A Role for Advanced Germ Cells in the Control of the Spermatogenic Wave

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Précis

Spermatogenesis occurs within the testis and is the process by which males produce viable sperm. This process involves the proliferation of spermatogonial stem cells and their subsequent differentiation and progressive maturation into sperm, resulting in male fertility. The timing of differentiation within the testis is an important aspect of spermatogenesis, and disturbing this timing can reduce the fertility of a male throughout its reproductive lifespan. Vitamin A (retinol, ROL) is required for successful spermatogenesis. Animals deficient in vitamin A (VAD) are infertile and do not contain any differentiated germ cells within their gonads. Recent reports have shown that active vitamin A, or retinoic acid (RA), is required for the differentiation of germ cells within the gonad, and acts to establish the pattern of germ cell differentiation seen in the normal testis. By exposing newborn male mice to a single, large dose of RA by injection at 2 days after birth, it is possible to change the pattern of spermatogenesis within the male gonad. This change in the pattern of differentiating germ cells is characterized by non-uniform production of sperm over time. Spermatogenesis in mice is a process that releases mature sperm from the testes every 8.6 days. In animals with a change in their spermatogenesis induced by RA treatment, sperm counts are higher during a two day window, and lower during the rest of this 8.6 day cycle. This change in number of sperm is uncharacteristic of males which are constantly fertile, and is detrimental to reproductive fitness. However, treatment of adult male mice with a single pulse of RA does not change their pattern of spermatogenesis. With these results we hypothesize that there is some ability of adult males to prevent a change in the timing of their spermatogenesis which is not yet established in neonatal males. To test this hypothesis animals were treated with RA at 4, 6, and

8 days post-partum (dpp) and analyzed for any change in spermatogenesis. Experiments revealed that the onset of this control of spermatogenic timing occurred between 4 and 8 dpp. This timing also correlates with the appearance of a more advanced germ cell population entering meiosis in the neonatal testis. Results indicate that this advanced germ cell population may somehow solidify the timing of spermatogenesis, making the testis and its maturing germ cells less susceptible to inappropriate activation of germ cell differentiation, thereby preserving constant male fertility.

With this preliminary data indicating that advanced germ cells somehow control the timing of germ cell differentiation within the testis, further studies are necessary to determine the mechanism by which this control is exerted. It would be of interest to determine if this mechanism of regulating differentiation has any homologous functions in other tissues with adult stem cell populations.

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I. Introduction

Spermatogenesis is the process by which the undifferentiated germ cells of the male gonad, spermatogonia, undergo proliferation and differentiation resulting in the formation of haploid spermatozoa. This process occurs within the seminiferous tubules of the testis and ultimately results in fertile males. Differentiation of spermatogonia is controlled both temporally and spatially throughout the testis. The transition of undifferentiated spermatogonia present in syncytia, or connected chains, termed A aligned (A_{al}) spermatogonia, into differentiated type A_1 spermatogonia occurs approximately every 8.6 days at a single point along the seminiferous tubules.[1] As germ cells differentiate, enter meiosis, and mature after meiotic division they are progressively moved from the basement membrane (toward the outside of each seminiferous tubule) toward the lumen (inside) of the seminiferous epithelium. These movements through the seminiferous epithelium, in concert with the recurring differentiation of spermatogonia, give rise to well-characterized, constantly cycling associations of germ cells in the adult testis.[2] Twelve of these associations have been identified in the murine gonad,[1] termed the stages of the cycle of the seminiferous epithelium (Fig. 1a). The spatial distribution of these stages along the length of the seminiferous tubules has been described as the spermatogenic wave, referring to the cycling of stages present along the length of the seminiferous tubules over time.[2] (Fig. 1b.) In the overall structure of the testis, this distribution gives rise to a full representation of all spermatogenic stages at any given point in time, resulting in constantly fertile males. Spermatozoa are being released at all times into the lumen of the seminiferous tubules at many different points throughout the testis.

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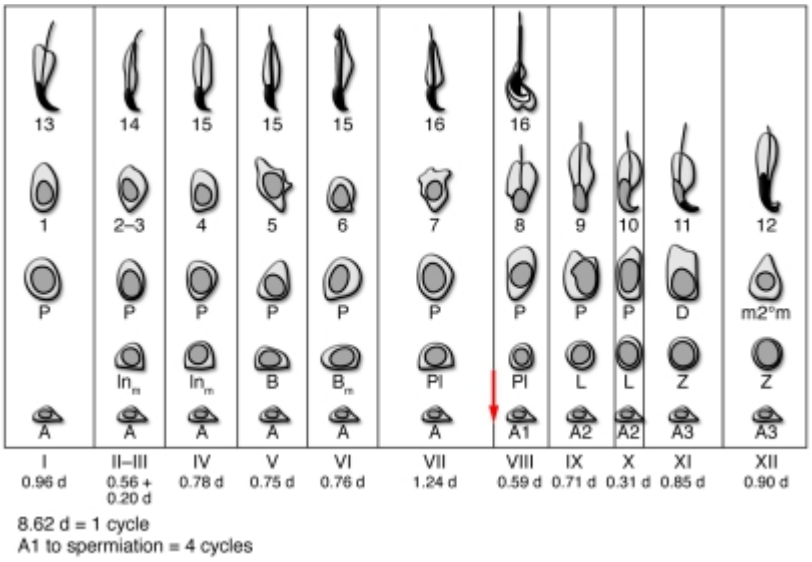

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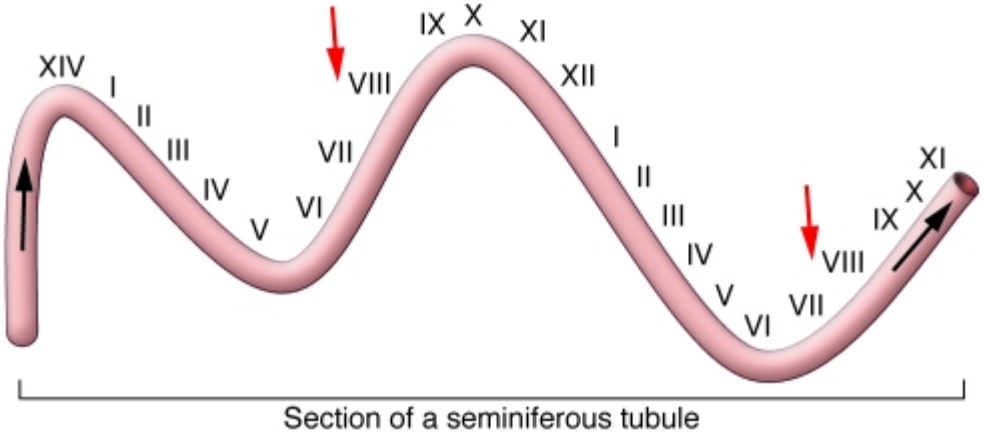


Fig. 1 The Cycle of the Seminiferous Epithelium and the Spermatogenic Wave

A) In the mouse there are twelve characterized stages of spermatogenesis (I-XII). Each of these stages is defined by the associations of differently progressed germ cells, organized from least to most developed from the basement membrane to the lumen, respectively. B) The spermatogenic wave is three-dimensional distribution of these stages along the length of the seminiferous tubules at a single point in time. Red arrows denote the stage at which spermatogonial differentiation occurs. Figures taken from Hogarth and Griswold, 2010.[3]

Differentiation of A_{al} to A_1 spermatogonia is dependent upon the presence of vitamin A, or retinol (ROL). This was first demonstrated through examination of vitamin A deficient (VAD)

male rats that completely lacked advanced germ cells in the seminiferous epithelium.[3] This same phenotype was later confirmed in VAD mice.[4] Further examination of the immature spermatogonia in VAD seminiferous tubules revealed that few, if any differentiated spermatogonia are present in the seminiferous epithelium of these animals. This indicates that ROL is required for the process of spermatogonial differentiation. Spermatogenesis can be rescued in these animals by re-introducing ROL or its active metabolite, retinoic acid (RA) into their diets.[5,5] However, after spermatogenesis has recovered in an animal after a VAD state, the spermatogenic wave is no longer present, as the full representation of the stages of the seminiferous epithelium has been ablated. A fraction of stages are represented in abnormally high frequency in these VAD-rescued animals and their spermatogenesis can be described as "synchronous", referring to the identical timing of germ cell progression throughout the gonad.[6] This phenotype results in changing levels of spermatozoa released into the lumen over the 8.6 day cycle of the seminiferous epithelium in mice, changing the fertility of an individual over this period (Fig. 2). It is for this reason that preservation of the spermatogenic wave is paramount to the successful maintenance of male fertility, as the preservation of asynchronous differentiation ensures a constant supply of viable spermatozoa at all times during the reproductive lifetime of males.

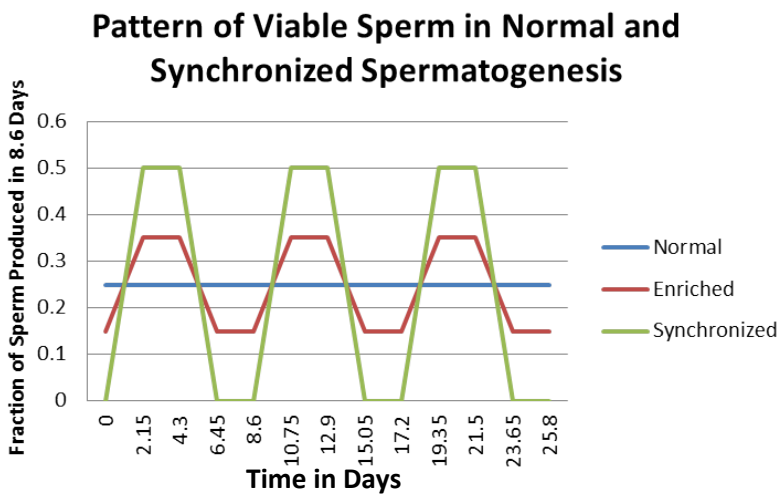


Fig. 2 A Graphical Representation of Sperm Production in Normal and Synchronized Spermatogenesis

Patterns of functional sperm production in normal and synchronized spermatogenesis. Normal spermatogenesis in which all stages of the cycle of the seminiferous epithelium are present at any given time results in a stable number of mature spermatozoa produced over the 8.6 day spermatogenic cycle in the mouse (blue). Enriched spermatogenesis (red) results in periodic over and under production of spermatozoa. Synchronized spermatogenesis (green) results in a recurring pulse of spermatozoa production for a portion of the cycle of the seminiferous epithelium and an absence of sperm produced for the rest of the cycle. This over and under accumulation of sperm results in a highly variable degree of fertility during an 8.6 day period.

The transition of A_{al} to A_1 spermatogonia has been associated with the expression of several genes including *Stra8* and *Kit*,[7,8] and loss of markers of undifferentiated spermatogonia, including *Plzf*,[9] a previously demonstrated repressor of *Kit* transcription,[10] and *Oct4*.[11] Both *Stra8* and *Kit* are known to be RA-responsive, showing increased expression at 8 and 24 hours after treatment of cultured testes *in vivo*.[12] Cultured germ cells treated with RA *in vitro* also result in a higher number of differentiated spermatogonia at these time points, indicating that RA is an essential component of spermatogonial differentiation both *in vitro* and *in vivo*.[7] RA is absent in the cords of the fetal testis, presumeably due to constitutive expression of the RA

degrading enzyme CYP26B1, and it is not until after birth that detectable levels of RA accumulate within the male gonad.[13] We have recently demonstrated that RA is responsible for establishing the pattern of spermatogonial differentiation in the neonatal gonad, as the first germ cells that are capable of responding to RA are the first cells to undergo differentiation. [8] This pattern of neonatal spermatogonial differentiation is preserved into adulthood, resulting in the previously described spermatogenic wave, and constant sperm production. It appears this pattern is established by local accumulation of RA in germ cells of the seminiferous epithelium.[8] Altering RA availability in the vitamin A sufficient (VAS) neonatal testis at 2 dpp, prior to significant RA accumulation, results in a wave of apoptosis in a subset of spermatogonia followed by synchronous initiation of spermatogonial differentiation. Long term analysis of vitamin A sufficient (VAS) males treated at 2 dpp with a single injection of RA reveals that established spermatogenesis is synchronous in the adult animal[14] This was the first study demonstrating perturbation of the asynchronous differentiation pattern of spermatogonia within a VAS testis without first depleting retinoid stores. This finding allows for the study of a synchronized model of spermatogenesis from its onset in the neonatal testis, whereas the VAD-rescue paradigm only allows for study of adult testes due to the time required to make an animal completely VAD before rescue of spermatogenesis.[3] More recently it has also been demonstrated that inhibiting aldehyde dehydrogenases within the testis results in a VAD-like state by blocking RA formation from retinaldehydes (the intermediary between ROL and RA) and RA-rescue in this paradigm also results in synchronous spermatogenesis,[15] supporting a model in which it is the RA moiety of retinoids in particular which is necessary for the differentiation of spermatogonia in the testis. While it is possible to ablate the spermatogenic

wave in a neonatal VAS testis with a single pulse of RA, it is not possible to reset the timing of differentiation in the VAS adult with an RA pulse.[14] While RA is still necessary for germ cells to successfully undergo maturation in the adult testis, it is not sufficient to alter the timing of spermatogonial differentiation. Recent work from other groups also indicates that control of RA and its metabolism is intricately linked to proper timing and maintenance of the spermatogenic wave.[16] This change in spermatogonial response to RA with age indicates that there is a mechanism to maintain timing of differentiation in the germ cells of adult mice which is not yet functional in the early neonatal mouse, and may serve a yet unexplored role in preservation of appropriate timing of spermatogonial differentiation in the mature male gonad.

In the testis, advanced germ cells are not present within the seminiferous epithelium immediately after birth. This is due to a lack of differentiating germ cells during embryonic development. As early as 3 dpp, the process of spermatogonial differentiation is evident within the cords of the testis. This process is detectable by the presence of proteins marking differentiating spermatogonia including KIT and STRA8. Recent work has demonstrated that this lack of differentiation in the prenatal male gonad is maintained through the high expression of *Cyp26b1.* By inhibition of RA accumulation within germ cells, germ cell differentiation is not achievable. Animals which are lacking this regulation of RA in the prenatal male gonad do have differentiation of germ cells prior to birth. This was determined through studies of animals which are lacking the *Cyp26b1* gene within the gonad.[13] Because RA is not present at detectable levels within the early male gonad, differentiation of spermatogonia does not occur prior to birth. This results in a gonad without an established pattern of differentiation in the neonatal male. As spermatogonia undergo subsequent steps of differentiation, the seminiferous

epithelium becomes host to a progressively larger number of germ cell populations during the first month of life. At approximately 35 dpp, males have begun to produce mature spermatozoa within the seminiferous epithelium. During this first month of life, the cellular composition of the male gonad is constantly changing. This changing environment within the male gonad offers a potential explanation for the difference in resulting spermatogenesis in response to RA treatment between neonatal and adult males.

It is with these recent studies in mind that we hypothesize the presence of advanced germ cells in the developing and adult testis are responsible for the maintenance of the spermatogenic wave, either by direct signaling to the undifferentiated spermatogonia population, or by acting through the somatic Sertoli cell population. These cells largely determine the microenvironment of developing germ cells. By characterizing changes in the spermatogenic wave in response to RA-treatment at multiple neonatal time points, we propose to elucidate at what point synchronous spermatogenesis is no longer inducible with a single RA treatment. Using this information we can identify what changes are occurring within the neonatal testis at this time point which may be related to the developing ability to control the spermatogenic wave in response to ectopic RA signaling within the seminiferous epithelium, ensuring that males are constantly fertile. RA metabolism and degradation is a cycling process within the seminiferous epithelium occurring in concert between multiple cell types, appearing to be synergistic in nature.[16] Maintaining the timing of RA-dependent events, including spermatogonial differentiation, in the cycle of the seminiferous epithelium is likely crucial for proper spermatogenesis. Mechanisms preserving this timing are important in male fertility and thus worth studying to better understand the patterning and establishment of spermatogenesis.

II. Materials and Methods

Neonatal Treatment of Animals

Animals were treated in accordance with WSU IACUC regulations, using the Griswold laboratory animal protocol 01519. Using a dose-response curve, optimal treatment doses of RA were determined in order to induce maximal protein response with minimal dose of RA. This was performed by treating males with varying amounts of RA, waiting 24 hours, and measuring gene expression and protein detectability for each treatment. Doses were 100 µg of RA for animals aged 4 days and 150 µg RA for both 6 and 8 dpp. Each bolus of RA was suspended in 10 µL of dimethyl-sulfoxide (DMSO). Control animals were treated at each time point with 10 µL of vehicle only. Sub-cutaneous injections for animals at 4 dpp were performed along the dorsal midline. Animals at 6 and 8 dpp were treated with an intra-peritoneal injection of RA on the left side of the abdominal cavity.

Collection and Fixation of Testes

Animals were humanely sacrificed using methods suggested by the Washington State University Institutional Animal Care and use Committee (IACUC). After sacrifice, testes were removed from the lower body cavity and fixed either in Bouin's fixative, 4% paraformaldehyde (pH = 7.4), or flash frozen on dry ice for Real Time-RT-PCR quantification of gene expression. Preserved testes for histological evaluation were then dehydrated and embedded in paraffin

wax. Cross sections of tissue were prepared 4 µm think and adhered to glass slides for analysis. In analyses requiring more than one cross-section of testis, at least 50 µm separated cross-sections analyzed in order to ensure accurate representation of the entire seminiferous epithelium during histological examination.

Histological Analysis of Spermatogenesis

Normal spermatogenesis has been described previously, including the frequencies of stages represented in the 129 inbred mouse line.[18] Using these values for comparison, we calculated the "synchrony factor" associated with the frequencies of stages represented in the seminiferous epithelium as previously described. [17]

Immunohistochemistry

4 µm sections of tissue were prepared as previously described and fixed to glass slides. Sections were deparaffinized in xylene and rehydrated. Sections underwent antigen retrieval using sodium citrate, bringing the buffer to a rolling boil for seven and a half minutes in a microwave. Sections were then treated with 3% hydrogen peroxide to neutralize endogenous peroxidases. Antibodies were used at a dilution from 1:1000 to 1:5000. Biotinylated secondary antibodies were used to recognize primary staining. Horse radish peroxidase (HRP) conjugated to streptavidin was incubated on sections, and a colorimetric reaction using diaminobenzoate (DAB) was performed after washing the tissue of excess reagent. Sections were counter-stained using Harris Hematoxylin Stain, dehydrated, and mounted.

Analysis of Apoptosis using TUNEL labeling

PFA fixed tissue was fixed to glass slides in 4 µm sections and labeled for cells actively undergoing apoptosis using the DEADEND TUNEL labeling system, which detects apoptotic (dying) cells by fluorescently labeling sheared DNA, following the protocol optimized by Bio-Rad. Sections were counter-stained with DAPI to visualize cell nuclei and observed under a fluorescent microscope. Quantification of apoptosis was performed by calculating the average number of TUNEL-positive cells per tubule cross section.

Statistical Analysis

All statistical analysis was performed using the JMP software package. Statistical significance for experimental results was determined using a student's t test with a p-value cutoff of 0.05.

III. Results

Analysis of Synchronized Spermatogenesis in Adults

Time Points for Analysis

Response to RA was first characterized in animals treated at 8 dpp. This time point corresponds to

the first entry of germ cells into meiosis in the male gonad. Germ cells undergoing meiosis are no longer termed spermatogonia, but spermatocytes. The earliest spermatocytes within the seminiferous epithelium are preleptotene germ cells. In the organization of the seminiferous epithelium, preleptotenes are present in the seminiferous epithelium at the same stages as differentiating spermatogonia and are known to express several RA-dependent genes including *c-kit* and *Stra8.*[19]

Preleptotenes are the advanced germ cell population closest in proximity to the differentiating spermatogonia located along the basement membrane of the seminiferous cords. 8 dpp was chosen as the first treatment age because if a developing germ cell population plays a role in the maintenance of the spermatogenic wave by regulating the timing of spermatogonial differentiation, the preleptotene population would be well suited to do so. 8 days post-partum (dpp) is the earliest time point when the majority of seminiferous tubule cross sections contains spermatocytes,[18] and thus would theoretically be under the control of advanced germ cells for maintaining the timing of spermatogonial differentiation. We hypothesize that if an advanced germ cell population exerts control on the timing of spermatogonial differentiation, RA treatment will no longer induce synchronous spermatogenesis at the same age when that germ cell population is present within the seminiferous epithelium. If this hypothesis is correct and preleptotene spermatocytes are the advanced germ cell population controlling the timing of differentiation, RA treatment at 8 dpp will not result in synchronous spermatogenesis.

8 dpp Treatment

Animals were first treated via intraperitoneal injection of either RA or vehicle at 8 dpp.

Doses were determined by dose-response studies, ensuring maximal response to treatment with minimal doses. 8 dpp males were treated either with 150 µg of RA dissolved in 10 µL of vehicle, or vehicle only. Neonatal males were allowed to recover for 30 days and sacrificed at 38 dpp. Cross sections of testes were then examined for the frequency of the most advanced germ cells present in seminiferous tubules, in order to determine if RA treatment at 8 dpp disturbs the pattern of spermatogenesis in the male gonad. There was no significant change in advanced germ cell frequency between animals treated with RA or vehicle. These data confirmed the selected time points for neonatal male treatment – 4, 6, and 8 dpp as the oldest treated age did not appear to result in a change of the temporal distribution of spermatogonial differentiation with a single pulse of RA.

Animals were again treated with RA or vehicle and allowed to recover to adulthood (65 dpp) before sacrifice. Cross-sections of adult male testes treated with either RA or vehicle were then analyzed for the distribution of all twelve stages of the spermatogenic cycle in at least 200 tubules for each sample. The frequency of stages represented in a testis cross-section is constant because the process of spermatogenesis is static in its timing. By counting the number of stages represented, the degree of spermatogenic "synchrony" can be interpreted by comparison to previously published stage frequencies in several inbred strains of mice. The degree of synchrony can be quantified through the calculation of a synchrony factor, utilizing these frequency values.[17] A calculated synchrony factor is useful to describe the pattern of spermatogenesis in males and if the pattern of their sperm production has been altered with treatment. Upon quantification there was no significant change in synchrony factor between animals treated with RA or vehicle (Fig. 3g, h). Synchrony factor values averaged approximately

1.4 for both vehicle and RA treatment. This deviation from 1.0, which would be considered completely normal, can be explained by the strain of inbred males used for these experiments, which were derived from a cross between 129 and C57-bl6 mice continually bred against the 129 strain. Different strains have altered timing in the progression through the stages of the seminiferous epithelium. Mixing strains has most likely altered the exact timing of each stage during spermatogenesis for this model. However, when determining any change in the timing of spermatogenesis, it is the change in synchrony factor between treatments, and not the absolute value of synchrony factor which is most informative

6 dpp Treatment

The second treatment age analyzed was 6 dpp. Animals at 6 dpp were also treated with 150 µg of RA or vehicle only and allowed to recover to adulthood at 65 dpp before sacrifice and histological analysis of their testes was performed. Cross-sections were again analyzed and average synchrony factors resulting from treatment were calculated for both vehicle and RA treated animals. There was a significant change in the average synchrony factor of testes from animals treated with RA when compared to those treated with vehicle (Fig. 3e, f). Previous work has established that a synchrony factor over 2.5 is significantly synchronized and suitable for studying the cyclical changing of gene expression on the global scale within the testis.[6] Average synchrony factors calculated for RA treated individuals were barely below this threshold. There was a significant perturbation in stage frequencies, with approximately double the number of stages represented within cluster 2 (stages II-VI) and a significantly lower representation of

clusters 1 and 4, corresponding to stages IX-I (Fig. 1a). This enrichment in a subset of stages indicates that at 6 dpp it is still possible to disturb the pattern of spermatogonial differentiation with a single treatment of RA. However, this disturbance is incomplete. This time point likely falls in the window of the changing response of germ cells to exogenous RA.

4 dpp Treatment

The final treatment age analyzed after recovery into adulthood was 4 dpp. Animals were allowed to recover to 65 dpp as previously performed at 8 and 6 dpp. Animals aged 4 dpp were treated with 100 µg of RA or vehicle only. Histological analysis and quantification of synchrony factor revealed that RA treatment at 4 dpp results in significantly synchronized spermatogenesis, with synchrony factors averaging at approximately 4.5. For all time points analyzed, animals treated with vehicle resulted in similar synchrony factors, just below 1.5 (Fig. 3c, d). This value of 4.5 closely resembles our previously published synchrony factor values for animals treated at 2 dpp,[14] which has been included in Fig. 3 to give more context to the data collected in this experiment (Fig. 3a, b). Every sample collected for this time point, as well as animals treated at 6 and 8 dpp, show similar patterning.

In summary, a single treatment with RA at 4 dpp resulted in ablation of the spermatogenic wave after recovery to adulthood in male mice, and testes of RA treated animals are considered strongly synchronized. Those treated at 6 dpp and allowed to recover resulted in a disturbance of the spermatogenic wave, but all stages were still moderately represented in cross-sections of adult testes, though most were at lower than normal levels. Animals treated at 8 dpp displayed no significant change in the distribution of stages after recovery to adulthood.

These data indicate that the ability of the murine testis to maintain the timing of spermatogenesis is gained early on in development. These results also implicate the appearance of preleptotene spermatocytes in the timing of this ability, as the appearance of these cells in the seminiferous epithelium and the ability of RA to change the patterning of spermatogenesis are inversely correlated.

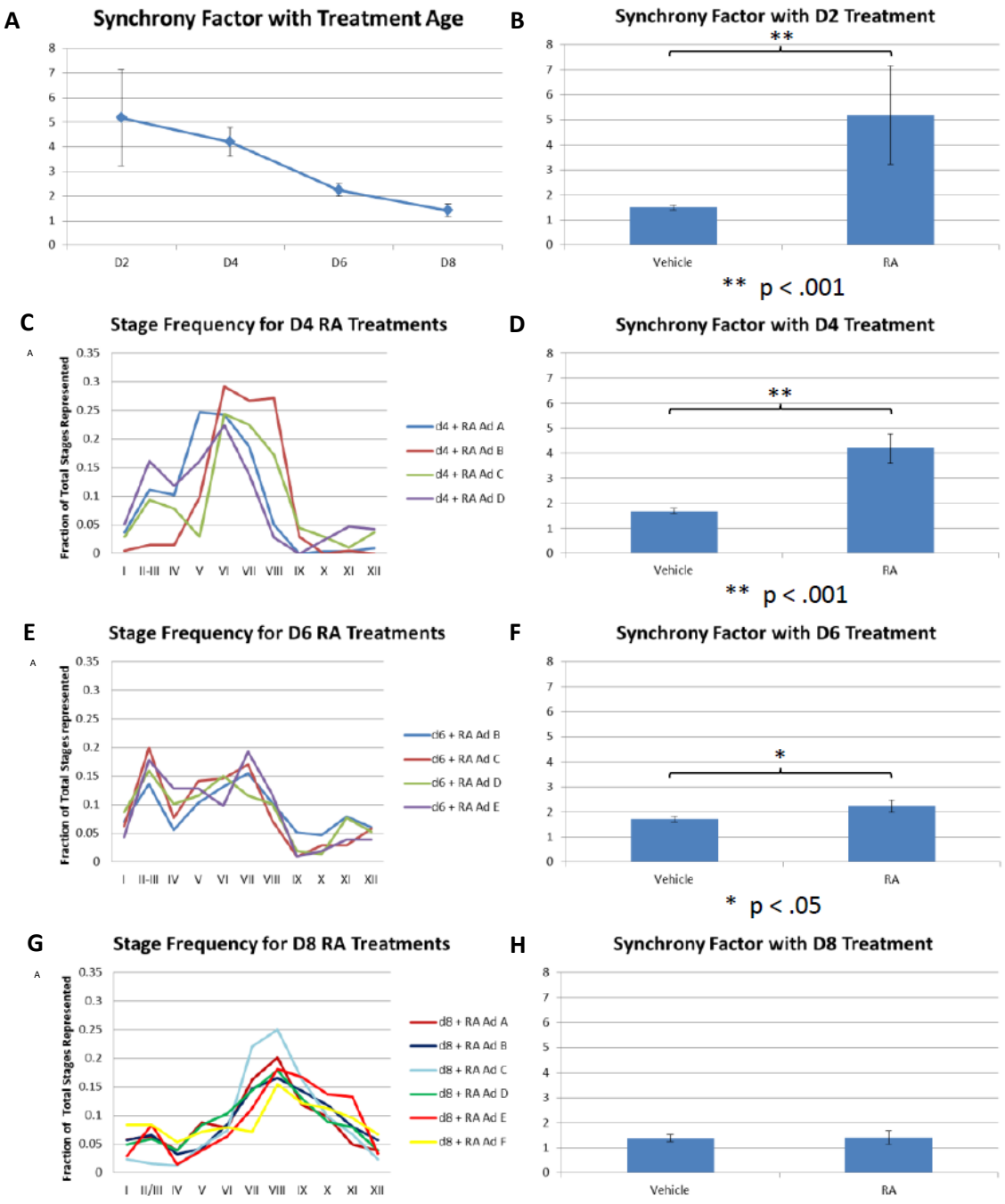


Fig. 3 Results from Long Term RA Treatments at Multiple Neonatal Time Points

Stage Frequency analysis of animals treated at 4, 6, and 8 dpp. A) Stage frequencies of adult testes from animals treated with RA at 4 dpp. B) Calculated Synchrony factors for animals treated at 4 dpp with either vehicle or RA. RA treated animals are synchronized at adulthood. C) Stage frequencies of testes from adults treated at 6 dpp with RA. D) Calculated synchrony factors for adults treated with vehicle or RA at 6 dpp. Adult testes are enriched but not synchronized. E) Stage frequencies for animals treated at 8 dpp with RA. F) Calculated synchrony factors for adults treated at 8 dpp with either vehicle or RA (no significant change).

Short Term Effects of RA Treatment

In order to gain insight into the changing response to RA in the developing gonad, animals treated at 4, 6, and 8 dpp were sacrificed at 24, 48, and 72 hours post-treatment. Germ cells in testis cross sections were examined for the marker of differentiation, STRA8 as well as rapidly degrading chromatin, an indicator of apoptosis, or cell suicide. Our initial report of RA-induced synchronous spermatogenesis included a wave of apoptosis approximately 48 hours post-treatment, followed by re-initiation of synchronous spermatogenesis. Any change in this profile may indicate how the maintenance of spermatogenic patterning is undergone.

8 dpp Treatment

Twenty four hours post-treatment, nearly every germ cell present within a testis cross section was immunopositive for STRA8 protein (Fig. 4a) in contrast to sections from animals treated with vehicle (Fig. 4b). Within 48 hours, the number of STRA8-immunopositive cells in testis cross-sections were identical between treatments (Fig. 4c,d) and this pattern continued through 72 hours post-treatment (Fig. 4e,f). It appears that when a single treatment of RA is no longer able to disturb the pattern of spermatogonial differentiation and as a result the pattern of spermatogenesis in the male gonad, the appropriate timing of differentiation is restored to the spermatogonia population within 48 hours post RA-treatment. This response is different from short term analysis of animals treated at 2 dpp when synchrony is achievable with RA treatment, as these animals contain almost no STRA8-positive germ cells within the seminiferous epithelium at 48 and 72 hours post-treatment.[14]

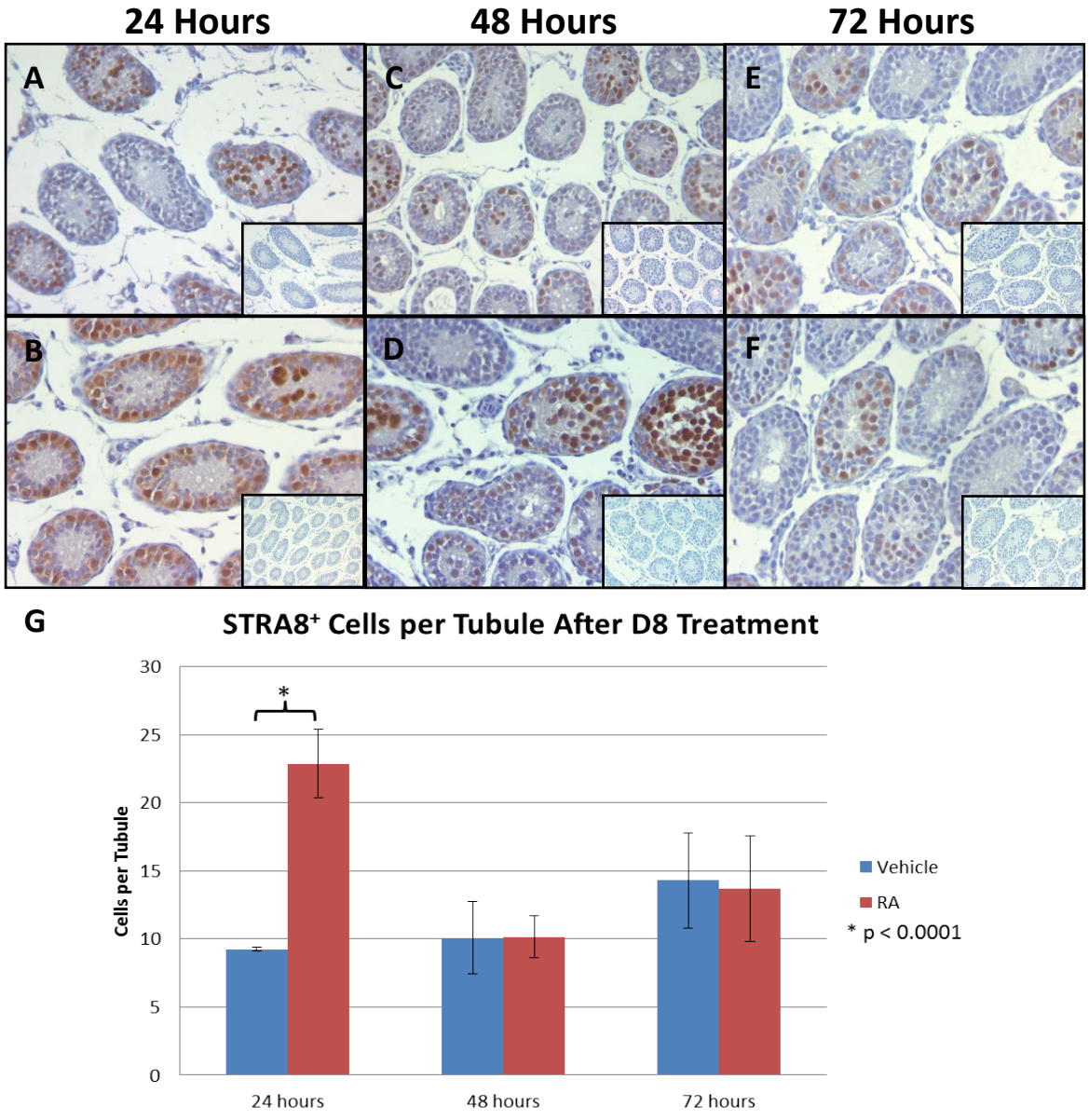
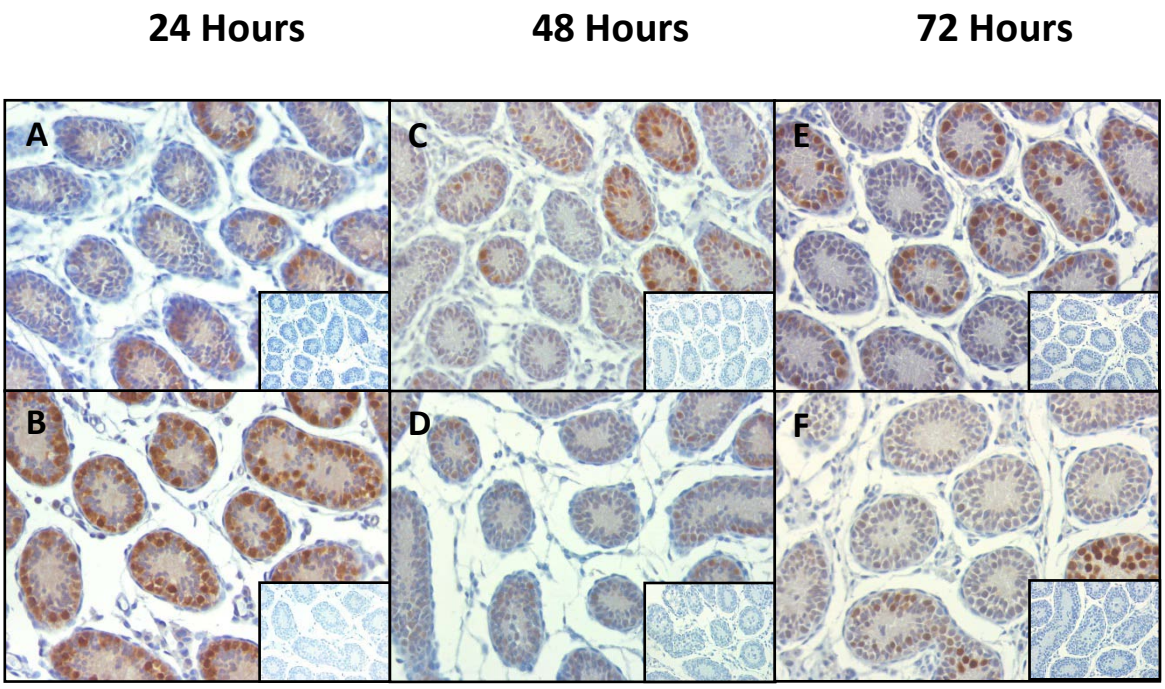


Fig. 4 STRA8 Immunohistochemistry after Treatment at 8 dpp

Immunohistochemical detection of STRA8 protein following RA treatment of 8 dpp males. Animals were allowed 24, 48 , and 72 hours to recover after treatment before sacrifice. A) Cross sections of vehicle treated animals at twenty four hours post-injection show heterogeneous distribution of STRA8 immunopositive cells (stained brown) (negative control inset) at 20, 10x magnification. B) In contrast, nearly every germ cell in RA treated animals at 24 hours post-injection stain positive for STRA8 protein. C) Testis cross section of animal treated with vehicle after 48 hours. D) The distribution and number of immunopositive cells at 48 hours post RA-treatment is identical to the pattern in vehicle treated animals. E) Vehicle treated animal after 72 hours. F) The distribution of STRA8 immunopositive germ cells continues to match that of vehicle treated animals after 72 hours. G) Quantification of cells immunopositive for STRA8 per tubule at each time point for each treatment. Only at 24 hours post-injection is the difference in STRA8 positive cells statistically different between treatments.

6 dpp Treatment

Animals were next injected at 6 dpp and again allowed to recover for 24, 48, and 72 hours after treatment with either RA or vehicle alone. 24 hour response to RA was detected with significantly higher ($p < 0.05$) numbers of STRA8 immunopositive spermatogonia in each seminiferous cord when compared with vehicle treated animals (Fig. 5a,b). At 48 hours post-treatment, STRA8 immunopositive cells in each tubule match the number of cells in vehicle treated animals, similar to results obtained for 8 dpp injected males (Fig. 5c,d). However, at 72 hours post-injection there is a significantly lower number of spermatogonia immunopositive for STRA8 in testes from RA-treated animals when compared to those of vehicle-treated samples (Fig. 5e,f) ($p < 0.05$).



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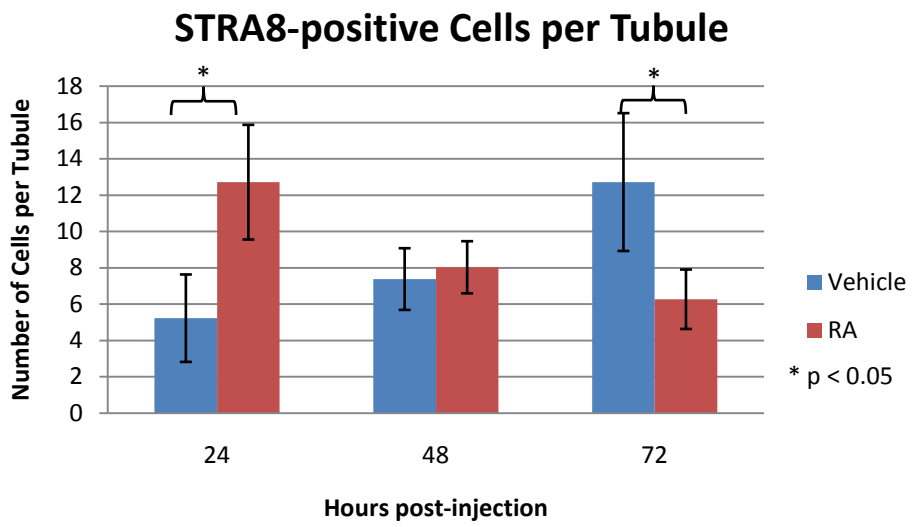


Fig. 5 STRA8 Immunohistochemistry after Treatment at 6 dpp

Immunohistochemical detection of STRA8 protein in germ cells after 6 dpp Treatment. A) Cross section of testis from vehicle treated animal 24 hours post-injection stained for STRA8 protein (brown) and negative control (inset) at 20x, 10x. B) Cross section of testis from RA-treated animal 24 hours-post injection. C) Vehicle treated animal after 48 hour recovery. D) RA treated animal after 48 hours. E) Vehicle treated animal after 72 hours. F) RA treated animal after 72 hours. G) STRA8 immunopositive cells per tubule at each time point and treatment. RA treatment results in significantly elevated numbers of STRA8 immunopositive germ cells at 24 hours, and significantly decreased numbers of STRA8 immunopositive cells 72 hours post-treatment.

4 dpp Treatment

Currently, only one time point has been analyzed for short term 4 dpp treatments. Cross sections of testes 48 hours post-treatment were analyzed for STRA8 immunopositive cells. There appears to be an increase in immunopositive cells 48 hours post-treatment at 4 dpp, although the difference is not statistically significant (Fig. 6a,b,c). This indicates that while Synchrony is achievable with RA treatment at 4 dpp, a wave of apoptosis does not precede delayed synchronous spermatogenesis. We expect to see elevated numbers of STRA8 positive cells per tubule from RA treated samples at 24 hours post-treatment as well. At 72 hours, there

may be a more significant decrease in STRA8 immunopositive cells per tubule in RA treated samples when compared of the same time point for animals treated at 6 dpp. This could be the result of a higher number of germ cells induced to differentiate at 4 dpp versus 6 dpp, resulting in a larger percentage of tubules with altered spermatogonial timing. The higher number of STRA8 immunopositive cells in these sections may indicate that the germ cells affected by this exogenous RA dose directly entered the differentiation pathway. By 72 hours a majority of germ cells in the first round of spermatogenesis may have progressed through the phase of high *Stra8* and *c-Kit* expression. This will be examined via use of STRA8 immunohistochemistry as well as real time reverse transcription PCR to analyze the intensity of gene expression for these markers of differentiation in all time points.

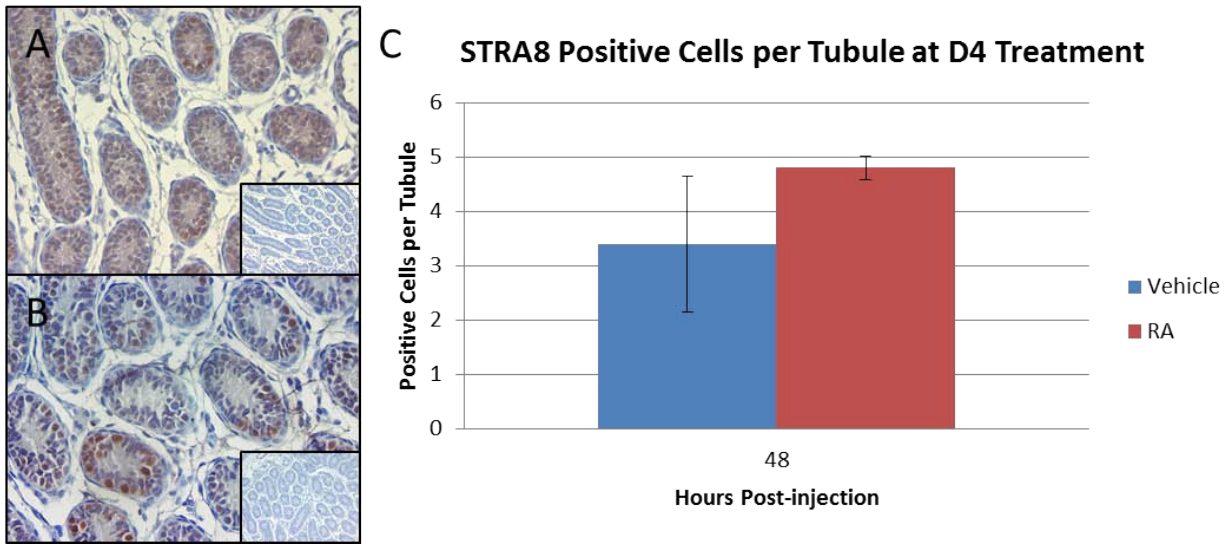


Fig. 6 STRA8 Immunohistochemistry after Treatment at 4 dpp

STRA8 detection via immunohistochemistry 48 hours post-treatment of animals at 4 dpp. A) A cross section of a testis from a vehicle-treated animal at 20x magnification (negative control inset). B) A cross section of testis from an RA-treated animal. C) Quantification of STRA8 immunopositive cells per tubule

for each treatment. There appears to be a higher number of STRA8 positive cells after RA treatment but these numbers are not statistically significant.

Detection of Apoptosis in Germ Cells After Treatment

Our initial report of RA-induced synchrony included an apparent delay in spermatogenesis following RA treatment of VAS males. This delay was hypothesized to be caused by a wave of apoptosis, detectable 48 hours post-treatment.[14] To determine if this apoptotic response is constant in all ages when the spermatogenic wave is disturbed, apoptotic cells were detected 48 hours post-treatment via TUNEL analysis. TUNEL staining detects dying cells via labeling fragmented DNA, which is an essential component of controlled cell death. Animals were treated at 4, 6, and 8 dpp and fragmented DNA was labeled. Significantly higher numbers of apoptotic cells were detected at both 4 and 6 dpp 48 hours post RA treatment when compared with vehicle treated animals at the same age(Fig. 7). However, there was no detectable change in apoptotic germ cells after 48 hours in testes from vehicle and RA treated males at 8 dpp. TUNEL analysis was also performed at 24 and 72 hours post-treatment, but no increased level of TUNEL-positive cells was found between any treatment groups at these ages. It appears that at 48 hours post-treatment RA exposure does lead to apoptosis in a subset of spermatogonia. However, because STRA8 protein staining indicates that the neonatal gonad does not need to undergo a delay in spermatogenesis for synchrony to occur, apoptosis may not be directly related to the alteration of the spermatogenic pattern in the gonad, but rather a side-effect of high RA levels in immature germ cells at 2 dpp.

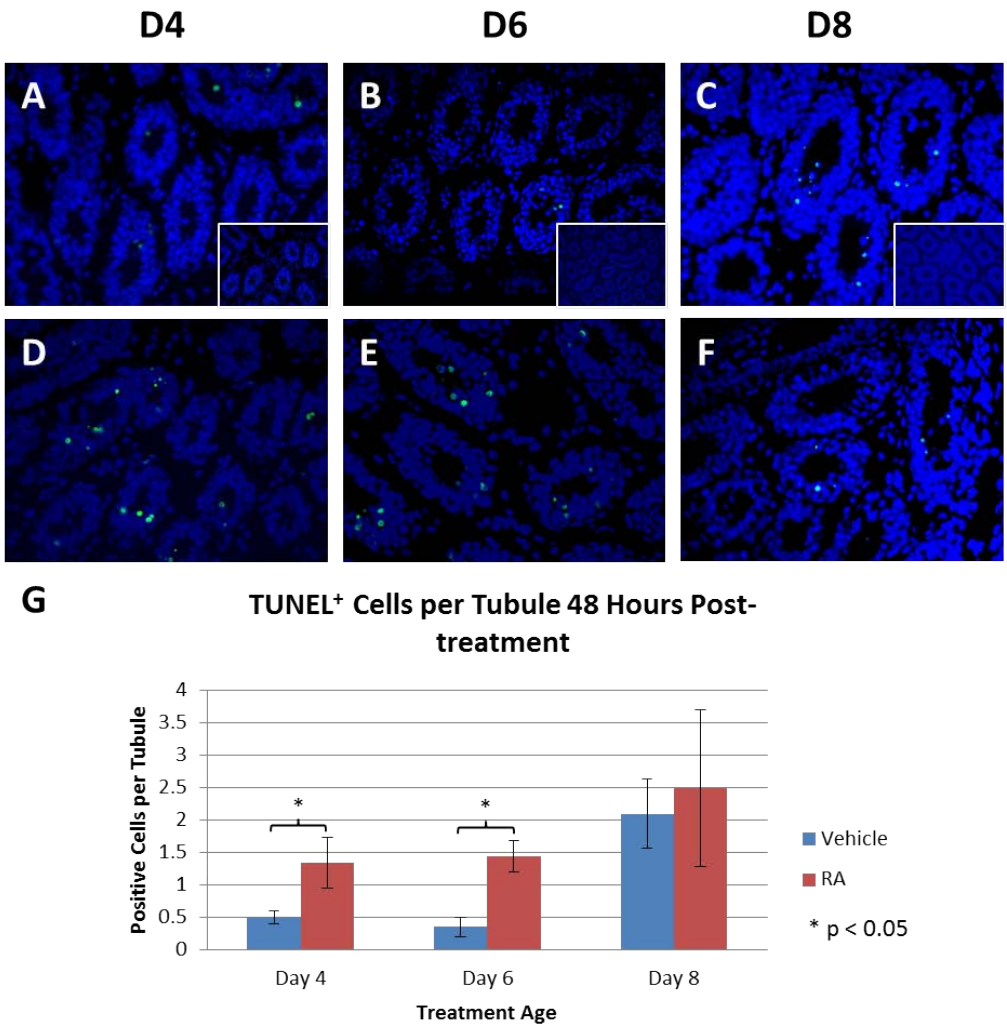


Fig. 7 Detection of Apoptosis 48 Hours Post-Treatment

Detection of Apoptotic cells 48 hours post-treatment. A) Cross sections of testes from vehicle treated animals contain fewer TUNEL positive cells than D) RA-treated testes at 4 dpp. A similar pattern is detected in vehicle (B) and RA-treated animals (E) at 6 dpp. However, there is no significant difference in STRA8 immunopositive cells per tubule between C) vehicle and F) RA-treated animals at 48 hours post-injection.

Discussion

Long Term RA Treatments

Analysis of murine spermatogenesis after RA treatment at these time points strongly indicates that RA is able to disturb the pattern of spermatogonial differentiation and as a result, the pattern of sperm production in the testis, until 4 dpp. Over the next 96 hours of gonadal development in male mice, the pattern of spermatogonial differentiation is cemented, and supplementation of exogenous RA is no longer able to disturb the pattern of spermatogenesis without first depriving males of retinoids for an extended period of time. Numerous changes in the testicular physiology are occurring during early neonatal development. These processes include development of the blood-testis-barrier, loss of the gonocyte germ cell population which transitions to the spermatogonia population immediately after birth, as well as the accumulation of more advanced germ cells as germ cell differentiation is initiation post-partum. The loss of gonocytes is a process which is completed before 6 dpp.[18] This time frame is too early to be consistent with our data, which indicates that 6 dpp is in the window of transition from a blank slate for patterning to a permanently timed differentiation program for the gonad. Formation of the blood-testis-barrier also does not correspond to the time points of interest which were identified by these long-term RA treatment studies. This structure, formed by tight junctions between adjacent Sertoli cells which functions to isolate haploid germ cells from the rest of the body to prevent an immune response to these distinct cells, is not fully formed until 15 dpp. This is far too late to match our window from four to eight dpp. The appearance of meiotic germ cells in the seminiferous epithelium, which occurs simultaneously with the

following round of spermatogonial differentiation, occurs during this change in response to RA in the neonatal male, and is worth investigating further to determine if this cell population is directly responsible for maintaining the pattern of spermatogonial differentiation in the testis which is originally established via RA accumulation in the neonatal testis.

Short Term RA Treatments

Response to RA at 24 hours post-treatment strongly resembled responses reported previously for treatments at 2 dpp. However, the 48 hour time point was significantly different from previous reports.[14] Animals treated with RA at 2 dpp do not contain a significant amount of STRA8 immunopositive cells at 48 or 72 hours post-injection. This was explained previously through the observation of a significant increase in apoptotic germ cells and an apparent delay in spermatogenesis which reinitiates in a synchronous manner. In the adult, a missing germ cell layer was also observed in a subset of seminiferous tubules, which corresponded to the germ cells which were undifferentiated spermatogonia at the time of treatment. However, at no time point 48 hours post-injection was a decrease in STRA8 immunopositive cells observed, either at 8 dpp injection when there is no observable change in spermatogenesis, or 4 and 6 dpp when the pattern of spermatogonial differentiation is altered. While these testes do contain elevated numbers of apoptotic germ cells, there is no apparent delay in spermatogonial differentiation after treatment, which would be observed as a significant drop in STRA8 immunopositive cells at 48 hours post-treatment. The drop in STRA8 immunopositive germ cells at 72 hours after RA treatment for 6 dpp animals is likely due to the majority of spermatogonia induced to differentiation with RA treatment progressing through the phase of high *Stra8* and other RA-responsive gene expression, and toward meiotic initiation. Continued analysis of short term treatments for 4 dpp males will most likely display a more dramatic effect of the 6 dpp treatments at 72 hours;

significantly lower levels of STRA8 immunopositive germ cells in each tubule compared with vehicle treated animals.

Apoptotic Germ Cells

A striking difference in response to RA between 2 and 4 dpp, both ages at which RA treatment results in synchronous spermatogenesis, is the delay of spermatogenesis resulting from treatment at 2 but not 4 dpp. The degree of synchrony which results from treatment is not significantly different, but how this synchrony is achieved appears to differ greatly. One explanation is that the majority of germ cells present within the seminiferous epithelium at 2 dpp are gonocytes. These are the germ cells whose response to RA accumulation in the testis first dictates the pattern of spermatogonial differentiation. However, these cells begin to transition to spermatogonia and by 6 dpp they are no longer detectable within the gonad. It may be these gonocytes are more sensitive to RA than their descendant spermatogonia. Levels of RA adequate to illicit a testis-wide response may be cytotoxic to these cells, but may not kill spermatogonia, instead successfully directing them to differentiate into type A_1 spermatogonia. Prolonged exposure to RA may result in increased germ cell apoptosis in spermatogonia, as was observed in adults treated with RA which resulted in a missing cell layer in the seminiferous epithelium corresponding to differentiating spermatogonia at the time of treatment. This was the effect of a three day treatment regimen of RA, in contrast to this experiment's single treatment, and may explain the lack of increased apoptosis between RA and vehicle treatment in 8 dpp males.

Control of the Spermatogenic Wave

The data collected in these experiments indicate that advanced germ cells may act to regulate the timing of spermatogonial differentiation. Curiously, in 8 dpp animals, the number of germ cells, as detected by GCNA immunohistochemistry does not change between vehicle and RA treatment at any of

these three time points (Supplemental Fig. 1). Without increased apoptosis or a change in the number of germ cells per tubule, the timing of differentiation appears to be corrected after RA treatment at 8 dpp without killing or losing an abnormal number of spermatogonia. This indicates that inappropriate STRA8 activation may be reversible, and that initiation of these RA-responsive genes which are considered markers of differentiation is not enough to drive spermatogonial differentiation within its environment in the testis. If this timing is controlled by advanced germ cells, as these data suggest, there may be communication between advanced germ cells and undifferentiated germ cells, most likely through the Sertoli cell population of the testis, as spermatocytes are separated from differentiating spermatogonia by the blood-testis-barrier.[2] This mechanism could be simply based on the detection of progressing advanced germ cells in the seminiferous epithelium. Advanced germ cells which are not associated with differentiating spermatogonia may be detected by Sertoli cells, which in turn may inhibit spermatogonial differentiation through a yet undetermined pathway. However, when preleptotene and leptotene spermatocytes are present within the epithelium, this may serve as a "differentiation checkpoint" for the Sertoli cell population which would then be permissive to the spermatogonial differentiation pathway (Fig. 8).

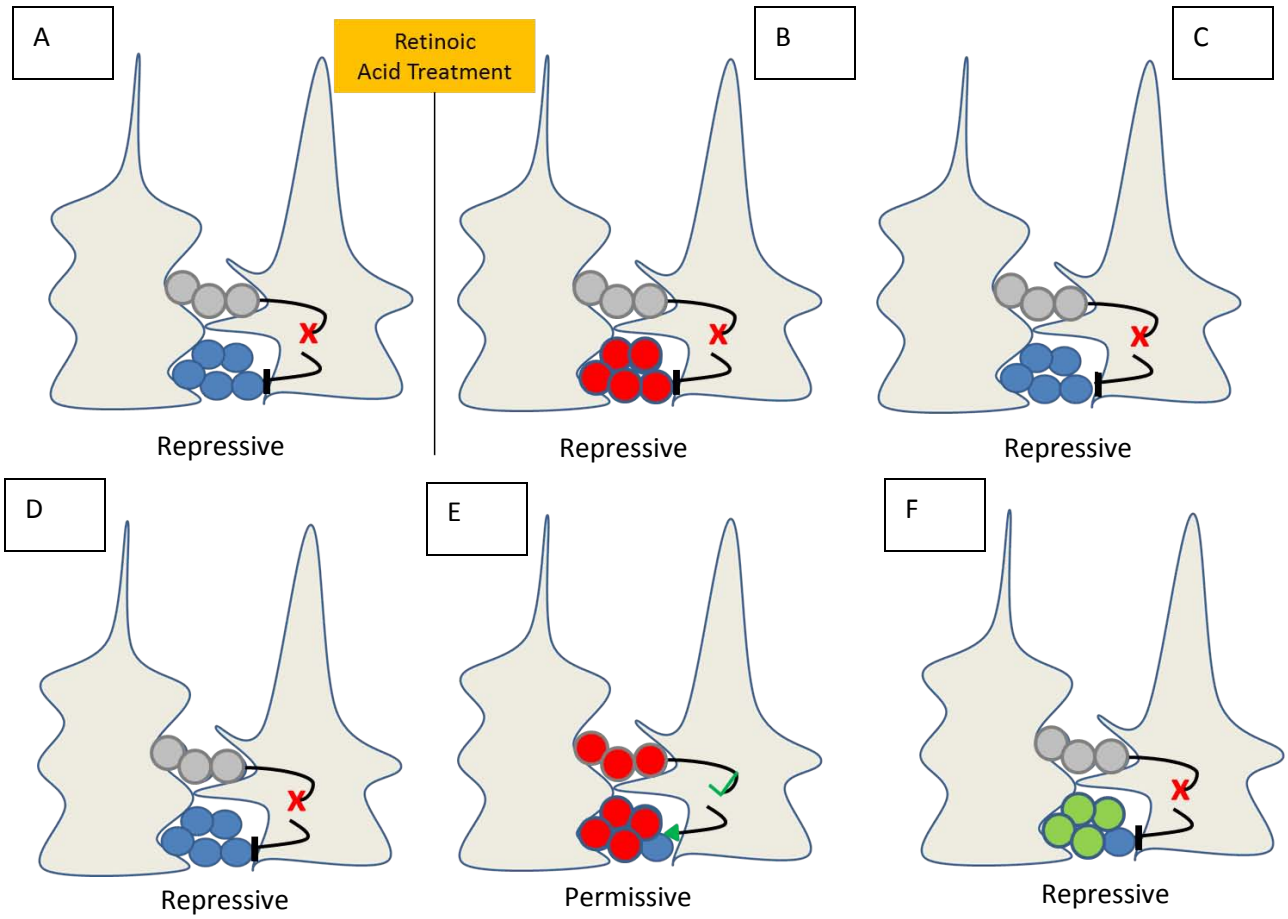


Fig. 8 A Proposed Model for the Control of Spermatogonial Differentiation

A model for the control of spermatogonial differentiation. A) The presence of advanced germ cells (in grey) are detected by the Sertoli cells within the seminiferous epithelium. When their progression does not correlate to proper spermatogonial differentiation, the Sertoli cell population inhibits spermatogonial differentiation. B) When the Sertoli cells are not permissive to differentiation, treatment of males with a full complement of advanced germ cells with RA results in a differentiation response of spermatogonia (in red). C) This response is repressed by the somatic component of the testis and spermatogonia revert to an undifferentiated state (in blue). D) These spermatogonia continue to remain undifferentiated until E) advanced germ cells have reached a developmental "checkpoint" detectable by Sertoli cells which then become permissive to spermatogonial differentiation. F) After this event differentiated spermatogonia continue their development (green cells) and advanced germ cells progress past the Sertoli cell checkpoint, resulting in repression of spermatogonial differentiation.

A model for the Control of Spermatogonial Differentiation.

Our resulting model from these experiments predicts that advanced germ cells, beginning at the preleptotene spermatocyte stage, aid the somatic component of the seminiferous epithelium to inhibit premature spermatogonial differentiation. While this is the first report in which advanced germ cells

may actively repress improper spermatogonial differentiation, the idea that advanced germ cells affect the timing of the seminiferous epithelium during spermatogenesis is not new. Recently it has been suggested that the role of RA metabolism is the driving force in establishing the timing of germ cell development and thus the cycling of the seminiferous epithelium.[15] This theory complements, rather than conflicts with our model of the regulation of spermatogonial differentiation. The timing of RA metabolism may very well be the clock which determines that every 8.6 days a germ cell develops from differentiation, to meiotic entry, and after 35 days, eventually into a mature spermatozoon in a precisely recurrent manner. Our model suggests that whatever the timing mechanism may be to keep the cells within the seminiferous epithelium on such a defined temporal pattern, advanced germ cells may actively regulate spermatogonial differentiation, and allow the possibly RA-driven timing of spermatogenesis to begin. A component of the RA metabolism/signaling pathway may even serve to release the block of spermatogonial differentiation in the mature seminiferous epithelium. Preleptotene cells express many similar markers of newly differentiated spermatogonia including STRA8 and c-Kit.[7,19] Similarities in gene expression, including RA-responsive genes, between spermatogonia undergoing differentiation and associated preleptotene spermatocytes makes this an attractively simple possibility.

Advanced germ cells also play a role in the timing of cellular differentiation in models distant from the mouse. In drosophila, a similar role exists for advanced germ cells to trigger the differentiation of the somatic cells which carry them through the fly gonad, in turn allowing for germ cell differentiation.[20] While there is no differentiation of Sertoli cells within the mammalian testis, these cells do cyclically express a large number of genes, correlating to increased and decreased RA metabolism[21] as well as other genes involved in hormone response and regulation pathways.[22] It may be that advanced germ cells, when detected by the somatic component of the epithelium, set the timing of some expressed genes, and aiding in the cycling expression of genes instead of inducing permanent differentiation as is observed in the *drosophila* model. It has been well established that the germ cell

component of the seminiferous tubules are responsible for the timing of spermatogenesis. This was proved via spermatogonial stem cell transplants from rats into mice in which mouse testes were completely evacuated of germ cells followed by introduction of rat spermatogonial stem cells into the testis of recipient mice.[23] Spermatogenesis with germ cells from rats took on the timing of the mouse cycle of the seminiferous epithelium instead of the timing of the mouse epithelium which takes significantly less time than the rat. If more developed spermatocytes were able to direct the timing of spermatogonial differentiation within these testes as well, that may explain the normal appearance of spermatogenesis, though the somatic component of the epithelium normally takes nearly half the time of the rat to undergo one full round of the spermatogenic cycle.

The model created from these data coincides well with previous studies investigating the timing of spermatogenesis. It also offers an explanation for the control of the initiation differentiation in spermatogonia to ensure that the cellular associations of spermatogenesis remain intact as well as the observation that germ cells alone are responsible for the timing of spermatogenesis.

Conclusion

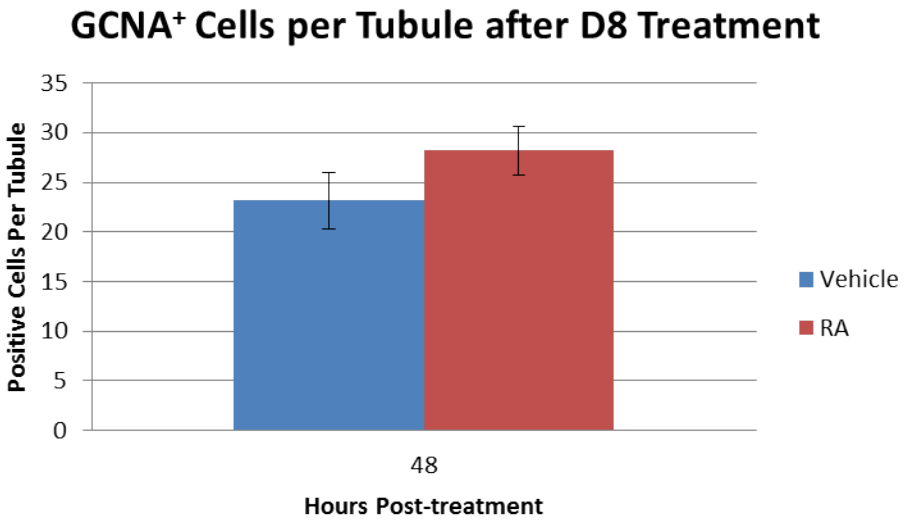
These described experiments offer an explanation for the precise timing of spermatogonial differentiation, which in mice occurs every 8.6 days at any given location within the seminiferous epithelium. Advanced germ cells have recently been suggested to determine the timing of spermatogenesis via the metabolism of RA. We propose that in addition to this role, they may also serve to prevent and allow spermatogonial differentiation at inappropriate and appropriate points during the cycle of the epithelium, respectively, in order to maintain this timing. Further investigation is warranted to determine the molecular mechanism for the control of the timing of spermatogonial differentiation as well as the potential of such a mechanism to act similarly in other adult stem cell models.

Works Cited

1. OAKBERG, E. F. A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *Am. J. Anat.* **99**, 391–413 (1956).
2. Russell, L. *Histological and histopathological evaluation of the testis*. (Cache River Press: Clearwater Fl, 1990).
3. McCARTHY, P. T. & CERECEDO, L. R. Vitamin A deficiency in the mouse. *J. Nutr.* **46**, 361–376 (1952).
4. van Pelt, A. M. & de Rooij, D. G. Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. *Biol. Reprod.* **43**, 363–367 (1990).
5. van Pelt, A. M. & de Rooij, D. G. Retinoic acid is able to reinitiate spermatogenesis in vitamin A-deficient rats and high replicate doses support the full development of spermatogenic cells. *Endocrinology* **128**, 697–704 (1991).
6. Morales, C. & Griswold, M. D. Retinol-induced stage synchronization in seminiferous tubules of the rat. *Endocrinology* **121**, 432–434 (1987).
7. Zhou, Q. *et al.* Expression of stimulated by retinoic acid gene 8 (Stra8) and maturation of murine gonocytes and spermatogonia induced by retinoic acid in vitro. *Biol. Reprod.* **78**, 537–545 (2008).
8. Snyder, E. M., Small, C. & Griswold, M. D. Retinoic acid availability drives the asynchronous initiation of spermatogonial differentiation in the mouse. *Biol. Reprod.* **83**, 783–790 (2010).
9. Buaas, F. W. *et al.* Plzf is required in adult male germ cells for stem cell self-renewal. *Nat. Genet.* **36**, 647–652 (2004).
10. Filipponi, D. *et al.* Repression of kit Expression by Plzf in Germ Cells. *Molecular and Cellular Biology* **27**, 6770–6781 (2007).
11. Pesce, M., Wang, X., Wolgemuth, D. J. & Schöler, H. Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. *Mech. Dev.* **71**, 89–98 (1998).
12. Zhou, Q. *et al.* Expression of stimulated by retinoic acid gene 8 (Stra8) in spermatogenic cells induced by retinoic acid: an in vivo study in vitamin A-sufficient postnatal murine testes. *Biol. Reprod.* **79**, 35–42 (2008).
13. Li, H., MacLean, G., Cameron, D., Clagett-Dame, M. & Petkovich, M. Cyp26b1 expression in murine Sertoli cells is required to maintain male germ cells in an undifferentiated state during embryogenesis. *PLoS ONE* **4**, e7501 (2009).
14. Snyder, E. M., Davis, J. C., Zhou, Q., Evanoff, R. & Griswold, M. D. Exposure to retinoic acid in the neonatal but not adult mouse results in synchronous spermatogenesis. *Biol. Reprod.* **84**, 886–893 (2011).
15. Hogarth, C. A. *et al.* Suppression of Stra8 expression in the mouse gonad by WIN 18,446. *Biol. Reprod.* **84**, 957–965 (2011).
16. Sugimoto, R., Nabeshima, Y. & Yoshida, S. Retinoic acid metabolism links the periodical differentiation of germ cells with the cycle of Sertoli cells in mouse seminiferous epithelium. *Mechanisms of Development* (2011).doi:10.1016/j.mod.2011.12.003

17. Siiteri, J. E., Karl, A. F., Linder, C. C. & Griswold, M. D. Testicular synchrony: evaluation and analysis of different protocols. *Biol. Reprod.* **46**, 284–289 (1992).
18. Desjardins, C. & Ewing, L. L. *Cell and molecular biology of the testis*. (Oxford University Press: New York, 1993).
19. Prabhu, S. M. *et al.* Expression of c-Kit receptor mRNA and protein in the developing, adult and irradiated rodent testis. *Reproduction* **131**, 489–499 (2006).
20. Sarkar, A. *et al.* Antagonistic roles of Rac and Rho in organizing the germ cell microenvironment. *Curr. Biol.* **17**, 1253–1258 (2007).
21. Johnston, D. S. *et al.* Stage-specific gene expression is a fundamental characteristic of rat spermatogenic cells and Sertoli cells. *Proceedings of the National Academy of Sciences* **105**, 8315–8320 (2008).
22. O'Donnell, L. *et al.* Transcriptional Profiling of the Hormone-Responsive Stages of Spermatogenesis Reveals Cell-, Stage-, and Hormone-Specific Events. *Endocrinology* **150**, 5074–5084 (2009).
23. França, L. R., Ogawa, T., Avarbock, M. R., Brinster, R. L. & Russell, L. D. Germ cell genotype controls cell cycle during spermatogenesis in the rat. *Biol. Reprod.* **59**, 1371–1377 (1998).

Supplemental Figures



Supplemental Fig. 1 GCNA-immunopositive Cells Per Tubule 48 Hours Post-Treatment

Detection of Germ Cells via immunohistochemical staining of GCNA (Germ Cell Nuclear Antigen) reveals no significant change in germ cell numbers between vehicle and RA treated testes 48 hours post-treatment.

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