Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Magee-Womens Research Institute and Foundation

2. **Reporting Period (start and end date of grant award period):** 1/1/2012-12/31/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Cheryl Richards, MBA

4. **Grant Contact Person's Telephone Number:** (412) 641-8932

5. **Grant SAP Number:** 4100057668

6. **Project Number and Title of Research Project**: Project 4 -Regulation of Spermatogenesis by Classical and Non-classical Testosterone Signaling

7. **Start and End Date of Research Project:** 01/01/2012-12/31/2012

8. **Name of Principal Investigator for the Research Project:** William Walker, Ph.D.

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ 269,848.26

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Walker	Principle Investigator	50%	$60,827.58
Shupe	Research technician	100%	$11,696.62
Nefjordova	Student worker	100%	$195.02
Toocheck	Research technician	100%	$5,079.20

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
Jacobus	Visiting professor	20%
Hoffman	Volunteer	30%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No_____X_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____X____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Nongenomic Androgen Signaling in Sertoli Cells	X NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	2/2012	$1,270,410	$0
Role of Non-classical Androgen Receptor Signaling in Spermatogenesis	X NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	6/2012	$1,163,651	$0
Selective activation of testosterone signaling pathways in transgenic mice	X NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	2/2013	$100,000	$ pending

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes___X_____ No_____

If yes, please describe your plans:

An RO1 proposal will be submitted to the NIH in June of 2013.

12. Future of Research Project. What are the future plans for this research project?

An RO3 application has been made to the NIH to develop transgenic animals to perform follow *in vivo* studies. An RO1 grant application is planned for June 2013. Both of these proposals will build on the progress from this funding to investigate the molecular mechanisms by which testosterone acts in Sertoli cells to support germ cell development.

13. **New Investigator Training and Development**. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes_____X____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male	1			
Female	1		1	1
Unknown				
Total	**2**		**1**	**1**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic	2		1	
Unknown				1
Total	**2**		**1**	**1**

	Undergraduate	Masters	Pre-doc	Post-doc
White	2		1	1
Black				
Asian				
Other				
Unknown				
Total	**2**		**1**	**1**

14. **Recruitment of Out-of–State Researchers**. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. **Impact on Research Capacity and Quality**. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes_____X____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

The project allowed a strengthening of a collaboration with Dr. Pancharatnam Jeyasuria at the University of Pittsburgh. The results of our studies provided needed preliminary data for a collaborative NIH RO1 proposal that was submitted with Dr. Jeyasuria in February 2012. In addition, the project permitted the retention of a research technician position that would have been lost without the funding.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes_____ No_____X____

If yes, please describe the collaborations:

16(B) Did the research project result in commercial development of any research products?

Yes_____ No_____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Project goals, objectives and specific aims

The major objective of this proposal was to identify the molecular mechanisms by which testosterone (T) supports male fertility. We focused on the processes that are required for spermatogenesis and fertility that are regulated by classical and non-classical T signaling. We hypothesized that each pathway regulates specific factors that are essential for maintaining spermatogenesis. We proposed that these studies would provide (1) information needed to treat specific male infertility conditions and (2) long-needed new targets for male contraceptive research. The specific aims were:

Aim 1: Determine whether inhibiting non-classical T signaling blocks sperm release.

Aim 2: Determine whether inhibiting classical or non-classical T signaling blocks spermatogenesis in testis explants.

Aim 3: Determine whether the classical and/or non-classical pathway is required to maintain spermatogenesis *in vivo*.

Summary of Research Completed:

Testosterone (T) is essential for fertility and regulates Sertoli cells in the mammalian testis through the androgen receptor (AR) to generate the environment required for germ cell proliferation and differentiation. Numerous processes that are critical for maintaining male fertility are dependent on T action, including (1) the completion of meiosis by germ cells, (2)

maintenance of the attachment of maturing spermatids to Sertoli cells, and (3) initiation of the release of mature spermatozoa. However, the molecular mechanisms by which T supports spermatogenesis and fertility are poorly understood. This lack of knowledge has prevented the diagnosis and treatment of infertile patients with T signaling defects. Also, it has not been possible to develop long sought male contraceptive strategies based on blocking specific T-dependent molecular events.

Progress has been made in that it is now known that T can act via two mechanisms in the Sertoli cell, the classical pathway and the non-classical pathway. In the classical pathway, T binds to the AR, resulting in migration of the receptor to the nucleus, where it binds androgen response elements in the promoters of T-responsive genes. In the non-classical pathway, T binding to AR recruits Src kinase that then activates a series of kinases to elicit numerous downstream kinase-dependent events, including transcription regulation by the cyclic adenosine monophosphate response element binding protein (CREB) transcription factor.

The relative contributions of the classical and non-classical pathways in maintaining spermatogenesis have not been investigated. We propose that characterization of these pathways will permit the identification of distinct T signaling control defects in infertility patients that can be targeted for therapies. Also, we expect to identify molecular mechanisms regulated specifically by each T signaling pathway that can be targeted to develop finely focused male contraceptive drugs that would block early steps of spermatogenesis before the production of sperm but not affect other T-dependent processes. The major goal of this study was to identify processes required for spermatogenesis that are regulated by the classical and non-classical pathways.

Aim 1: Determine whether inhibiting non-classical testosterone signaling blocks sperm release.

T initiates the induction of the non-classical signaling pathway by causing the receptor (AR) to interact with and activate Src kinase in Sertoli cells and other cell types via a proline-rich domain in AR that binds to the SH3 domain of Src. The 10–amino acid S1 peptide, including the proline-rich region of AR, blocks AR and Src interaction and inhibits androgen-mediated activation of Src and the non-classical pathway. We synthesized the S1 peptide (aa 377-386 in hAR). The peptide was acetylated at the N-terminus to inhibit degradation, and a fluorescein isothiocyanate (FITC) fluorescent tag was placed on the C-terminus. The S1 peptide did not inhibit classical T signaling as S1 had no effect on transcription of a T-inducible reporter gene driven by the prostate-specific antigen (PSA) promoter (Fig. 1A). In contrast, addition of the S1 peptide blocked phosphorylation of extracellular regulated kinase (ERK), a target of non-classical signaling in primary Sertoli cells (Fig. 1B). A scrambled S1 peptide (S1s) had no effect on T-mediated transcription or ERK phosphorylation. These data indicate that S1 is a selective inhibitor of the non-classical pathway.

We tested the hypothesis that the S1 inhibitor of non-classical T signaling could block T-dependent release of mature sperm. To test this hypothesis, individual seminiferous tubule fragments containing fully developed sperm that are poised to be released (stage VII-VIII tubules) were isolated from adult rats and cultured. The tubule fragments were cultured for 20 hours in the presence of T (100 nM). Addition of the S1 peptide inhibitor of the non-classical T

signaling pathway (5 mM) reduced sperm release by 50% compared to the S1s scrambled control peptide (Fig. 1C).

We also found that the S1 inhibitor disrupts tight junctions that are essential to form the blood-testis barrier (BTB), which is required to maintain spermatogenesis and male fertility. Sertoli cells cultured on membrane supports in bicameral units were treated with S1 peptide in the presence of T (100 nM). Measurements of transepithelial resistance were used to assay the integrity of tight junction formation in the cultures. The S1 peptide (5 mM) decreased tight junction integrity by 52% compared to T stimulation alone (Fig. 1D), which was similar to the 46% inhibition by the MAP kinase inhibitor PD98059 (50 μM), a known disruptor of tight junctions. These data indicate that the S1 peptide is an effective inhibitor of the non-classical pathway and that inhibition of the pathway disrupts the tight junctions and sperm release that are essential for fertility.

Milestones and significance of results

Aim 1 was 80% completed. We found that a peptide inhibitor of non-classical T signaling inhibited sperm release from cultured rat seminiferous tubules by 50%. This finding fulfilled the major goal of this aim and provides the important conclusion that that non-classical T signaling contributes to the release of sperm. An additional finding that was not planned was that inhibition of non-classical signaling disrupts the Sertoli-Sertoli junctions that are required to maintain the BTB. Our findings from Aim 1 indicate that non-classical T signaling contributes to the release of sperm as well as to maintaining the BTB, two processes that are essential to maintain male fertility. These findings are significant because inhibitors of the non-classical pathway may be developed as targets for male contraception development.

Studies intended to determine the efficiency of inhibitor uptake by Sertoli cells in the cultured tubule fragments were not completed (20% of the goals).

Aim 2: Determine whether inhibiting classical or non-classical T signaling blocks spermatogenesis in testis explants.

Development of a testis explant culture model

We developed an organ culture system to maintain testis tissue fragments in culture for at least 7 days. These testis explant cultures contain all of the cell types of the testis and maintain functional cell-to-cell connections and interactions as *in vivo* and, thus, are a useful model to study non-classical T effects on spermatogenesis.

To optimize the organ culture system, we conducted an experiment to determine whether the size of cultured testis explants affected seminiferous tubule morphology. Adult rat testis tissue was cut into fragments either 2 or 4 mm in diameter and cultured for 2 to 4 days on agarose platforms that provided media by diffusion and access to oxygenated atmosphere. Testis tissue that was not cultured was included as a control. Testis tissue sections were stained with periodic acid-Schiff's base-hematoxylin (PAS-H) and analyzed for tubule morphology using light microscopy. These studies revealed that the morphology of the seminiferous tubules is maintained similarly in 2-mm and 4-mm testis tissue fragments through 4 days in culture (Fig. 2). Further studies showed that testis tissue integrity could be maintained through at least 7 days of culture with little cell death

(data not shown).

The Non-Classical Actions of Testosterone in Explants Can be Disrupted by Inhibitors.
To determine whether the non-classical pathway can be inhibited in testis explants, a peptidomimetic inhibitor of AR-Src interactions named D1 was incubated with the explants for 2 hours, after which the explants were placed on agarose platforms and cultured for 2 days. The testis explants were then fixed in 4% paraformaldehyde and probed with antisera against phosphorylated-Src, which is the first protein to be activated in the non-classical pathway. The levels of Src phosphorylation did not appear to be decreased by D1 near the Sertoli cell–elongated spermatid cell junction at the interior (lumen) of seminiferous tubules (Fig. 3). However, the D1 inhibitor caused a decrease in the levels of phosphorylated Src observed in the Sertoli cell cytoplasm near the basement membrane and at junctions of Sertoli cells with less mature germ cells. The sites of Src inhibition mirror that for localization of the D1 inhibitor. The D1 inhibitor was localized in the testis explant tissue sections by probing for a covalently attached FITC tag. Probing of the sections with anti-FITC antiserum, revealed that after addition to the explants the peptidomimetic D,1 as well as the S1s peptide, were localized primarily near the basement membrane; however, some FITC staining from inhibitors is present toward the lumen (Fig. 4).

Testis explants treated with D1 or controls were also probed with antisera against phosphorylated CREB, a transcription factor that is a target of non-classical pathway kinase activity. The phosporylation of CREB results in the activation of CREB-inducible genes that we have found to be essential in Sertoli cells to maintain spermatogenesis. Phosphorylated CREB was observed in the nuclei of Sertoli cells in testis explants that were untreated or treated with the D2 negative control peptidomimetic (Fig. 5). In contrast, testis explants that were treated with D1 exhibited decreased activation of CREB in the nuclei of Sertoli cells. These data suggest that the D1 inhibitor blocks T activation of CREB via the non-classical pathway.

We have also made progress in the use of adenoviral vectors to deliver inhibitors of classical and non-classical T signaling to Sertoli cells within testis explants. Testis explants isolated from 28-day-old rats treated with no adenovirus or adenovirus expressing control β-galactosidase protein contained seminiferous tubules with normal morphology (Fig. 6). In contrast, treatment with an adenovirus expressing the S1 inhibitor resulted in seminiferous tubule cross sections that contained vacuoles due to the loss of germ cells. Treatment with an inhibitor of classical T signaling (HDAC-KRAB-AR122) caused germ cells to be mislocalized away from the basement membrane toward the lumen, an indication that the germ cells were released prematurely. Treatment with both classical and non-classical inhibitors also caused vacuoles to appear; however, mislocalized germ cells were not as frequently observed as with HDAC-KRAB-AR122 alone. These results indicate that inhibition of either the classical or non-classical pathway disrupts spermatogenesis by different mechanisms in testis explants.

Pathway selective activators can induce gene expression in testis explants.
During the second 6-month period, we built upon the success of our studies of inhibitors in testis explants to expand our studies to determine whether activators of classical and non-classical signaling could alter T signaling and spermatogenesis in testis explants. For these studies, explants were cultured from AR-defective testicular feminized (tfm) rats in which T-mediated

gene expression is inhibited and spermatogenesis is blocked during meiosis. The explants were infected on the first day of culture with adenovirus constructs expressing wild-type AR or mutant ARs that selectively activate either only the classical pathway or only the non-classical pathway. Adenovirus constructs do not efficiently infect germ cells, but they do infect Sertoli cells with high efficiency. RNA was extracted from the explants 7 days after adenovirus infection and reverse transcription-polymerase chain reaction assays were performed to determine the expression levels of the housekeeping gene GAPDH and Rhox5, a homeobox gene that is highly inducible by T via the classical signaling pathway. Rhox5 expression in the tfm explants was below the level of detection in the absence of exogenous AR expression. In contrast, Rhox5 was stimulated after infection of adenovirus constructs expressing wild-type AR or the classical pathway activator (AdARΔ372-385) (Fig. 7). As expected, an adenovirus expressing an activator of the non-classical pathway (AdARC562) did not stimulate Rhox5 expression. Together, these data confirm that our AR mutants can be used to selectively activate pathway-specific T signaling in testis tissue.

Further studies were performed using explants isolated from wild-type rats and adenovirus constructs expressing selective inhibitors of the classical or non-classical pathways. In these studies, Rhox5 expression was observed in uninfected explants and explants infected with a control adenovirus expressing enhanced green fluorescent protein (EGFP) (Fig. 7). Infection with adenoviruses expressing either classical or non-classical inhibitors resulted in decreased Rhox5 expression and both inhibitors together synergized to decrease Rhox5 expression further. These data again indicate that T signaling in explants can be regulated by inhibitors of the classical and non-classical pathways. Although inhibition of classical pathway–regulated Rhox5 gene expression with the classical pathway inhibitor was expected, the decreased expression of Rhox5 in the presence of the non-classical inhibitor was not anticipated. The non-classical inhibitor did not alter the expression of the GAPDH control gene, suggesting that the inhibitor does not have a general poisoning effect on gene expression or Sertoli cells' health. It is possible that inhibition of non-classical T signaling may alter regulatory pathways in Sertoli cells such that T-mediated gene expression is indirectly inhibited.

The findings that activators and inhibitors of T signaling can regulate gene expression in explants raised the possibility that pathway-specific activators could be used to alter Sertoli cell functions that regulate germ cell development. To test this hypothesis, the expression of genes expressed specifically in spermatogonia germ cells was assayed in testis explants isolated from tfm rats in which spermatogenesis is halted. T and AR are known to not directly regulate expression of genes in germ cells.

Expression of the spermatogonia-specific genes PLZF and c-kit in the cultured explants was assayed by quantitative PCR (qPCR). Infection of the explants with an adenovirus expressing wild-type AR resulted in a fivefold increase in expression of the PLZF gene (Fig. 8). Activation of only the classical pathway with the ARΔ372-385 mutant was as effective as wild-type AR in increasing PLZF expression, whereas, the non-classical pathway activator ARC562G increased PLZF expression in spermatogonia by two-fold. Expression of the classical and non-classical pathways together further increased PLZF expression, but the increase was not statistically significant. The c-kit gene was stimulated to a similar extent after infection of Sertoli cells with wild-type AR, the classical pathway activator, the non-classical pathway activator, or the

combination of the classical and non-classical pathway selective AR mutants. These data indicate that activation of the classical pathway in Sertoli cells is more effective than the non-classical pathway in supporting the expression of PLZF, which is a marker for undifferentiated spermatogonia. Activation of either the classical or the non-classical pathway in Sertoli cells increased c-kit expression, which is a marker for the differentiated spermatogonia. Together, these results support the idea that the classical and non-classical pathways both contribute to providing the factors that are required for the progression of spermatogenesis. These data provide important support for the funding of future studies in which transgenic mouse models expressing only the classical or only the non-classical pathway will be created to identify the spermatogenesis processes that are regulated by each pathway.

Milestones and significance of results

Aim 2 was 90% completed. Our studies have shown that we can use testis explants as a model system in place of performing studies on testes *in vivo*. Using our explant culture system we found that peptide and petidomimetic inhibitors of the non-classical pathway could be used to block the non-classical T signaling pathway in an intact testis culture system. Furthermore, we found that adenovirus constructs expressing classical or non-classical pathway inhibitors caused specific disruptions of spermatogenesis in the explants. We proved that expression of pathway-selective activators and inhibitors could regulate Sertoli cell–specific T-mediated expression in the explants. Finally, our studies show that activation of the classical or non-classical pathway in Sertoli cells results in altered transcription of genes in undifferentiated and differentiated spermatogonia that are essential for the progression of spermatogenesis.

Plans to assay the activation of members of the non-classical pathway in testis explants after blocking all T signaling using flutamide, followed by washing away the flutamide and then stimulating with T, were not performed. However, the goals of this experiment were fulfilled using an improved strategy in Aim 3. The rationale for this decision was that we were able to replace the explant culture model with an *in* vivo system in which T signaling could be rapidly blocked and restored in testes (See the Cetrorelix model in Aim 3).

Aim 3: Determine whether the classical and/or non-classical pathway is required to maintain spermatogenesis *in vivo*.

Deletion of AR exon 3 blocks classical and non-classical signaling.

It has been proposed that only the classical pathway is required for spermatogenesis because spermatogenesis was halted during meiosis in transgenic mice in which exon 3 of AR, containing a portion of the DNA binding domain, was removed. However, we have found that the removal of exon 3, encoding more than 50 amino acids of AR, also abolished non-classical T signaling in Sertoli cells. A Sertoli cell line (15P-1) that does not express AR was infected with adenovirus constructs expressing wild-type AR (AdARwt) or the exon 3-deleted mutant (AdARΔexon3). T stimulation induced ERK phosphorylation through wild-type AR but not AdARΔexon3 (Fig. 9). These results indicate that deletion of AR exon 3 disables both the classical and non-classical signaling pathways and that further studies are required to determine the relative contributions of each pathway toward maintaining spermatogenesis and fertility.

Use of the Cetrorelix model to show that non-classical T signaling increases ERK phosphorylation in Sertoli cells in vivo.
To test whether non-classical T signaling occurs in Sertoli cells *in vivo*, we developed a rat model in which T levels could be rapidly manipulated. Adult rats were injected intraperitoneally with the gonadotropin-releasing hormone antagonist Cetrorelix (50 μg/100 μl) to transiently decrease gonadotropin (luteinizing hormone and follicle-stimulating hormone) secretion and T levels. After 7 hours, testicular T levels fell to 16% of control levels, in agreement with previous studies (Fig. 10A). Subsequent injections of T propionate (TP, 5 mg, ip) plus T enanthate (TE, 10 mg, im) first elevated testicular T levels after 30 minutes, and T increased to 137% of control levels after 1 hour. These initial studies determined that intratesticular T levels could be rapidly decreased and increased in rats.

To determine whether intratesticular T levels correlated with ERK kinase phosphorylation, a key component in the non-classical T signaling pathway, phosphorylated ERK (p-ERK) levels were assayed in T-sensitive stage VII seminiferous tubule cross sections. p-ERK immunostaining decreased to 34% of normal levels 7 hours after Cetrorelix injection but, then, was restored to 103% of control levels 1 hour after injection of TP and TE (Fig. 10B). Inspection of stage VII cross sections revealed that, in control and Cetrorelix + T-treated rats, p-ERK immunostaining was present in the Sertoli cell cytoplasm associated with the BTB region and Sertoli–elongated spermatid adhesion sites (Fig. 10C). In contrast, p-ERK staining was reduced at both sites after Cetrorelix treatment alone.

The Cetrorelix model data show that testicular T levels can be rapidly decreased and then restored to normal levels within 1 hour. Importantly, ERK kinase phosphorylation is rapidly increased within 1 hour after administration of TP and TE to restore T levels. Because at least 30 minutes is required to elevate T levels in the testis after TP and TE injections (data not shown), and T-stimulated transcription requires 30 to 45 minutes to be productive, with protein production occurring even later, the increase in p-ERK levels observed within 1 hour is not consistent with a classical pathway model. The rapid increase in T-mediated p-ERK levels supports the hypothesis that the non-classical pathway activates kinase signaling in Sertoli cells *in vivo*.

Spermatogenesis is disrupted after inhibition of either classical or non-classical signaling.
To determine whether inhibition of the classical or non-classical pathways disrupts spermatogenesis, adenovirus constructs expressing pathway-selective inhibitors were injected into the testes of 14-day-old mice via the rete testis. We and other groups have shown that adenovirus constructs at this concentration (50 μl of 1 x 10^{10} particles/ml), injected by this protocol, infect only Sertoli cells and do not cause an immune response. Testes were isolated 4 days after injection and fixed in Bouin's solution and embedded in paraffin. Staining of testis sections with periodic acid-Schiff's base-hematoxylin revealed that injection of an adenovirus expressing EGFP (AdEGFP) did not affect testis morphology or spermatogenesis (Fig. 11A**)**. In contrast, after injection of an adenovirus expressing the S1 inhibitor of the non-classical pathway, spermatogenesis was disrupted, with spermatocytes (the most advanced germ cell at this age) being absent in at least 50% of the tubules (Fig. 11B).

Further studies of adult mice injected with adenoviruses via the rete testis revealed that the

adenovirus-mediated expression of the EGFP control protein had no effect (Fig. 11C), but the S1 non-classical inhibitor (AdS1) caused vacuoles in the seminiferous epithelium due to the loss of spermatocytes (Fig. 11D). AdH-K-AR122, which inhibits classical signaling, caused immature germ cells to be released prematurely into the lumen of seminiferous tubules (Fig. 11E). The combined inhibition of both pathways resulted in an additive effect including the loss of spermatocytes and the detachment of immature germ cells (Fig. 11F). These data indicate that both T signaling pathways are required to maintain spermatogenesis and that alteration of either signaling pathway contributes to a distinct defective spermatogenesis phenotype. Notably, these results were very similar to those observed using the testis explant models, thus providing further support for the hypothesis that inhibition of each pathway results in specific disruptions of spermatogenesis.

To determine the integrity of the BTB after inhibiting the non-classical pathway, 50 μl of EZ-Link Sulfo-NHS-LC-Biotin was injected into the tubules of adenovirus-infected testes 30 minutes before sacrifice. Testis sections were probed for the presence of the biotin tracer. These studies revealed that, in testes injected with adenovirus constructs expressing a control peptide, the BTB was intact as the biotin was excluded from all but the basement membrane portions of seminiferous tubules (Fig. 12**)**. In contrast, the biotin tracer was found throughout numerous tubules in testes injected with adenovirus constructs expressing a non-classical inhibitor. This finding indicates that inhibition of the non-classical pathway disrupts the integrity of the BTB.

Milestones and significance of results

Aim 3 was 70% completed. The results of the studies in Aim 3 provide solid evidence that the non-classical T signaling pathway is activated *in vivo* and that inhibition of either the non-classical or the classical pathway in Sertoli cells *in vivo* causes distinct disrupted spermatogenesis phenotypes. Specifically, we found that inhibiting the non-classical pathway caused the loss of spermatocytes undergoing meiosis, whereas inhibition of the classical pathway cause the detachment of most germ cells after the spermatogonia stage of development. These findings will contribute to the identification of T signaling control defects in infertility patients that can be targeted for therapies. Also, our results provide new directions for the development of male contraceptive drugs that would block early steps of spermatogenesis before the production of sperm but not affect other T-dependent processes.

Due to time constraints, we were unable to perform assays of actin expression and localization as well as p-ERK and p-CREB expression in tissue sections from testes infected with adenovirus constructs expressing pathway-specific inhibitors. We were unable to determine whether inhibition of the classical or non-classical pathway *in vivo* blocked sperm release because the extensive disruption of the seminiferous epithelium did not allow identification of the stages during which the sperm are released.

Published abstracts, poster presentations

Walker, WH, Shupe, J, Puri, P, Toocheck, C, Paranzino, P and Raj, G (2012) Regulation of Spermatogenesis by Non-classical Testosterone Signaling. Society for the Study of Reproduction Annual Meeting. July, State College, PA

Figures:

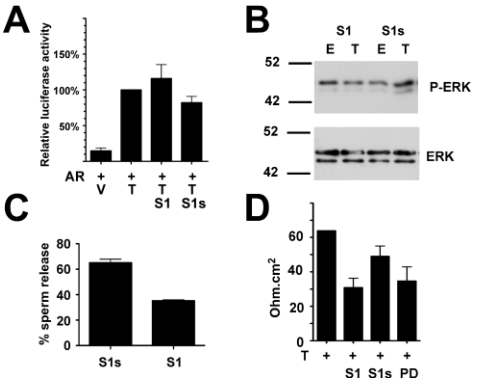

Fig. 1. The S1 peptide inhibits non-classical T signaling. (A) 15P-1 Sertoli cells transfected with the PSALuc reporter plasmid and an AR expression vector were stimulated with vehicle (V) or T for 24 h in the presence of 5 μM S1 or S1s. The relative luciferase levels were determined from whole cell lysates (n=3). (B) S1 or S1s (S1 scrambled) peptides were added to Sertoli cell cultures 2 h prior to stimulation with ethanol (E) control or T (100 nM). Western analysis of whole cell extracts are shown for p-ERK and total ERK (T-ERK). (C) The percentages of sperm released from stage VII-VIII tubule fragments are shown for a 20 h incubation with S1 or S1s, (n=3). (D) Sertoli cells isolated from 20-day-old wild-type rats were incubated with V, T, T + S1, or S1s peptides (5 μM) or the MAPK inhibitor PD98059 (PD, 50 μM). Transepithelial resistance was assayed (n=3).

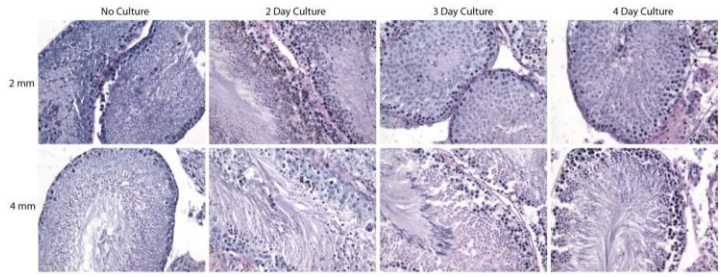

Fig. 2: Analysis of tubule morphology of adult rat testis explant fragments. Images of PAS-H stained testis explant tubules 2 mm in diameter (top) or 4 mm in diameter (bottom) are shown (40x magnification). Tissue was fixed immediately after isolation or 2, 3, or 4 days in culture. Images are representative of three replicate experiments.

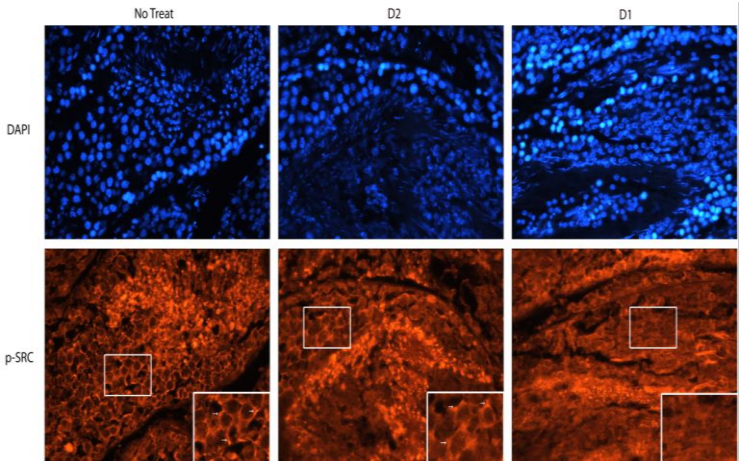

Fig. 3: Adult rat testis tissue explants incubated in media containing D1, D2, or media only (no treat control) were probed using DAPI nuclear stain (blue, top) or antisera against phosphorylated-Src (p-Src, bottom) to determine whether D1 inhibited the AR-mediated activation of the non-classical pathway in approximately stage VII seminiferous tubules. The levels of p-Src (red) in the Sertoli cytoplasm between germ cells (boxes) were decreased in D1-treated testis tissue. Areas within the boxes were enhanced to show detail (bottom right corner of each picture).

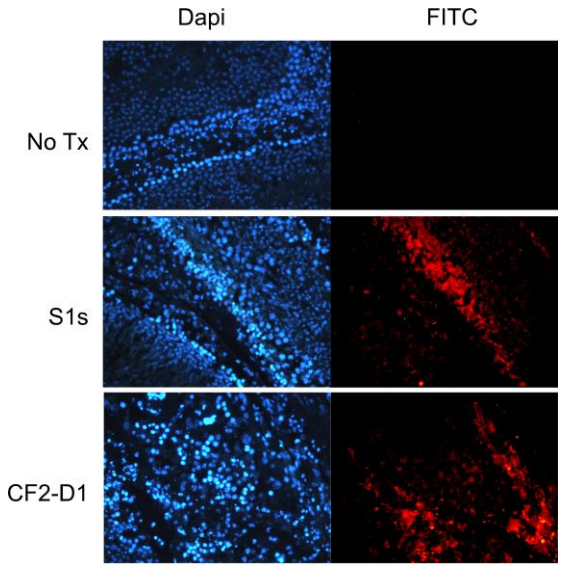

Fig. 4: Inhibitors are localized at the basement membrane of treated testis tissue explants. Explants were treated with culture media only (No Tx) or fluorescently labeled S1 scrambled (S1s) or D1 (CF2-D1). Inhibitors were probed using antisera against FITC (red). Nuclei were stained with DAPI (blue). Pictures were taken at 40x magnification.

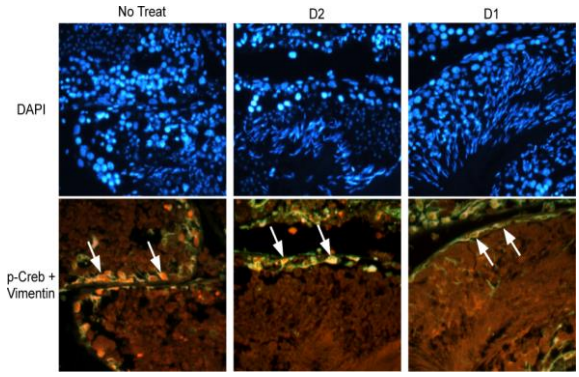

Fig. 5: D1 inhibits the phosphorylation of CREB in the nuclei of Sertoli cells in testis tissue explants. Adult rat testis tissue incubated in media containing D1, D2, or media only (No Treat) were probed using antisera against p-CREB (red) and Sertoli cell–specific vimentin (green). Nuclei were stained with DAPI. Sertoli cell nuclei are indicated by white arrows. Images were taken at 60x magnification and are representative of two experiments.

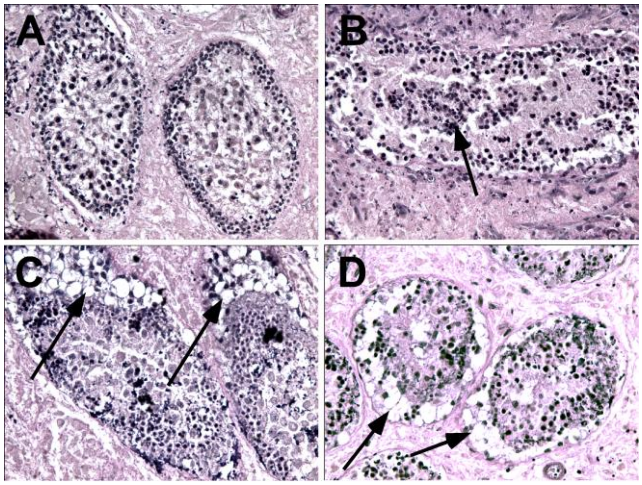

Fig. 6: Inhibitors of classical and non-classical T signaling disrupt spermatogenesis in testis explants. Explants were incubated with adenovirus expressing (A) control β-galactosidase, (B) classical pathway inhibitor HDAC-KRAB-AR122, (C) non-classical pathway inhibitor S1, or (D) HDAC-KRAB-AR122 + S1. Arrows point to germ cells mislocalized to the center of the tubule that have lost adhesion to Sertoli cells (B) or to vacuoles where germ cells have been lost (C, D). Images were taken at 40x magnification and are representative of two experiments.

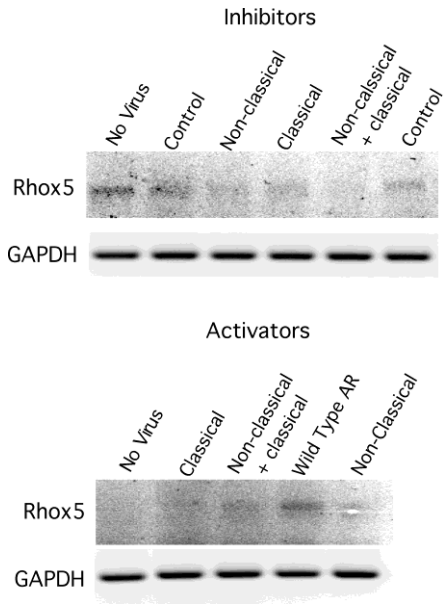

Fig. 7. Rhox5 is expressed in testis explants and is regulated by classical and non-classical signaling. (A) Testis explants from 35-day-old wild-type rats were exposed for 2 h to media alone (no virus), adenovirus constructs expressing control (β-galactosidase), non-classical inhibitor S1, classical inhibitor H-K-AR122, or classical + non-classical inhibitors or control inhibitor S1 scrambled. (B) Testis explants from 35-day-old AR-defective (tfm) rats were exposed for 2 h to media alone (no virus), adenovirus constructs expressing AR that activates only classical, non-classical, or classical + non classical pathways or wild-type AR.

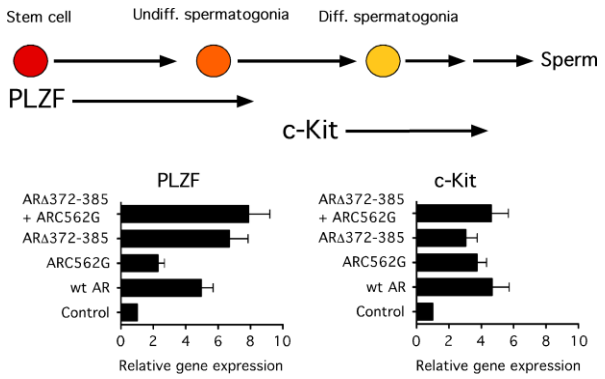

Fig. 8. Pathway-selective AR mutants increase spermatogonia-specific gene expression in testis explants from tfm rats. Top: The initial steps of spermatogenesis are provided, with the expression patterns of the PLZF and c-kit genes. Bottom: The results of qPCR assays of testis explants from tfm rats. The relative gene expression is provided for the PLZF and c-kit genes after infection of the explants with adenovirus construct expressing EGFP (control), wild-type AR (wt-AR), non-classical pathway activator (ARC562G), classical pathway activator (ARΔ372-385), or both activators (ARC562G + ARΔ372-385) (n=3).

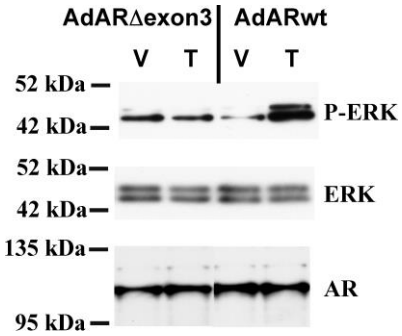

Fig. 9. Deletion of exon 3 eliminates non-classical AR activity. 15P-1 Sertoli cells were infected with adenovirus constructs expressing wild-type AR (AdhisAR) or exon 3–deleted AR (AdARΔexon3). p-ERK, ERK, and AR levels were determined by Western blot after stimulation (10 min) with vehicle (V) or T.

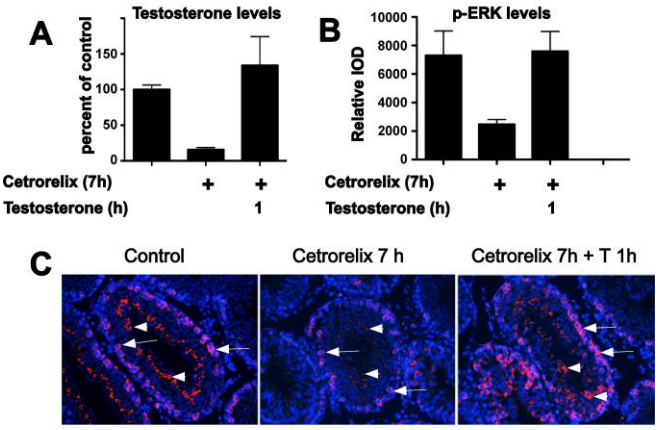

Fig. 10. T stimulates rapid ERK phosphorylation in Sertoli cells *in vivo*: The Cetrorelix model for deprivation and restoration of T. (A) T levels in control testes and after 7 h of Cetrorelix treatment or 7h Cetrorelix followed by 1 h T (n=3). (B) p-ERK immunostaining was quantified as relative integrated optical density (IOD) in stage VII tubules without treatment and 7h after Cetrorelix treatment and Cetrorelix (7h) + T for 1 (n=5). (C) Representative immunofluorescence microscopy images of p-ERK (red) staining in stage VII tubules in control, and after Cetrorelix (7h) or Cetrorelix + T (1h) treatments. Arrows indicate p-ERK staining in the BTB region, and arrowheads denote p-ERK staining associated with elongated spermatids. Nuclei are stained blue with DAPI.

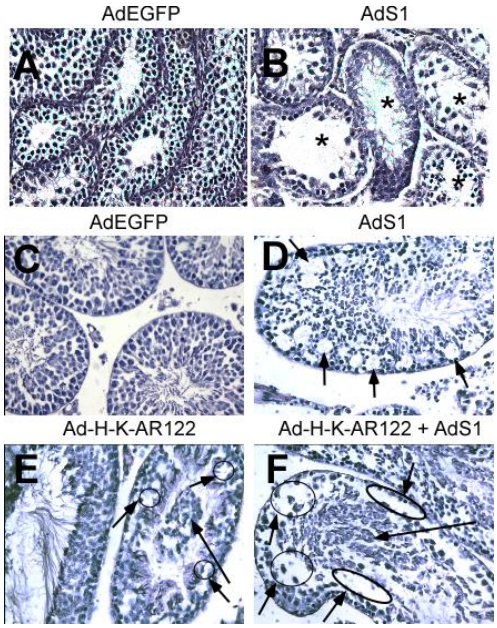

Fig. 11. Peptides inhibiting classical or non-classical signaling disrupt spermatogenesis. A+B**:** Hematoxylin staining of 18-day-old mouse testis 4 days after injection of (A) control AdEGFP (B) non-classical pathway inhibitor AdS1. C-F**:** Hematoxylin staining of adult mouse testis 4 days after injection of (C) AdEGFP (D) AdS1, (E) classical pathway inhibitor Ad-H-K-AR122, and (F) AdS1 + Ad-H-K-AR122. Asterisks (*) indicate tubules with disrupted spermatogenesis in panel B. Short arrows depict vacuoles lacking germ cells. Long arrows depict prematurely detached germ cells in the lumen. Circles and ovals indicate regions lacking germ cells.

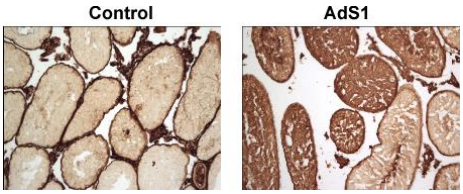

Fig. 12. The S1 inhibitor disrupts the BTB. Adult mouse testes were injected with adenovirus constructs expressing EGFP (control) or the S1 non-classical pathway inhibitor. Prior to sacrifice a biotin tracer was injected into the testes. A colorimetric staining for biotin (brown) showed that biotin lined the perimeter of control injected testes, but biotin penetrated the BTB and was present throughout about70% of seminiferous tubules in testes injected with adenovirus expressing S1.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
____X_No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
__X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two

publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. None				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____X_____ No_____

If yes, please describe your plans:

A manuscript summarizing our results for the funding period will be submitted to Molecular Endocrinology within the next 3 months.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

This proposal will benefit the 10% to15% of all couples that are unable to have children due to infertility. In half of the infertile couples, there is a male defect. The major objective of this proposal was to identify new mechanisms and factors through which testosterone acts to support spermatogenesis and fertility. Our studies determined that testosterone acts via both the classical and non-classical signaling pathways to support spermatogenesis and male fertility. We found that each pathway is required for distinct processes that are essential to

maintain spermatogenesis. With the finding that the non-classical pathway is required for the survival of developing germ cells, we have now identified the members of the non-classical pathway cascade of kinases and proteins the proteins and genes that they affect as novel targets for the development of male contraceptive drugs. In addition, our findings will facilitate the design of therapies to cure specific defects in testosterone-mediated signaling that cause infertility.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:

Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No___

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes__ No___

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
William H. Walker	Associate Professor of Obstetrics, Gynecology and Reproductive Sciences

EDUCATION/TRAINING

Univ of North Carolina, Chapel Hill	B.S.	1983	Biology
Univ of Tx Health Science Center, Houston	Ph.D.	1990	Biochem/ Mol. Biol.
Duke University Medical Center, Durham, NC	Post-doc	1990-1991	Mol Immunology
Massachusetts General Hospital, Harvard Medical School, Boston, MA	Post-doc	1992-1995	Molecular Endocrinology

A. Personal Statement

The goal of the proposed research is to determine the molecular mechanisms by which testosterone regulates spermatogenesis and male fertility. We will investigate the effects of selectively blocking classical and non-classical testosterone signaling in Sertoli cells *in vivo*. These studies will utilize the expertise that I have accumulated from previous studies of the hormonal regulation of gene expression, signal transduction, regulation of spermatogenesis and testis structure and function. As a postdoctoral fellow at Duke, I studied the structure and function of NF-χB transcription factors in immune cells. During my fellowship at Massachusetts General Hospital, I carried out studies of the regulation of members of the CREB family of transcription factors by follicle-stimulating hormone and other stimuli in Sertoli cells. As a P.I. at the University of Pittsburgh on several previous NIH-funded grants, I have characterized the expression of NF-χB transcription factors in the rat testis and determined that CREB expression in Sertoli cells is essential for the survival of spermatocyte germ cells and fertility. I have also determined that the ID2 transcriptional repressor regulates expression of the follicle-stimulating hormone receptor and that USF transcription factors are differentially regulated during Sertoli cell development. Finally, I have found a new (non-classical) mechanism of action for testosterone in the Sertoli cell such that testosterone activates a cascade of kinases that contribute to maintaining spermatogenesis.

B. Positions and Honors.

Positions and Employment

1990-91	Postdoctoral Associate, Duke University Medical Center, Durham, NC
1991	Research Associate, Howard Hughes Medical Institute, Duke Univ. Medical Center, Durham, NC
1992-95	Research Associate, Howard Hughes Medical Institute, Massachusetts General Hospital, Harvard Medical School, Boston, MA
1995-96	Instructor in Medicine, Harvard Medical School, Boston, MA
1996-2004	Assistant Professor, University of Pittsburgh School of Medicine, Cell Biology and Physiology Department, Pittsburgh, PA
2004-present	Associate Professor, University of Pittsburgh School of Medicine, Cell Biology and Physiology Department, Pittsburgh, PA

C. Selected peer-reviewed publications (selected from 37 peer-reviewed publications).

Most relevant to the current project

1. Fix CA, Jordon C, Cano P, **Walker WH,** (2004) Testosterone activates mitogen-activated protein kinase and the cAMP response element binding protein transcription factor in Sertoli cells. Proc Nat Acad. Sci. USA 101:10919-10924. PMID: 15263086
2. Cheng J, Watkins, SC, **Walker, WH**, (2007) Testosterone activates mitogen-activated protein kinase via Src kinase and the epidermal growth factor receptor in Sertoli cells. Endocrinology 148: 2066-2074. PMID: 17272394
3. Viswanathan P, Wood MA, **Walker WH**. (2009) Follicle-stimulating hormone (FSH) transiently blocks FSH receptor transcription by increasing inhibitor of deoxyribonucleic acid binding/differentiation-2 and decreasing upstream stimulatory factor expression in rat Sertoli cells. Endocrinology150: 3783-3791. PMID 19423764
4. Shupe J, Cheng J, Puri P, Kostereva N, **Walker WH.** (2011) Regulation of Sertoli-germ Cell Adhesion and Sperm Release by FSH and Non-classical Testosterone Signaling. Molecular Endocrinology 25:238-252 PMID: 21177760
5. Wood MA, Mukherjee, P, Toocheck, C. **Walker WH** (2011) Upstream Stimulatory Factor (USF) Induces *Nr5a1* and *Shbg* Gene Expression During the Onset of Rat Sertoli Cell Differentiation. Biology of Reproduction. 85: 965-976. PMID: 21734262

Additional publications of importance to the field (in chronological order)

1. **Walker WH**, Stein B, Ganchi PA, Kaufman PA, Ballard DW, Hannink M and Greene WC (1992) The v-Rel oncogene: insights into the mechanism of transcriptional activation, repression and transformation. J Virol 66:5018-5029. PMID: 1321284
2. **Walker WH**, Sanborn BM, Habener JF (1994) An isoform of transcription factor CREM expressed during spermatogenesis lacks the phosphorylation domain and represses cAMP-induced transcription. Proc Natl Acad Sci USA 91:12423-12427. PMID: 7809053
3. **Walker WH**, Fucci L, Habener JF (1995) Expression of the gene encoding transcription factor cyclic adenosine 3',5'-monophosphate (cAMP) response element-binding protein (CREB): regulation by follicle-stimulating hormone-induced cAMP signaling in primary rat Sertoli cells. Endocrinology 136:3534-3545. PMID 7628390
4. Delfino F, **Walker WH** (1998) Stage-specific nuclear expression of NF-κB in mammalian testis. Mol. Endo. 12:1696-1707. PMID: 9817596
5. Delfino F, **Walker WH** (1999) NF-κB induces cAMP-response element binding protein gene transcription in Sertoli cells. J. Biol. Chem. 274:35607-35613. PMID: 10585438
6. Scobey J, Sommers J, Butera S, Graham-Humphrey N, Watkins S, Zeleznik AJ, **Walker WH** (2001) Delivery of a cyclic adenosine 3’, 5’-monophosphate response element-binding protein (CREB) mutant to seminiferous tubules results in impaired spermatogenesis. Endocrinology. 142:948-954. PMID: 11159868
7. Wood MA, **Walker WH**. (2009) USF1/2 transcription factor DNA-binding activity is induced during Sertoli cell differentiation. Biol Reprod 80:24-33. PMID: 18768914