) with indicated treatment for 2 days. Scale bars, 10 μ m. (D) Immunostaining for DMC1 (red), DDX4 (green), and DAPI

(blue) in Spo11^{+/+} and Spo11^{-/-} 407 primary spermatogonial culture. Scale bars, 10 μ m. (E) (Left) UHC and heatmap of gene expression in cultured primary spermatogonia with indicated treatments for 2 days. (Right) Top GO enrichments with representative genes in each cluster. (F) GSVA analysis for indicated treatments. In the heatmap, rows are defined by the selected gene sets, and columns by consensus scores for each treatment. Group enriched gene sets are highlighted by different color. (G) Bar plot shows p-value of GO functions between RA and NRRA treatment. (H) UHC and heatmap for the expression of 165 early meiosis-associated genes. STRA8-dependent genes characterized in a previous study in ref. 2 are shown in red.

Figure 2

scRNA-seq analysis of primary spermatogonial culture during NRRA-induced meiotic initiation and progression. (A) Workflow of scRNA-seq experiment. Number of cells collected: 6,884 (0 day), 6,936 (2 day), 6,200 (3 day), and 5,587 (4 day). (B) A t-distributed Stochastic Neighbor Embedding (tSNE) plot for analyzed cells. Cluster 0 – 3 were germ cells and Cluster 4 was somatic cells. Number of cells selected for analysis: Cluster 0 (6,487 cells), Cluster 1 (5,286 cells), Cluster 2 (3,860 cells), Cluster 3 (2,170 cells),

Cluster 4 (285 cells). (C) Violin plots showing the expression level of representative genes in each cluster. (D) A bar plot showing the proportion of the different cell clusters at different time points. (E) A heatmap showing the expression of marker genes and GO functions in each cluster. (F) Gene expression patterns of indicated genes on tSNE plots and violin plots. (G) PCA analysis showing the relationship between single cell clusters from NRRA-treated culture in vitro (n = 4 clusters) and early mouse spermatogenesis in vivo (n = 6 clusters) based on the transcriptional profiles of commonly expressed genes (n = 7,803 genes). (H) Hierarchical clustering showing the relationship between single cell clusters from NRRA-treated culture in vitro and early mouse spermatogenesis in vivo. (I) Scatter plots comparing marker genes expression profile between in vitro clusters and in vivo clusters. (J) (Left) Heatmap of sex chromosome genes in Clusters 0 – 3. (Right). Violin plots showing percentage of X and Y chromosome genes profiles in Clusters 0 – 3. The median is shown by a red line. (K) Scatter plots showing cells along the projected pseudo-time in Cluster 0 – 3 (upper) and in indicated clusters from early mouse spermatogenesis in vivo (lower)

Figure 3

Stra8 is required for NRRA-induced meiotic initiation. (A) Immunostaining for DMC1 (red), DDX4 (green), and DAPI (blue) in Stra8^{+/+} and Stra8^{-/-} primary spermatogonial culture. Scale bars, 10 μ m. (B) (Left) UHC and heatmap of gene expression in cultured primary spermatogonia with indicated genotypes for 2 days. (Right) Top GO enrichments with representative genes in each cluster. (C) GSVA analysis for indicated genotypes. In the heatmap, rows are defined by the selected gene sets, and columns by consensus scores for each genotype. Group enriched gene sets are highlighted by different color. (D and E) Venn plots with top GO enrichments and representative genes for NRRA-upregulated (D) and -downregulated (E) genes in Stra8^{+/+} and Stra8^{-/-} cultures.

Figure 4

Number and distribution of meiotic DSB formation in vitro. (A) Representative chromosome spreads stained by RAD51, DMC1, MEIOB, SPATA22, and SYCP3 from juvenile mice (day 14 of age) or primary spermatogonial culture following NRRA induced meiotic initiation and progression are shown. (B) Quantification of foci for zygonema early pachynema stage. Error bars, mean $\pm$ SD. Each solid circle indicates the total number of foci from a single nucleus. Total number of cells quantified from three independent cultures are shown on each graph. n.s., not significant. (C) Immunofluorescence staining for SYCP3 and PRDM9 on chromosome spreads in testicular germ cells from juvenile mice (day 14 of age)

or in cells from primary spermatogonial culture following NRRA-induced meiotic initiation and progression. Scale bars, 10 μ m. (D) Enrichment of the DNA corresponding to several published hotspots by anti-DMC1 ChIP estimated by qPCR. ChIP was performed from primary spermatogonial culture following NRRA-induced meiotic initiation and progression. Enrichment of the DNA corresponding to genomic regions on Chr17, Chr5, Chr8, Chr11, Chr13, Chr14, and the b-actin gene was estimated by qPCR. Standard deviation from duplicate reactions are shown.

Figure 5

Nutrient restriction induces a network of TF genes involved in early meiosis. (A) A workflow showing filters to identify genes of interest. (B) Heatmap of 120 TF genes upregulated by nutrient restriction. 11 identified TF genes involved in early meiosis are shown on the right. (C) Gene expression patterns of 3 representative TF genes during early spermatogenesis from a published scRNA-seq database 24. (D) GTEx database showing correlation of representative TF genes (Sohlh1, Pou2f2, Bcl11a) with selected meiotic genes in 165 testis samples. (E) Schematics of nutrient restriction in combination with retinoic acid in inducing meiotic gene programs. Nutrient restriction alone is sufficient to activate a class of meiotic genes that do not require retinoic acid (e.g., Rad21l). Retinoic acid alone is sufficient to activate Rec8, an essential component of cohesion, and Stra8. Nutrient restriction and retinoic acid synergistically induce key meiotic genes for synapsis and recombination.