RETINOIC ACID REGULATES KIT PROTEIN EXPRESSION IN PERITUBULAR MYOID CELLS IN THE MAMMALIAN TESTIS

by

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Abstract

Spermatogenesis (production of sperm) takes place in the testis, which is composed of seminiferous tubules. Within mammalian seminiferous tubules, there are two cell types: germ cells and supporting somatic cells. The somatic cells found inside the tubules are Sertoli cells, and those surrounding the tubules are peritubular myoid cells (PTMs). Evidence supports roles for PTMs in multiple critical functions, however, almost nothing is known regarding how PTMs are instructed to regulate these distinct functions. Recent data from our lab are beginning to shed light on this – we made the exciting discovery that expression of the essential 'KIT protooncogene receptor tyrosine kinase' (KIT) is regulated in PTMs by retinoic acid (RA) signaling. As a morphogen, RA signals in discrete portions of the testis to drive germ cell differentiation, and we observed that RA dramatically modified the regional expression of KIT. In germ cells, RA upregulated KIT protein expression but did the opposite in PTMs; KIT protein was downregulated in PTMs in vivo by exogenous RA and upregulated by WIN 18,446/BDAD mediated inhibition of RA synthesis. We are currently investigating the extent to which RA regulates the regional and temporal expression of KIT in PTMs. We treated mice with WIN 18,446 for ten days and then injected exogenous RA. At different time points after RA injection, mice were euthanized and tissues were collected for immunostaining to detect KIT in PTMs, which express 'actin, alpha 2, smooth muscle, aorta' (ACTA2/a-SMA). We next determined the percentages of ACTA2+ PTMs that were KIT+. Our preliminary results suggest that numbers of KIT+ PTMs declines drastically, as early as two days in response to RA.

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Introduction and Literature Review

Peritubular Myoid Cells:

Most people dream of having their own children and family. While the process of conception is easy for some, for others it is not. According to clinical data, the current natural fertility rate for a healthy, fertile 30-year-old woman is only 20%. This is why it is important to not only understand how babies are made, but also to uncover the systems that create the two main components of making a baby: sperm and eggs.

Reproduction has and always will be a prevalent topic in science and understanding mechanisms guarding reproduction is of the upmost importance. According to clinical data, sperm quality has been continuously decreasing for the last 50 years in developing countries. Due to these findings, there is a larger emphasis on answering questions about spermatogenesis regulation and the importance of somatic cells in development and maintenance of the germ line. The goal of this project is to investigate the regulation of a specific somatic cell type present in the mammalian testis.

The mammalian testis contains several somatic cell types including Sertoli cells, Leydig cells and peritubular myoid cells. PTMs can be found surrounding the seminiferous tubules and have been found in all mammalian species. In rodents, such as rats, mice and hamsters, the cells make up a single layer connected by complexes similar to those in epithelial cells. Conversely, in humans there are several layers of PTMs. These cells, regardless of the organism, exhibit a flatten shape with a central nucleus giving them an appearance to a sunny side up egg. They also be identified by their actin filament arrangement which exhibits a lattice-work pattern in rats and mice (Maekawa M. et al.) Interestingly, PTMs are the only cell type in the testis for which no counterpart has been identified in the ovary (Jeanes A. et al.) The presence of PTMs in the adult and developing mouse testis suggests a

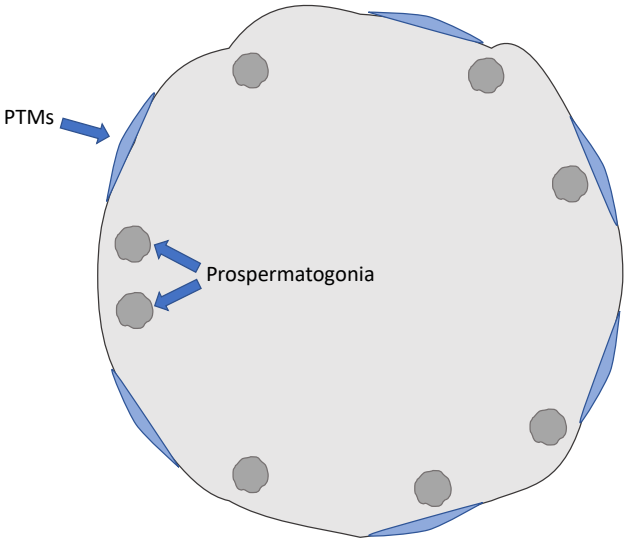

Figure 1. Structure of Interest. Cartoon representation of a seminiferous tubule within the mammalian testis. Displays prospermatogonia residing within the tubule and peritubular myoid cells wrapping around the outside of the tubule.

specific function in spermatogenesis. While it has been proposed that PTMs have several functions such as providing key paracrine signals for developing germ cells, providing structure for the seminiferous, and are thought to contract their cytoskeleton to extrude newly produced testicular sperm from the testis, mechanisms regulating these functions are still poorly understood (Maekawa M. et al.)

KIT Protein:

On other hand, the receptor tyrosine kinase c-Kit (KIT) is a heavily studied stem cell factor and is also expressed in PTMs. c-Kit was first discovered in 1986 as a transforming gene of Hardy-Zuckerman feline sarcoma virus and has further been cloned and located within other species such as mice and human (Besmer et al.) In both species the gene is expressed as a single, 5-kb transcript, but it is localized to human chromosome 4 and mouse chromosome 5 (Yarden et al.) KIT, and its ligand stem cell factor (SCF) are expressed in many tissues of the body where they control key cellular functions. These functions include mediating cell survival, migration, and proliferation. It has been shown to have increased importance in systems such as hematopoiesis, pigmentation, and fertility. Excessive c-Kit signaling has also been shown to result in cancers such as leukemia and tumors in the gastrointestinal tract as well as in germ cells (Lennartsson et al.) In terms of fertility, c-kit has shown to be essential in maintenance of primordial germ cells and is essential for spermatogonial differentiation during spermatogenesis (Rossi et al.)

Retinoic Acid:

Retinoic acid (RA) is an intermediate compound in the metabolism of vitamin A (Xuan et al.) and has been shown to be required for spermatogenesis. In a study by Griswold et. al., authors showed that rats deficient in vitamin A experienced a gradual loss in sperm. They also showed this could be recovered by giving a pulse of RA (Griswold et al.) The importance of RA in the induction of spermatogenesis is believed to be tied to a need for balance in the antioxidant defense system in the male reproductive tract and RA levels have been shown to increase during four major transitions during spermatogenesis (Malivindi et al, Endo et al.) While there have been many studies relating to spermatogonia and RA, there are very few looking at how RA interacts with PTMs. From the existing studies, Vernet et. al. suggested that PTMs express RA-degrading enzymes, CYP26, that create a catabolic barrier around the seminiferous tubules making it unlikely for low doses of RA to enter the tubules. Regardless,

none of this accounts for any signaling caused by RA in PTMs.

Project Overview:

The goal of this study is to investigate retinoic acid (RA) as a potential regulator of protein expression within peritubular myoid cell (PTM) in mice. Specifically, expression and signaling involving the 'KIT protooncogene receptor tyrosine kinase' (KIT). Within the literature, there is little known about how PTMs regulate their functions. Using our system in mice we are able to control retinoic acid metabolism and therefore begin to shed light on retinoic acid and its potential to regulate PTMs in the developing and adult mouse testis.

Materials and Methods

WIN 18,446/BDAD Mouse Treatment:

WIN 18,466 (also known as BDAD) is an established compound that blocks the metabolism of retinoic acid (RA) in the developing testis. We employ this compound to create a RA deficient environment. Between the ages of P1 and P14 mice are treated with WIN 18,446 through oral feeding and are treated by injection at any age past this point.

One cohort of mice was treated with WIN 18,446 from P1 through P10 to synchronize germ cells to undifferentiated spermatogonia. These mice were then given one pulse of RA on P11, BDAD was discontinued, and animals were sacrificed on P12, P14, P16, P18, and P20. Tissues were collected and prepared for IIF staining. This treatment plan would allow us to examine PTMs in an RA-sufficient environment and synchronized testis (Fig. 2).

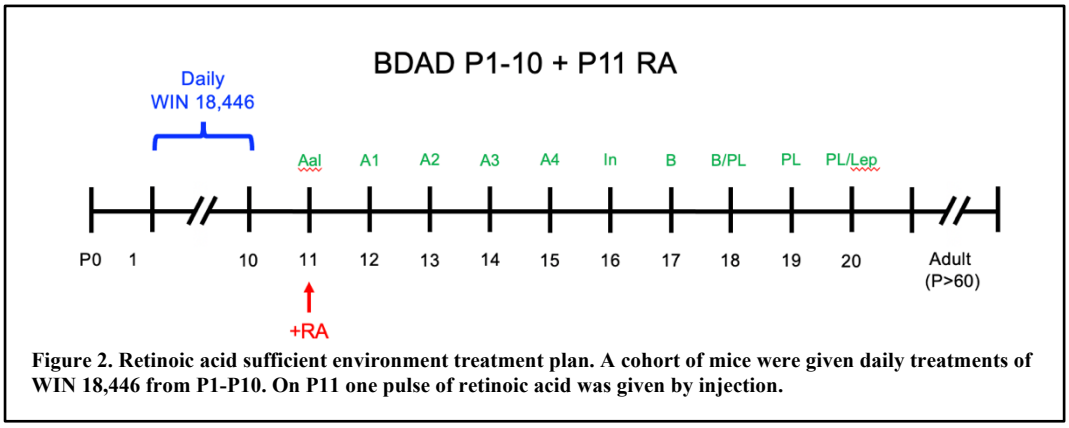

Figure 2. Retinoic acid sufficient environment treatment plan. A cohort of mice were given daily treatments of WIN 18,446 from P1-P10. On P11 one pulse of retinoic acid was given by injection.

To create an RA-deficient environment to compare to, another cohort of mice was treated with daily WIN 18,446 P1 - P10 to establish synchronization and were given RA on P11. This cohort continued to receive WIN 18,446 from P12 until day of sacrifice. These

timepoints were P12, P14, P17, P19, and P21. Tissues were then collected and prepared for IIF staining.

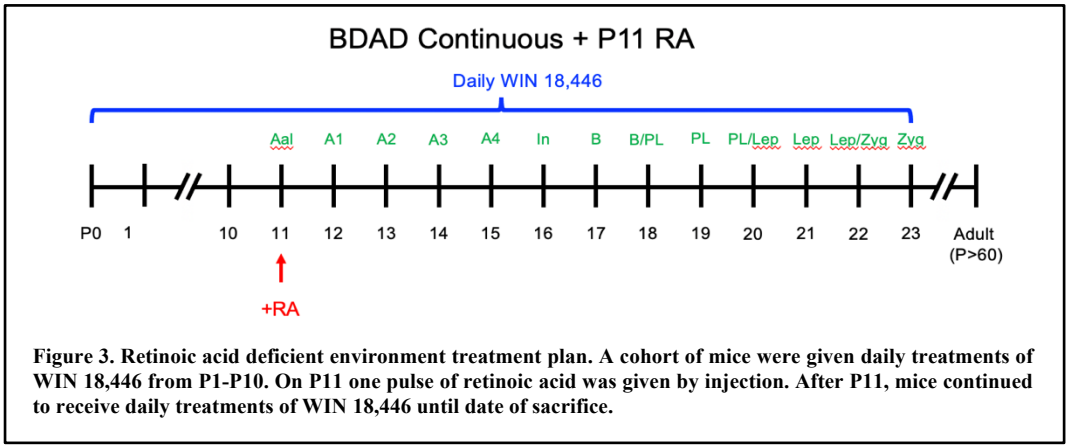

Figure 3. Retinoic acid deficient environment treatment plan. A cohort of mice were given daily treatments of WIN 18,446 from P1-P10. On P11 one pulse of retinoic acid was given by injection. After P11, mice continued to receive daily treatments of WIN 18,446 until date of sacrifice.

Adult Mouse Treatment:

Since we were also interested in how KIT protein levels in PTMs respond to RA in adult mice, we established a different treatment plan for adult males. Adult mice were exposed to RA for 6, 12, 24, and 48 hours before being sacrificed. This treatment method was used for adults because without constant WIN 18,446 treatment since birth there is no way to ensure a synchronized testis. Therefore, we decided to examine how PTMs in the adult testis respond to a surplus of RA. A vehicle treatment of DMSO was completed on another cohort of mice to mimic RA treatment. Those tissues were then collected and prepared for IIF staining.

IIF Staining and Quantification:

Testis tissue was dissected and prepared for cryosectioning. They are first fixed in 4% PFA overnight, then washed and incubated overnight again in 30% sucrose in 1X PBS. Finally, tissues are transferred from 30% sucrose to O.C.T. to be frozen and sectioned at 5um thickness. Once the tissues were sectioned, slides were stained using the Protein Tech rabbit 'actin, alpha 2, smooth muscle, aorta' (ACTA2/a-SMA) at a concentration of 1:250. This was used to visualize PTMs. A goat c-KIT antibody by R&D Systems was used at 1:1000 to identify cells expressing c-KIT. Lastly all of these slides were co-stained and mounted with DAPI to identify nuclei. Images were taken of each age for both RA deficient and RA sufficient neonatal mice as well as adult mice treated for RA or DMSO and then quantified using ImageJ to identify the number of KIT+ PTMs.

Results

RA Sufficient Neonatal Mice:

Analysis of tissue from mice with RA sufficient environments found significantly higher numbers of KIT+ PTMs at P12 when compared to just 72 hours after RA injection on P14 and P16 (Figure 4C). On P12, nearly 75% of PTMs expressed KIT protein and by P14 and P16 this percentage decreased to just under 20%. This decrease was also present when the number of KIT+ PTMs were compared to the tubule perimeter (Figure 4D). When observing stained tissues for KIT, SMA, and DAPI in P9 testis, most KIT expression can be seen surrounding the tubules where PTMs are also located (Figure 4A). This is different when looking at the same staining on a P14 tissue where KIT expression is localized inside of the tubules and is being expressed in germ cells (Figure 4B). These results demonstrate that RA plays a role in the expression of KIT protein in PTMs, but the next step was to determine if continuous treatment of WIN 18,446 could maintain KIT expression in PTMs after exposure to RA.

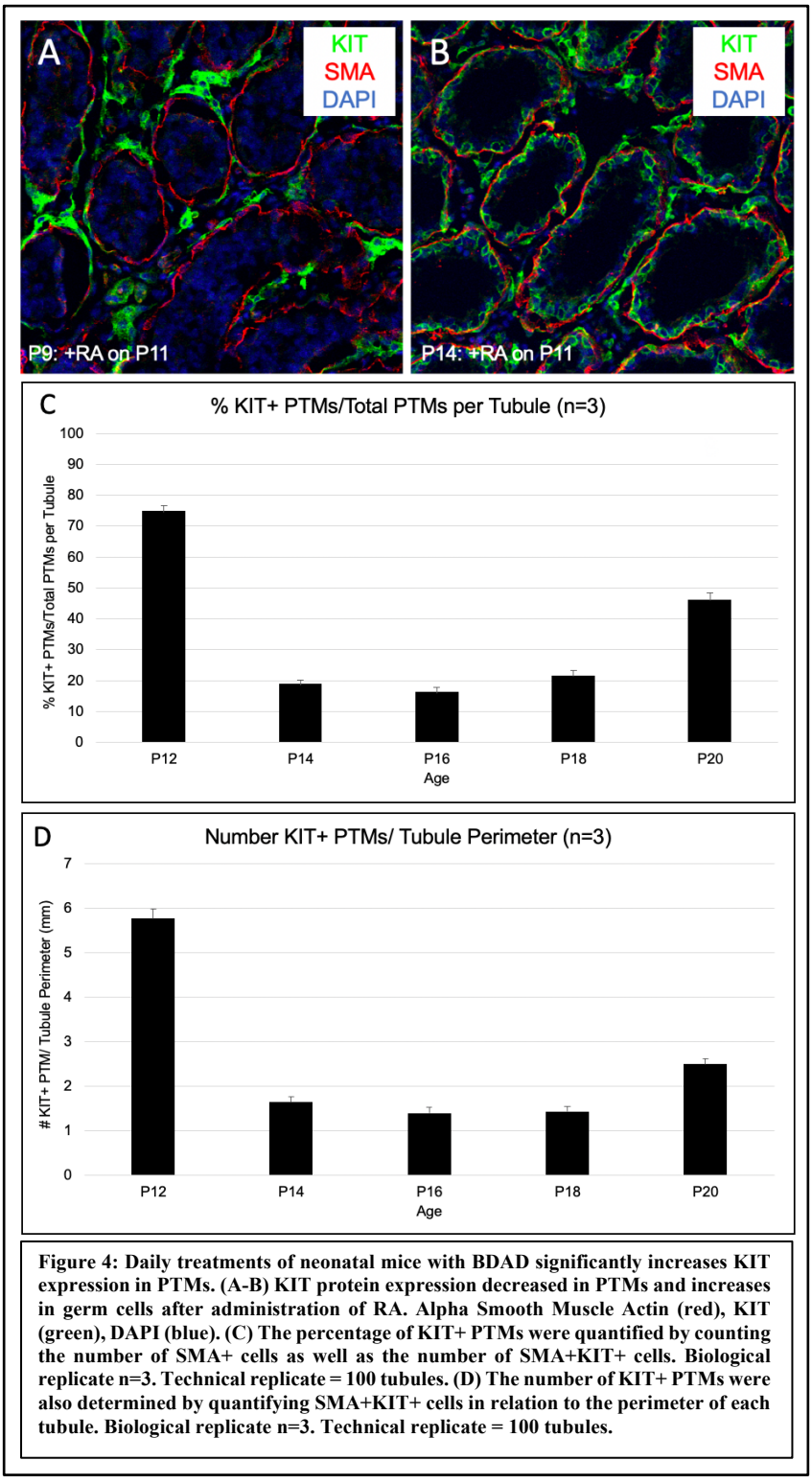

Figure 4: Daily treatments of neonatal mice with BDAD significantly increases KIT expression in PTMs. (A-B) KIT protein expression decreased in PTMs and increases in germ cells after administration of RA. Alpha Smooth Muscle Actin (red), KIT (green), DAPI (blue). (C) The percentage of KIT+ PTMs were quantified by counting the number of SMA+ cells as well as the number of SMA+KIT+ cells. Biological replicate n=3. Technical replicate = 100 tubules. (D) The number of KIT+ PTMs were also determined by quantifying SMA+KIT+ cells in relation to the perimeter of each tubule. Biological replicate n=3. Technical replicate = 100 tubules.

RA Deficient Neonatal Mice:

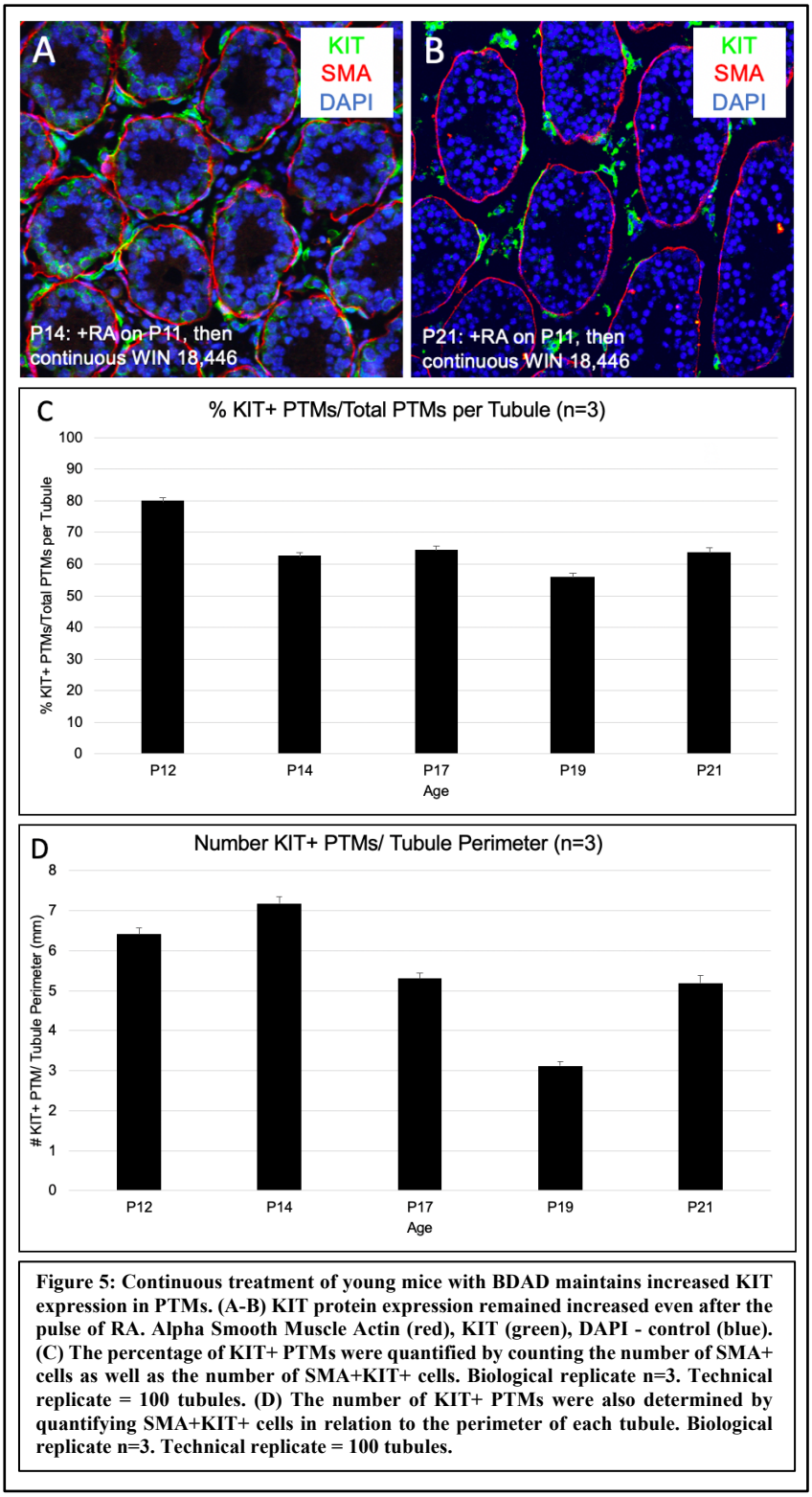

Figure 5: Continuous treatment of young mice with BDAD maintains increased KIT expression in PTMs. (A-B) KIT protein expression remained increased even after the pulse of RA. Alpha Smooth Muscle Actin (red), KIT (green), DAPI - control (blue). (C) The percentage of KIT+ PTMs were quantified by counting the number of SMA+ cells as well as the number of SMA+KIT+ cells. Biological replicate n=3. Technical replicate = 100 tubules. (D) The number of KIT+ PTMs were also determined by quantifying SMA+KIT+ cells in relation to the perimeter of each tubule. Biological replicate n=3. Technical replicate = 100 tubules.

Analysis of tissue from mice in RA deficient environments found that KIT expression could remain localized just outside of the tubules through continuous treatment of WIN 18,446. This can be seen in P14 mice stained for KIT, SMA, and DAPI, where KIT expression is still surrounding the tubules 72 hours after RA injection on P11 (Figure 5A). This suggests that KIT expression is primarily taking place in PTMs. This expression pattern is consistent through P21 when similar stains were conducted (Figure 5B). This increased expression in PTMs can also be seen in the percentage of PTMs that are KIT+. On P12, around 80% of PTMs express KIT and this percentage is maintained through P21 (Figure 5C). Given that the percentage of KIT+ PTMs in RA deficient mice is the same as RA sufficient mice, it can be concluded that continuous treatment of WIN 18,446 can maintain elevated

KIT expression in PTMs after exposure to RA. This conclusion is echoed when looking at the number of KIT+ PTMs per tubule perimeter (Figure 5D).

RA/DMSO Treated Adult Mice:

Analysis of tissues from adult mice found that regardless of the length of RA treatment, there was a decrease in the percentage of KIT+ PTMs when compared to DMSO-treated mice. Control DMSO-treated mice displayed that 50% of PTMs expressed KIT protein while mice treated with RA for the same amount of time expressed between 20-25%. This conclusion was consistent across 6,12-,24-, and 48-hour timepoints (Figure 6A). There was also a decrease in the number of KIT+ PTMs per tubule perimeter when comparing control DMSO-treated mice to RA-treated mice. This decrease was consistent across all time point, but the most drastic occurred at the 24- and 48-hour treatments (Figure 6B). From these results it can be concluded that exposure to RA also decreases the percentage of KIT+ PTMs and KIT+ PTMs per tubule perimeter in adult mice as well.

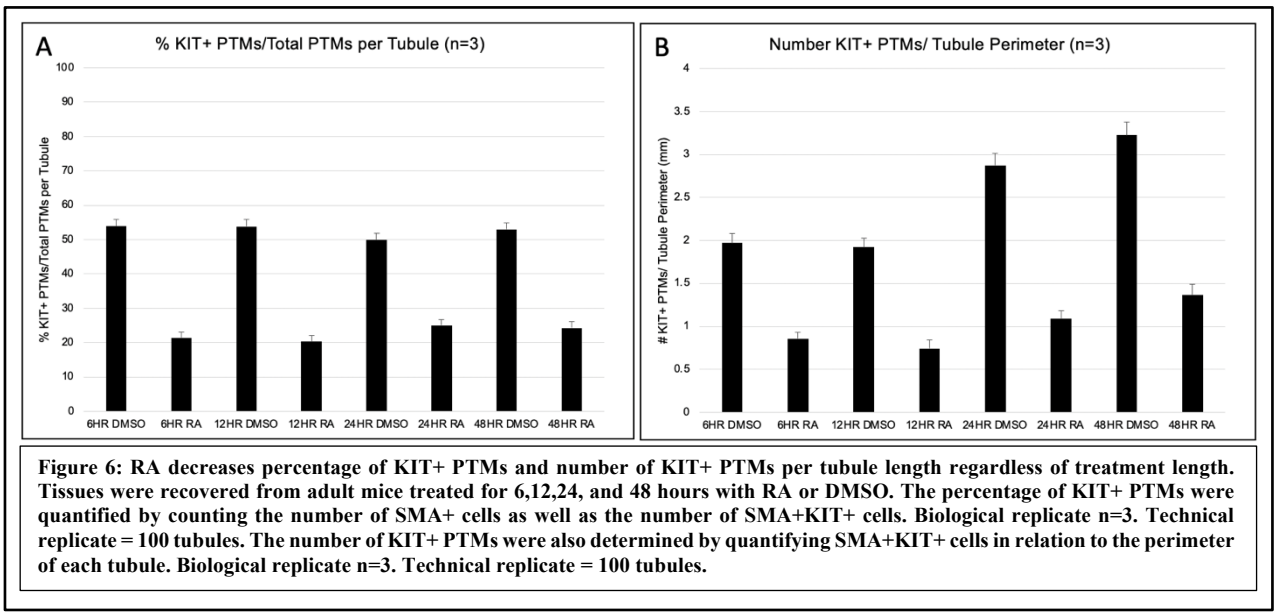

Figure 6: RA decreases percentage of KIT+ PTMs and number of KIT+ PTMs per tubule length regardless of treatment length. Tissues were recovered from adult mice treated for 6,12,24, and 48 hours with RA or DMSO. The percentage of KIT+ PTMs were quantified by counting the number of SMA+ cells as well as the number of SMA+KIT+ cells. Biological replicate n=3. Technical replicate = 100 tubules. The number of KIT+ PTMs were also determined by quantifying SMA+KIT+ cells in relation to the perimeter of each tubule. Biological replicate n=3. Technical replicate = 100 tubules.

Discussion

After analysis, we can conclude that RA decreases the percentage of KIT+ PTMs in neonatal mice. This can be seen mainly by comparing the percentage of KIT+ PTMs after P12 of mice in an RA sufficient environment to mice in an RA deficient environment. RA sufficient mice contain display around 80% of PTMs being KIT+. This percentage decreases to 20% by P14 and doesn't increase again until P20 when it reaches approximately 45% (Figure 4C). In comparison, around 80% of PTMs are KIT+ in RA deficient mice at P12 and this percentage remains elevated

between 60-70% until P21. There is no dramatic decline in KIT+ PTMs like is seen in the RA sufficient mice (Figure 5C). This means exposure to RA does in fact decrease KIT expression in PTMs in the neonatal mouse testis. This conclusion doesn't explain how RA regulates KIT protein expression and the answer to questions concerning this regulation will take further experiments and studies, some of which might include isolating PTMs from mice treated with WIN 18,446 and determining if RA regulates PTMs in a similar capacity in vitro or determining with form of KIT is expressed in PTMs of the mouse testis.

In addition, it can be concluded that RA also decreases the percentage of KIT+ PTMs in the adult mouse testis. This can be best observed in the quantification of KIT+ PTMs when treated with 6-,12-,24-, and 48-hours of RA or DMSO. In mice treated with RA, the percentage of KIT+ PTMs remained between 20-25% regardless of the amount of time treated with RA. In comparison, DMSO-treated mice displayed between 50-55% of PTMs being KIT+ regardless of the amount of time treated with DMSO. This means that even without WIN 18,446 treatment and a synchronization of the testis RA decreases the percentage of KIT+ PTMs in the adult mouse testis. Once again, this conclusion does not explain how RA regulates KIT protein expression and the answer to questions concerning this regulation will take further experiments and studies. Future studies might consider treating adult mice with WIN 18,446 to determine if prolonged treatment can induce elevated levels of KIT+ PTMs in the unsynchronized adult testis of mice.

While this study begins to explore how RA regulates KIT expression in PTMs, it does highlight the potential RA has as a regulator of PTM function in the testis. Our hope is that through future studies we will learn more about regulation of PTM function and how those regulators play role in overall spermatogenesis.

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