Award Number: W81XWH-12-1-0192

TITLE: Cell of Origin: Exploring an Alternative Contributor to Ovarian Cancer

PRINCIPAL INVESTIGATOR: Bo R. Rueda, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, MA 02114-2696

REPORT DATE: December 2015

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2015	Final	1Sep2012 - 31Aug2015

4. TITLE AND SUBTITLE

Cell of Origin: Exploring an Alternative Contributor to Ovarian Cancer

5a. CONTRACT NUMBER
W81XWH-12-1-0192

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Bo R. Rueda, Ph.D.

E-Mail: BRUEDA@PARTNERS.ORG

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Massachusetts General Hospital
55 Fruit Street
Boston, Massachusetts 02114-2696

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our studies to date have determined that human oogonial stem cells, while far less stable than their murine counterparts, can be successfully expanded and propagated in culture. This has allowed us to begin testing the potential tumorigenicity of these cells with the ultimate goal of comparing the DNA signature of the oogonial stem cell-derived tumors to that of primary human ovarian cancer. We have also successfully introduced in human oogonial stem cells genetic alterations commonly detected in ovarian cancer. We are now generating tumors from these altered oogonial stem cells and will compare the histologic features in the tumors formed from the modified oogonial stem cells to those of primary tumors collected from women diagnosed with serous ovarian cancer. Also of importance is the identification of Ddx4-positive cells in xenografts derived from primary human ovarian serous tumors. The biological significance of this apparent rare population is yet to be determined. We have designed and implemented initial studies to test the relative tumorigenicity of Ddx4-positive, CD133-positive and Ddx4 CD133 double positive fractions. Demonstrating that these highly specialized human oogonial stem cells have the capacity to form ovarian tumors would be a major paradigm shift.

15. SUBJECT TERMS

Serous Ovarian Cancer, Cell of Origin, Human Oogonial Stem Cells, Tumorigenicity, Ddx4

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	16	

Table of Contents

INTRODUCTION:

High grade ovarian cancer remains one of the most deadly gynecologic cancers. The high mortality rate is often attributed to our inability to detect early stage disease, the high incidence of recurrent chemoresistant disease and our limited understanding of the biology underlying early events in ovarian cancer development as reflected in continued debate surrounding the cell and site of origin of ovarian cancer. Our preliminary data generated using oogonial stem cells derived from the mouse ovary suggested that these rare cells have the capacity to form tumors in immunocompromised mice when 'misplaced' outside of the ovary. Specifically, while no tumor developed when oogonial stem cells were injected directly into the ovary, injection of the same cells into the mouse abdominal cavity resulted in tumors that histologically resemble ovarian cancer. Subsequent studies have identified the presence of a similarly rare oogonial stem cell population in the human ovary. We hypothesize that human oogonial stem cells either shed from the ovarian surface during ovulation or naturally present in an aged ovary have tumorigenic potential. Furthermore, we believe a genetic misprint will allow these cells to undergo additional changes common to the different ovarian cancer subtypes. Our objectives, therefore, were to determine the tumorigenic capacity of human oogonial stem cells and compare the DNA signature of the oogonial stem cell-derived tumors to that of primary human ovarian cancer. In addition, our objective was to induce in human oogonial stem cells genetic alterations commonly detected in ovarian cancer and compare the histologic features in the tumors formed from the modified oogonial stem cells to those of primary tumors collected from women diagnosed with serous ovarian cancer. Demonstrating that these highly specialized human oogonial stem cells have the capacity to form ovarian tumors would be a major paradigm shift.

Recently, the idea that these OSCs can be isolated by the cell surface marker DDX, also known as VASA, has become controversial. In 2012 Tilly et al. reported DDX4 had a carboxyl terminal domain that was expressed on the extracellular membrane and could be used to isolate OSCs. More recent data has challenged this finding. In addition, our own validation studies led us to question the surface expression of DDX4. Our original DOD proposal was predicated on the fact that these rare OSCs expressed DDX4 on the surface and DDX4 expression could be validated by semi-quantitative PCR using methods published by Tilly and colleagues. Given the recent controversy, we conducted additional validation experiments using more extensive methodology. We have completed these studies, which revealed some very interesting findings detailed below. While effort on our original aims was suspended until we could validate the identity of the cells we were working with, we now have resumed our original objective, which is to assess the tumorigenic capacity of the DDX4 positive OSCs. More recently, we requested and were granted a no cost extension to complete our objectives. Both our positive and negative results are described below.

BODY:

Task 1: Tumorigenic assessment of OSCs isolated from reproductive age and menopausal women.

Subtask 1a: In Aim 1 human OSCs isolated from a total of 10 patients (5 reproductive age women and 5 peri-/post-menopausal women) will be expanded in vitro under conditions established in our laboratory.

As mentioned in the previous report, both the ovarian samples and oogonial stem cell preparations/cultures isolated from pre- and post-menopausal ovaries obtained prior to receiving this funding did not survive long-term cryopreservation. We therefore modified our cryopreservation methods to ensure viability of both the ovarian samples and more importantly, the isolated oogonial stem cell cultures. Following optimization, we actively worked with MGH GYN Tissue Repository staff to accrue new samples and to restock the ovarian tissue required for the proposed studies. Since receiving the award, we have acquired, via the MGH GYN Tissue Repository, human ovarian tissue from nine different patients who fit the relevant clinical criteria. A subset of these ovarian samples has been processed to generate oogonial stem cell cultures. The remainder has been cryopreserved for future isolation of oogonial stem cells. The collected samples comprise ovarian tissue from pre- and post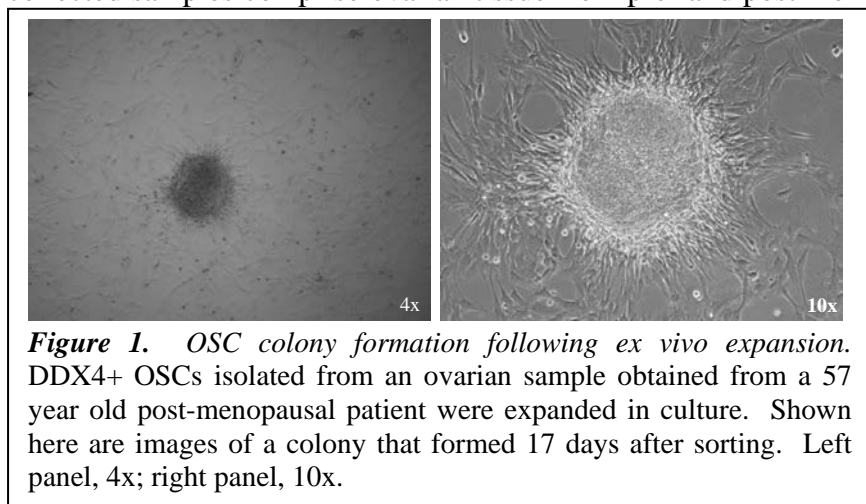-menopausal women. As expected, the majority of these samples are from post-menopausal women. We will continue to accrue samples in order to obtain sufficient numbers of ovaries from pre-menopausal women.

Figure 1. *OSC colony formation following ex vivo expansion.* DDX4+ OSCs isolated from an ovarian sample obtained from a 57 year old post-menopausal patient were expanded in culture. Shown here are images of a colony that formed 17 days after sorting. Left panel, 4x; right panel, 10x.

Three of the newly acquired ovarian samples were subjected to cell sorting to generate DDX4-positive oogonial stem cells. Cells obtained from two of the three sorted ovarian samples were healthy and could be successfully passaged and expanded for validation studies (Figure 1). In RT-PCR analyses, primers corresponding to *PRDM1*, *DPPA3*, *IFITM3*, *TERT*, *DDX4* and *beta –actin* as published by Tilly et al were used to confirm the oogonial stem cell profile of the cultured cells. Relevant controls included oocyte preparations, whole ovary tissue, testicular tissue (positive control) and fibroblasts (negative control).

Recent controversy over the isolation of OSCs via expression of the cell surface marker DDX4 (also known as VASA) is due in part to the historic consideration of DDX4 as an exclusively intracellular protein. In 2012 Tilly et al. reported DDX4 had a carboxyl terminal domain that was expressed on the extracellular membrane and could be used to isolate OSCs. Although current evidence supports both classifications of DDX4, our original DOD proposal was built on the assumption that these rare cells of interest expressed DDX4 on the surface and this expression could be validated by PCR using the methods published by Tilly and colleagues. Given the recent controversy, we sought to conduct additional validation steps using more extensive methodology.

Flow cytometric analysis and sorting

To further characterize the cellular expression of DDX4, we subjected fresh or fresh frozen human and mouse ovarian tissue to cell sorting to generate DDX4 positive and DDX4 negative populations using the antibody and sorting protocol published in White et al. The relative frequency of DDX4 antibody positive cells was highly variable with DDX4 antibody positive cells comprising 4.5-24% of human ovarian cells and 1.9-3.7% of mouse ovarian cells. Surprisingly, isolated antibody negative cells became highly antibody positive in culture (Figure 2). Furthermore, antibody positive, antibody negative and unsorted cells expressed similar levels of antibody positivity over sequential passages (Figure 2).
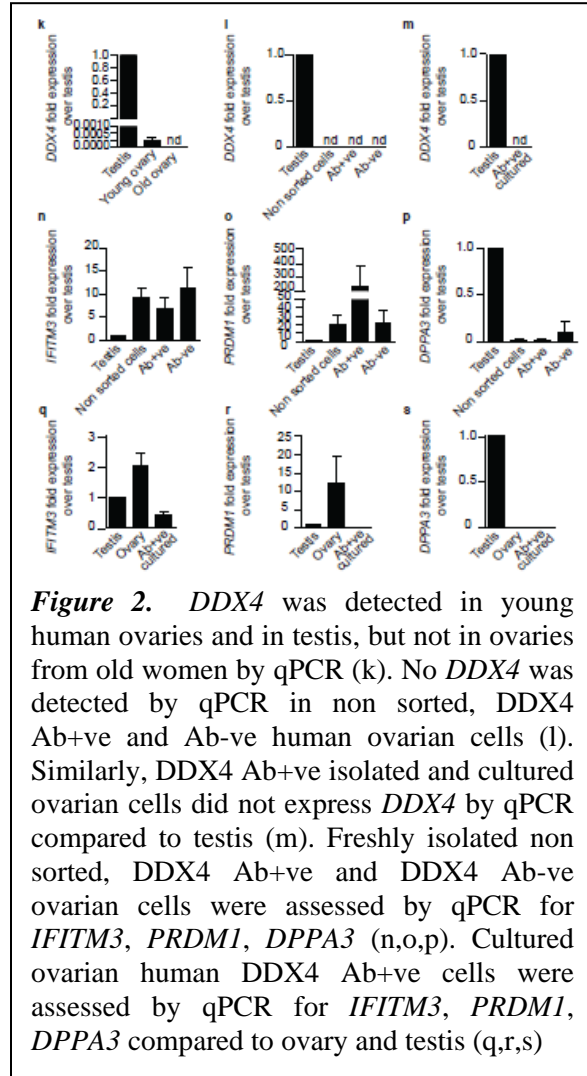

Figure 2. *DDX4* was detected in young human ovaries and in testis, but not in ovaries from old women by qPCR (k). No *DDX4* was detected by qPCR in non sorted, DDX4 Ab+ve and Ab-ve human ovarian cells (l). Similarly, DDX4 Ab+ve isolated and cultured ovarian cells did not express *DDX4* by qPCR compared to testis (m). Freshly isolated non sorted, DDX4 Ab+ve and DDX4 Ab-ve ovarian cells were assessed by qPCR for *IFITM3*, *PRDM1*, *DPPA3* (n,o,p). Cultured ovarian human DDX4 Ab+ve cells were assessed by qPCR for *IFITM3*, *PRDM1*, *DPPA3* compared to ovary and testis (q,r,s)

Expression of DDX4

As observed in the mouse, the level of *DDX4* expression in the human testicle is overwhelming when compared to expression in the ovary. Low *DDX4* expression was detected in the ovary derived from a younger woman (38 years of age) with no expression detected in the ovary of an older woman (57 years of age) consistent with the lack of oocytes observed on histology. Freshly isolated ovarian DDX4 antibody positive and negative cells were assessed by qPCR and compared to the unsorted populations. Neither population showed evidence of *DDX4* expression when compared to the positive control. Again mRNA expression levels of *PRDM1, IFITM3,* and *DPPA3,* were assessed immediately after sorting and again after culturing. With the exception of *PRDM1,* no clear pattern or differences were observed in *IFITM3,* and *DPPA3* among the sorted or cultured antibody positive and antibody negative cells.

Subtask 1b: The cells will be virally transduced to stably express luciferase, and then injected into the intraperitoneal cavity of immunocompromised (NOD/SCID) mice. The injections will be done in a dilution series, ($1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$ and $1x10^1$ cells, 2 mice per patient per dilution for a total of 100 mice). Cells will also be injected ($1x10^5$) directly into the ovaries of NOD/SCID mice (2 mice per patient for a total of 20 mice) using a 10 µL NanoFil syringe and a beveled 35gauge needle.

We generated a retrovirus that expresses both GFP and luciferase and infected pools of oogonial stem cells. The cell cultures were expanded to sufficient numbers for cell sorting to isolate GFP-positive infected cells injected into immunocompromised NOD/SCID mice by intraperitoneal injection as described in Subtask 1b at the highest dilutions to assess tumorigenicity. Even at the highest dilutions we observed no tumor formation. This was in direct contrast to what we had previously observed in our mouse studies. This was again repeated, at even higher cell concentrations, yet unlike the concurrent murine studies failed to develop any measureable tumor. The original objective required these cells to make measurable tumor to allow the completion of Subtasks 1c-1e. It is not clear why we failed to observe tumor formation. We speculate that either there is a species differences, the cells required additional genetic "hit(s)" or the DDX4 antibody positive cells we isolated were not pure oogonial stem cells. The last option is possible given that we determined that DDX4 antibody positivity did not ensure that the ovarian derived cells were in fact, all oogonial stem cells.

Subtask 1c: Tumor development, growth and metastatic potential will be evaluated by the Mouse Imaging Program (MGH/Harvard core facility). The rate of tumorigenesis as well as metastatic spread will be assessed. When tumors have reached a sufficient size for evaluation, mice will be sacrificed and tumors harvested. Each tumor will be divided into 3 equal pieces, one will be preserved in paraformaldehyde and processed for histological analysis, one snap-frozen for genomic analysis, and one vitrified and stored in liquid nitrogen for further analysis at a later date.

This subtask was not initiated.

Subtask 1d: For histological analysis as well as immunohistochemistry, PFA-fixed tumors will be embedded in paraffin, sectioned, placed onto slides and 5 sections per tumor will be stained with hemotoxylin and eosin for phenotypic analysis. Additionally, sections will be analyzed for p53, cytokeratin (CK) 7, CK20, PAX-8, WT-1, and ER by immunohistochemistry using commercially available antibodies.

This subtask was not initiated.

Subtask 1e: Genomic Assessment of Tumors. Gene array analysis will be conducted on Affymetrix gene chips

SAMPLE	PRIMARY SITE	HISTOLOGY	GRADE	DDX4- CD133-	DDX4+ CD133-	DDX4- CD133+	DDX4+ CD133+
OVCA1	Peritoneum	Serous	3	83.27	7.43	8.89	0.41
OVCA2	Peritoneum	Serous	3	73.1	10.6	9.04	7.26
OVCA3	Peritoneum	Serous	3	92.7	6.41	0.77	0.078
OVCA4	Ovary	Serous	3	69.1	26.1	3.69	1.05
OVCA5	Peritoneum	Serous	3	92.7	3.02	4.08	0.14
OVCA6	Peritoneum	Serous	3	86.9	11.9	0.71	0.41

Table 1. Flow cytometric analysis of DDX4 and CD133 expression in human ovarian cancer xenografts. The relative frequency (indicated as percent of the total viable population) of cells with the indicated DDX4 and CD133 expression phenotypes was determined in xenografts derived from six independent primary ovarian serous cancers. Note the consistent presence of a DDX4+CD133- population in all analyzed samples.

by our institutional core facility. The resulting information will be compared to published data

sets.

We also proposed to compare the genomic profile of the tumors generated from the oogonial stem cells with the profile of xenografts derived from primary human serous ovarian cancer and/or primary human serous peritoneal cancer. Toward this goal, we have generated xenografts from such primary tumors which have been harvested and frozen for future nucleic acid isolation.

	5×10^4	5×10^3	5×10^2
Bulk	4	4	4
DDX4-CD133-	4	4	4
DDX4+CD133-	4	4	4
DDX4-CD133+	2	6	4
DDX4+CD133+	1	3	8

Table 2. Assessing the relative tumorigenicity of the isolated cell populations. Immunocompromised NOD/SCID mice were injected with the indicated cell numbers of each purified cell population in a limiting dilution assay. The numbers in each column represent the total number of mice injected with each cell number. The information reported here comprises three separate injection series using cells derived from three independent patient xenografts. The bulk population represents unsorted tumor cells.

We previously reported that in parallel analyses, we used flow cytometry to determine the relative frequency of DDX4-positive cells in a subset of xenograft tumors derived from primary human ovarian serous cancer. We detected a DDX4-positive population within every analyzed tumor (Table 1). The presence of this rare sub-population within human ovarian tumors was not reported at that time and its biological significance remains to be determined.

We initiated experiments to test the relative tumorigenicity of tumor-derived DDX4+ cells. Specifically, we isolated purified populations of DDX4+CD133-, DDX4-CD133+, DDX4+CD133+ and DDX4-CD133- cells and have injected them into NOD/SCID mice in a limiting dilution assay (Table 2). Tumor formation was monitored in the injected animals. While there initially appeared to be differences in tumor formation following injection of cells with specific DDX4 and CD133 expression profiles, no consistent difference in the onset of tumor formation as a result of the injection of the different cell populations (DDX4+CD133-, DDX4-CD133+, DDX4+ CD133+ and DDX4-CD133- cells) was evident at the end of the experiment.   Interestingly, other investigators have used immunohistochemistry to show that DDX4 is over expressed in a significant number of epithelial ovarian cancers and its expression was positively correlated with advancing age and serous histology.   DDX4 positive staining was also observed in ovarian inclusions cysts with metaplastic changes, but absent in cysts without metaplastic changes. More recently published data suggests that DDX4 is co-expressed with the cancer stem cell marker CD133 in ovarian cancer tissue samples and cell lines whereas CD133 negative ovarian cancer cells do not express DDX4. This study is similar to what we had proposed and were actively testing when the above manuscript was published online. Given that the reported results were different than what we were discovering and the report lacked validation of true DDX4 expression, we sought to verify their findings as well as our own.

Characterization of DDX4 in ovarian cancer and established ovarian cancer cell lines

As mentioned, it was previously shown that DDX4 is expressed in ovarian carcinomas and its expression is associated with age and the serous histophenotype. Thus, we analyzed a subset of patient derived xenografts (PDXs) established from primary human ovarian cancer samples. FACS analysis of these tumors revealed a significant percentage (5-20%, Figure 3a) of DDX4 positive cells that were easily isolated. These purified cells, however, showed no evidence of DDX4 expression by qPCR, Western blot and mass spectrometry (not shown).

Freshly isolated ovarian tumor antibody positive and negative cells also did not express DDX4 as assessed by qPCR (Figure 3b). Non-sorted, antibody negative and antibody positive cells were injected into NOD/SCID mice. Tumors were harv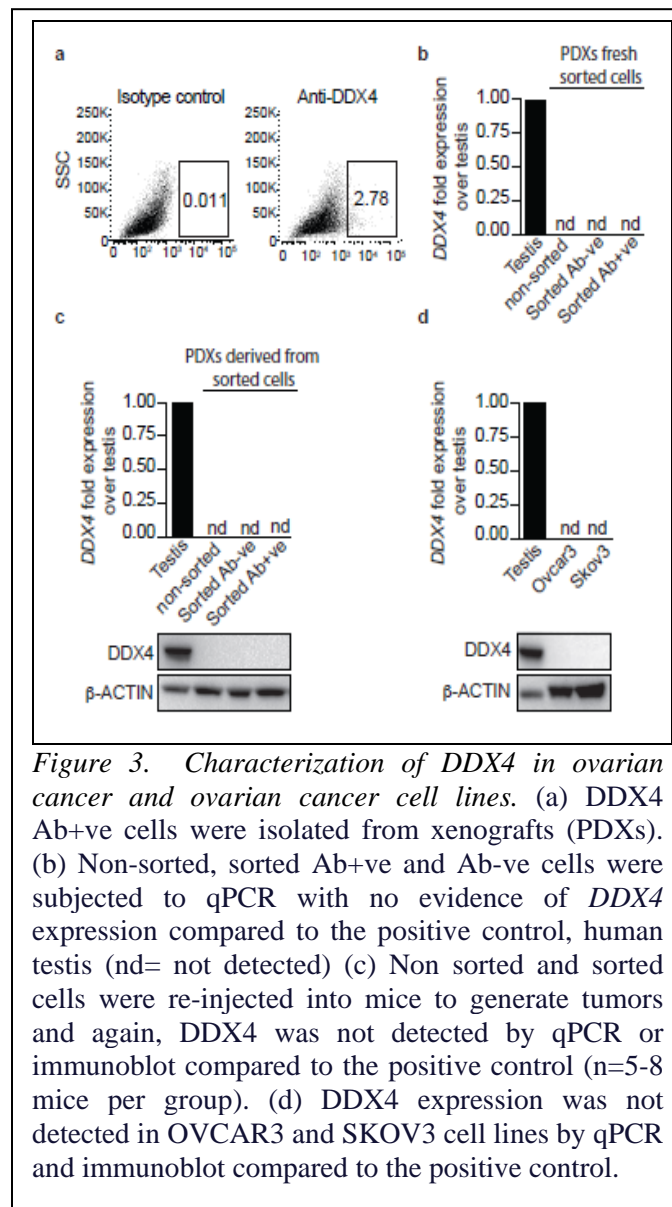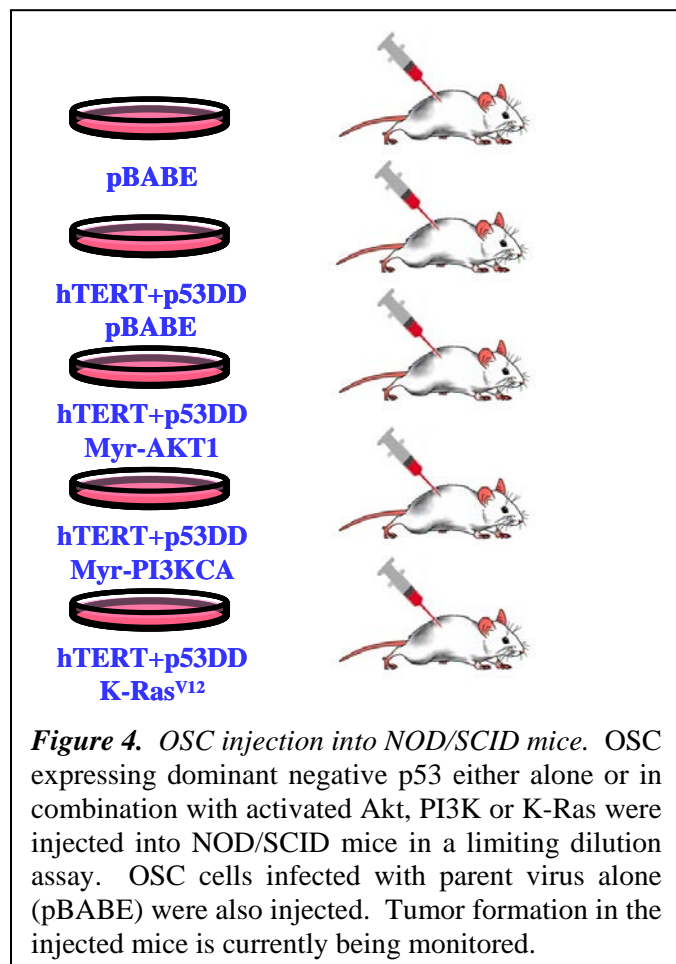ested after 2 months and analyzed by qPCR and western blot, with no evident DDX4 expression (Figure 3c). Mass spectrometry revealed no positivity for DDX4 in the tumor cells (negative data not shown).

As discussed above, a recent publication provided some evidence to suggest that specific ovarian cancer cell lines expressed significant levels of DDX4 by protein analysis. To this end we assessed the expression of *DDX4* mRNA and protein in the OVCAR3 and SKOV3 established ovarian cancer cell lines. Consistent with the results obtained in human ovarian cells from our PDX model, we detected no expression of either *DDX4* mRNA or protein in OVCAR3 or SKOV3 cells (Figure 3d).

To further test DDX4 protein expression in antibody negative and antibody positive samples, a mass spectrometry approach was utilized. Rhesus testis was used as a positive control to ensure that DDX4 protein could be recognized by mass spectrometry. Next, we analyzed antibody positive cells from murine and human ovaries. In addition to analysis of our benign primary ovarian cells, we also analyzed cells derived from primary ovarian cancer xenografts (PDXs). We detected no DDX4 peptides in any of these murine or human ovarian samples despite easy detection of DDX4 peptides in Rhesus testis. Detailed results can be found in Hernandez et al., 2015 *Nature Medicine*.

Figure 3. Characterization of DDX4 in ovarian cancer and ovarian cancer cell lines. (a) DDX4 Ab+ve cells were isolated from xenografts (PDXs). (b) Non-sorted, sorted Ab+ve and Ab-ve cells were subjected to qPCR with no evidence of *DDX4* expression compared to the positive control, human testis (nd= not detected) (c) Non sorted and sorted cells were re-injected into mice to generate tumors and again, DDX4 was not detected by qPCR or immunoblot compared to the positive control (n=5-8 mice per group). (d) DDX4 expression was not detected in OVCAR3 and SKOV3 cell lines by qPCR and immunoblot compared to the positive control.

Task 2: Assessment of induced genetic mutations in human OSCs. Human OSCs will be expanded in vitro (see Subtask 1a). These cells will be retrovirally transformed to stably express either genetic mutations or constitutively active oncogenes. The genetically altered cell lines will be injected into immunocompromised mice and evaluated for changes in tumorigenic characteristics.

Figure 4. OSC injection into NOD/SCID mice. OSC expressing dominant negative p53 either alone or in combination with activated Akt, PI3K or K-Ras were injected into NOD/SCID mice in a limiting dilution assay. OSC cells infected with parent virus alone (pBABE) were also injected. Tumor formation in the injected mice is currently being monitored.

Subtask 2a: Retroviral transduction of human OSCs. OSCs transformed with luciferase in Subtask 1b will be stably transduced using the pBABE retroviral vectors already produced in our laboratory (dominant negative TP53, KRAS mutant, constitutively active PIK3CA and AKT1, and control vector; see preliminary data) using the PLAT-A packaging cell line.

Retroviral vectors expressing oncogenic alleles of human *TP53*, *AKT1*, *KRAS*, and *PIK3CA* were constructed and initially validated in both a human endometrial cancer cell line and mouse oogonial stem cells. The expected effect on the expression of relevant genes/proteins was confirmed by RT-PCR or immunoblotting. The viruses were then used to infect a human oogonial stem cell line. Cells infected with the *AKT1*, *KRAS* or *PIK3CA* viruses were generated by puromycin selection five days post-infection and then maintained in a low concentration of puromycin to ensure a stable pure population of virus-containing cells. Since the p53-expressing retrovirus construct does not confer puromycin resistance, successful infection with this virus was confirmed by RT-PCR using primers designed to specifically detect exogenous *TP53* gene expression.

Although co-infection of human oogonial stem cells with the oncogenic *TP53* and *KRAS* retroviruses was highly efficient, co-infection of the *TP53* virus with either the oncogenic *AKT1* or *PIK3CA* viruses was unsuccessful. We determined that initial infection with the TP53 retrovirus followed by subsequent re-infection with the *KRAS*, *AKT* or *PIK3CA* retrovirus was required to generate human oogonial cells that co-expressed the relevant oncogenes as validated by either RT-PCR or immunoblotting.

Subtask 2b: The transformed cell lines (5 lines) will be injected into the intraperitoneal cavity

of immunocompromised (NOD/SCID) mice. The injections will be done in a dilution series, ($1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$ and $1x10^1$, 3 mice per mutation, for a total of 60 mice).

We previously reported that we conducted simple preliminary studies to assess the potential impact of the retroviral infections on human oogonial stem cell proliferation. Surprisingly, human oogonial stem cells expressing exogenous oncogenic p53, KRAS, AKT1 or PI3K reached confluency in culture 3-fold faster than cells infected with the control retrovirus. We have since subcutaneously injected varying cell numbers (1×10^6, 1×10^5, 1×10^4, and 1×10^3) of human oogonial stem cells infected with the various retroviruses into NOD/SCID to pre-assess their ability to generate tumors in mice (Figure 4). This was expected to inform our choice of the post-injection time points at which we will assess tumor formation in mice using the more expensive IVIS imaging system. Concurrently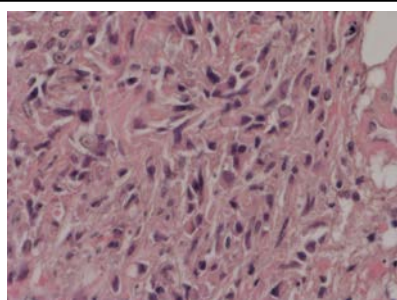, we have introduced a retrovirus which co-expresses GFP and luciferase into the human oogonial stem cell lines expressing the various oncogenes. Following infection, the cells were to be sorted based on GFP expression to generate pure populations of infected cells. Those cells were subsequently to be injected into the intraperitoneal cavity of NOD/SCID mice and tracked *in vivo* via IVIS imaging of luciferase activity. We had initiated this task but suspended further work once we realized that the isolation of the OSCs via the DDX4 antibody may be not as specific and efficient as previously believed. More specifically, we determined that although the DDX4 antibody could be used isolate a small population of cells that could give rise to oocytes, the cells were not truly DDX4 positive. That is, the DDX4 antibody was indirectly enriching for OSCs but the resulting populations were not pure. Therefore, we delayed further experiments until we could confirm that these cells could give rise to tumors in our corresponding mouse model. We have determined that modified mouse OSCs can generate tumors following injection (Figure 5), albeit with poor efficiency.

Figure 5. *Histological evidence of tumor formation.* Given the previous challenges we wanted to verify that the OSCs in culture could induce tumor an aliquot of the cells were injected into immunocompromised mice. Over time a poorly differentiated tumor with adenocarcinoma features formed.

Despite the presumed lack of specificity of DDX4 resulting in the inability to isolate a pure population of oogonial stem cells we elected to move forward by injecting what the mutant cells we had prepared into immunocompromised NOD/SCID mice by intraperitoneal injection as described in at 1×10^6 dilution to assess tumorigenicity. It was believed that if we observed tumors at this dilution we would proceed with the limiting dilution studies. However, even at 1×10^6 we failed to observe any measureable tumor formation. To be sure we repeated the experiment with similar results. This forced us to generate new lines. This was initiated under the no cost extension period. Infection of the new DDX4 positive cell lines with GFP containing viruses that conferred puromycin resistance failed to establish stable cell lines, which could be expanded in culture. Despite the fact that the award has ended we are generating all new viruses to be injected in OSC lines established by other labs.

Subtask 2d: Tumor development, growth and metastatic potential will be evaluated by the Mouse Imaging Program (MIP, MGH/Harvard core facility). All mice will be assessed weekly in the IVIS imaging system (each run takes approximately 20-30 minutes). The rate of tumorigenesis as well as metastatic spread will be assessed by the core facility software. When tumors have reached a sufficient size for evaluation, mice will be sacrificed and tumors harvested. Each tumor will be divided into 3 equal pieces, one will be preserved in paraformaldehyde and processed for histological analysis (including immunohistochemistry), one snap-frozen for genomic analysis, and one vitrified and stored in liquid nitrogen for further analysis at a later date.

This subtask was not initiated due to the inability to generate tumors from mutant cells.

Subtask 2e: As in task 1, tumors will be subjected to histological analysis as well as immunohistochemistry. PFA-fixed tumors will be embedded in paraffin, sectioned, placed onto slides and 5 sections per tumor will be stained with hemotoxylin and eosin for phenotypic analysis. Additionally, sections will be analyzed for p53 signature, CK7, CK20, PAX-8, WT-1, and ER by immunohistochemistry using commercially available antibodies.

This subtask was not due to the inability to generate tumors from mutant cells.

KEY RESEARCH ACCOMPLISHMENTS:

1. The oogonial stem cell preparations/cultures isolated from both pre- and post-menopausal ovaries collected prior to receiving funding did not survive long term cryopreservation. Consequently, we have been restocking our resource of ovarian tissue.

2. We acquired human ovarian tissue from 11 different patients via the MGH GYN Tissue Repository.

3. A subset of these has been cryopreserved for future isolation of oogonial stem cells.

4. It was ascertained that the samples collected represent ovarian tissue from women considered to be pre- and post-menopausal. As expected, the majority of the samples are post-menopausal ovaries.

5. Newly collected samples were subjected to flow cytometric sorting. Only some of the sorted samples yielded viable cells.

6. The viable oogonial stem cell cultures have been successfully passaged and expanded for validation studies.

7. We originally validated the oogonial stem cell nature of the isolated cultures, by using previously published primers for *PRDM1*, *DPPA3*, *IFITM3*, *TERT*, *DDX4* and *beta – actin* in RT-PCR analyses. Relevant controls included oocyte preparations, whole ovary

tissue, testicular tissue (positive control) and fibroblasts (negative control). We since incorporated more specific primer sets for qPCR as opposed to RT-PCR, and performed additional methodologies such as mass spectrometry, Image Stream and RNAseq. Taken together, our results showed that the cells of interest do not express DDX4 but can be isolated by the anti-DDX4 antibody. This observation suggests that indirect binding of the antibody to the surface will enrich for these cells. The efficiency at which this occurs is not yet known though it is expected to be low.

8. Retroviral vectors expressing oncogenic alleles of human *TP53*, *AKT1*, *KRAS*, or *PIK3CA* were successfully constructed.

9. The viral constructs were initially validated in a human endometrial cancer cell line and mouse oogonial stem cells. The expected effect of each on expression of appropriate genes/proteins was confirmed by RT-PCR or immunoblot analysis.

10. Once validated, the viruses were used to infect a human oogonial stem cell line. Cells infected with the *AKT1*, *KRAS* or *PIK3CA* viruses were generated by puromycin selection five days post-infection and then maintained in a low concentration of puromycin to ensure a stable pure population of virus-containing cells. As described, the expected effect on the expression of relevant genes/proteins was confirmed by RT-PCR or immunoblotting.

11. Since the p53-expressing retrovirus construct does not confer puromycin resistance, successful infection with this virus was confirmed by RT-PCR using primers designed to specifically detect exogenous *TP53* gene expression.

12. Simple preliminary studies assessing the potential impact of exogenous expression of oncogenic alleles of human *TP53*, *KRAS*, *AKT1* and *PIK3CA* have determined that human oogonial stem cells expressing any of these proteins reach confluency in culture 3-fold faster than cells infected with the control retrovirus.

13. Based on the validation findings we temporarily suspended our tumorigenicity studies until we could validate the nature of the cells isolated with the anti-DDX4 antibody. We also wanted to repeat the correlative mouse OSC studies (not funded by this award) until we confirmed we could repeat what we observed in our earlier studies. We have determined that mOSCs could indeed induce tumor formation although our more recent experiments required injection of higher numbers of cells expressing oncogenic alleles of *TP53* and *AKT1* to generate tumors at a much lower frequency.

14. In order to compare the genomic profile of tumors generated from human oogonial stem cells to the profile of xenografts derived from human primary serous ovarian or peritoneal tumors, we generated xenografts, which have been harvested and frozen for future nucleic acid isolation. Despite previous reports, our preliminary findings suggest that our ovarian tumors samples do not express DDX4 as evidenced by qPCR, western blot and mass spectrometry analyses. These data were confirmed following more detailed studies including RNAseq analysis

15. Using flow cytometry, we reported that we detected a population of DDX4-positive cells in xenografts derived from primary human serous ovarian and peritoneal tumors. Others have recently reported that this rare sub-population overlaps with a CD133 fraction. We have since disproven this using more extensive validation assays. This work detailed in our publication in Nature Medicine 2015.

16. We have reinitiated new infections with new viruses, into OSCs derived from another source. Again, we will introduce mutations described.

REPORTABLE OUTCOMES:

o *manuscripts, abstracts, or presentations*

Our findings regarding DDX4, OSCs and ovarian cancer cells was submitted and reviewed by Nature Medicine in 2014. In 2015 it was finally accepted in a Communication with the bulk of the finding being allowed as supplemental data.

Characterization of extracellular DDX4- or Ddx4-positive ovarian cells.

Silvia F Hernandez, Nima A Vahidi, Solji Park, R Patrick Weitzel, John Tisdale, Bo R Rueda & Erin F Wolff *Nature Medicine* **21**, 1114–1116 (2015) doi:10.1038/nm.3966 Published online 07 October 2015

o *licenses applied for and/or issued;*

No licenses have been applied for or issued

o *degrees obtained that are supported by this award;*

N/A

o *development of cell lines, tissue or serum repositories;*

As part of the proposal we have collected samples of ovaries and developed short term cell lines from the oogonial stem cells. Due to IRB restrictions these are not available for distribution.

o *informatics such as databases and animal models, etc.;*

RNA sequence analysis for DDX4 expression on human tissue was conducted. Due to IRB restrictions this information is protected and cannot be distributed.

o *funding applied for based on work supported by this award;*

N/A

○ *employment or research opportunities applied for and/or received based on experience/training supported by this award*

N/A

CONCLUSION:

By utilizing more extensive validation methods, we have determined that the finding that DDX4 is expressed both intracellularly and extracellularly may not be entirely correct. This discovery led to a temporary hold on our progress related to the tumorigenic potential of OSCs until we could confirm the cells we had isolated for these studies were the same as those previously described by White et al. In a collaborative effort with Dr. Erin Wolff (NIH based scientist), we re-characterized both freshly isolated and cultured OSCs that were purified using the anti-DDX4 antibody. In short, our recent work published in Nature Medicine summarizes our findings from RNA sequencing and mass spectrometry data as well as PCR and immunoblot analysis to provide evidence suggesting that DDX4 antibody positive cells isolated from the ovary and the ovarian tumor samples by FACS had no evidence of DDX4 expression. Moreover, even if DDX4 is over expressed in ovarian stem cells, our results suggest that DDX4 cannot be expressed at the cell surface. It is important to note that Dr. Wolff did provide evidence that these cells could generate oocyte like structures confirming the original findings by White et al. Thus, these data suggest that the antibody is non-specifically binding something novel and this may provide the basis for the indirect identification of stem populations such as OSCs. Given the lack of antibody specificity and low efficiency of isolating true OSCs, the human DDX4-positive sorted cell populations likely only contain a subset of the true cells of interest. We therefore predicted we would likely need to increase the number of cells injected into immunocompromised mice in order to test our original hypothesis that OSCs have the potential to become malignant and take on a phenotype similar to that of ovarian cancer as was observed with murine OSCs.

We were granted the opportunity to continue our experiments under a no cost extension. Unfortunately, we were not able to re-initiate the project for several months due to administrative and IACUC related issues they were out of our control. As requested, we submitted our newly approved MGH IACUC protocol for review to the DOD in December of 2014. Approval of our animal studies by the Director, Office of Research Protections Animal Care and Use Review Office was not received until May of 2015. At this time we took previously infected cells as well as newly generated cells and injected them into immunocompromised mice to determine their tumorigenicity. Unlike what was observed in the mouse studies the DDX4 antibody positive cells injected into the mice never formed any measurable tumor. This was repeated with more cells at higher dilutions with the same negative results. Moving on to the second aspect of our proposal required that we generate DDX4 positive lines that *TP53*, *AKT1*, *KRAS*, and/or *PIK3CA* were mutated. We successfully generated lines that expressed variations of *TP53*, *AKT1*, *KRAS*, and *PIK3CA*. *Interestingly while the mutant cells proliferated much faster then their wild type controls they too failed too produce any measurable tumors*

It was not clear again why the mutant cells failed to generate tumors, especially since it has been previously shown in other cell type that these mutations could result in tumor formation. Consequently, we again went back to generate new lines. Unlike the first round infection of the new lines with GFP containing viruses that conferred puromycin resistance failed to establish stable cell lines, which could be expanded in culture.

In August of 2015 we received notice that Award was ending and we were delinquent with respect to submission of the Closure report and no longer had access to the funds. Since there was such a delay due to bureaucracy on both sides our Institutional representative was working with DOD representatives to determine if we would be allowed to continue the study, utilizing the remaining funds under a no cost extension (correspondence available upon request). This opportunity was finally granted albeit time was limited. We re-initiated the establishment of virally infected lines that expressed the oncogenic mutations until the award ended. Regardless of the challenges, some of the results culminated in a publication in Nature Medicine and we are continuing this line of research.

REFERENCES:

N/A

APPENDICES:

N/A

SUPPORTING DATA:

Figures have been included within the text.