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TITLE: Coexpression of the Follicle Stimulating Hormone Receptor and Stem Cell Markers: A Novel Approach to Target Ovarian Cancer Stem Cells

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**14. ABSTRACT**

Purpose: The purpose of this project was to determine whether the Follicle-stimulating Hormone Receptor (FSHR) is co-expressed with a sufficient number of ovarian cancer stem cell markers to consider it as a new experimental target for novel nanotechnology approaches capable of destroying ovarian cancer stem/progenitor cells (OCSCs).

Scope: We examined individual cells obtained from ovarian cancer patients to determine whether they expressed recognized ovarian cancer stem cell markers and the FSHR on the surface membrane of the same cell. Cells co-expressing the markers and the FSHR (plus appropriate controls) were then tested in mice to determine whether any tumors formed also co-expressed the same markers.

Major findings: 1) Analysis by Fluorescence Activated Cell Sorting (FACS) showed that the FSHR is definitively co-expressed with the OCSC markers CD24, CD44, CD133 and Notch 2. 2) The number and type of post-FACS cells available for *in vivo* studies to meet the criteria of cancer stem cells was very limiting however: 1) they demonstrated ovarian cancer-like nodule formation at the macroscopic level, but 2) we were not able to establish a firm answer to the question of co-expression of the FSHR and ovarian cancer stem cell markers at the cellular level. The results did, however, demonstrate proof-of-principle.

**15. SUBJECT TERMS**

ovarian cancer stem cells; Follicle-stimulating Hormone receptor; cancer stem cell markers; fluorescence-activated cell sorting (FACS)

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## Table of Contents

	<u>Page</u>
Front cover.....	1
Abstract.....	3
Introduction.....	5
Keywords .....	5
Accomplishments.....	5
Conclusion.....	16
Impact.....	17
Changes/Problems.....	17
Products.....	18
Participants/other collaborating organizations.....	18
Special Reporting Requirements.....	18
Appendices.....	19

## INTRODUCTION:

Subject. The project was designed to determine whether or not the Follicle-stimulating Hormone Receptor (FSHR) is co-expressed on the same cell as various candidate ovarian cancer stem/progenitor cell markers (OCSCM).

Purpose. The project's purpose was to determine whether the FSHR is co-expressed with a sufficient number of ovarian cancer stem cell markers to consider it as a new experimental target for novel nanotechnology approaches capable of destroying OCSCMs. Our working hypothesis is that a subset of ovarian cancer ascites cells are stem cells that co-express the FSHR and several candidate ovarian cancer stem/progenitor cell markers.

Scope. The work scope involved Fluorescence Activated Cell Sorting (FACS) analysis of single human ascites cells of ovarian cancer patients to establish whether OCSCMs are simultaneously expressed with the FSHR. The second portion of the project involved Injection of such cells into nude mice to determine whether intraperitoneal nodules resembling those of human ovarian cancer at the macroscopic level would develop *in situ* and whether co-expression of the FSHR and ovarian cancer cell stem cell markers could be demonstrated at the cellular level.

**KEYWORDS:** ovarian cancer; ovarian cancer stem cells; stem cells; Follicle-Stimulating Hormone (FSH); Fluorescence-Activated Cell Sorting (FACS); nude mice; ascites fluid

## ACCOMPLISHMENTS:

The Statement of Work subtasks are reproduced here from page 40 of the SFS 424 Application Form.

### MAJOR GOALS: Statement of Work (SOW)

Description and timeline of the tasks:

Subtask 1: Experiments testing whether FSHR and the various candidate stem/progenitor cell markers are co-expressed on ovarian cancer ascites cells. This task will involve working with frozen and fresh ascites cells samples, preparing them for FACS and storing the sorted cells for further analyses, i.e., culture for spheroid formation, RT-PCR, immunofluorescence, and injection into nude mice. Timeframe: 1-12 months.

Subtask 2: Develop spheroids in cell culture and tumors in nude mice. Utilizing the FACS cells above, culture to develop spheroids and also inoculate cells into nude mice plus/minus FSH. Then analyze by FACS, immunofluorescence, H&E staining, and the metrics of measuring tumor response. Timeframe: 6 - 24 months.

## ACCOMPLISHMENTS UNDER THE GOALS

### Year 1: experimental summaries, both subtasks.

FACS analysis results and culture of FACS-isolated cells. By FACS analysis, FSHR was expressed in 85.7% of the patients (18 /21). Definitive co-expression of FSHR and OCSC markers by FACS analysis and/or mRNA expression was shown for Notch 2, CD24, CD44, and CD133. Marginal co-expression was observed for Oct4, ALDH1, and LGR5 at the mRNA level. Candidates not showing co-expression at the membrane protein and/or mRNA level were Notch 1, Notch 4, CD117, and ABCG2.

Stem cell characteristics: non-cloned and cloned cells. Expression of FSHR and Notch 2 by FACS-isolated cells was maintained over several generations in primary culture. However an attempt at monoclonal growth and expansion of cells representing FSHR/Notch 2 co-expressing cells (+/+, “double positives”), FSHR or Notch 2 “single positives” (+/-) and “double negatives” (-/-) was not successful. Spheroid formation was not used as evidence of stem cell maintenance/function. Our collaborators have shown that aggregation rather than monoclonal expansion explains ovarian cancer cell spheroid formation *in vitro* to a large extent and that spheroid formation should only be used very cautiously as a functional end point characteristic of ovarian cancer stem cells.

Results *in vivo*: Tumor formation from FACS-purified cells. Solid tumors surrounded by a significant degree of fluid formed from two out of the three cell inocula in the Notch-2-single-positive arm of an ovariectomized group. Ascites was not evident in any of the animals. Note: The tumors were analyzed in more detail in year 2 by our veterinary staff and determined to be lipomas, a condition not uncommon in this particular strain of nude mice.

### Year 1: Detailed experimental results.

1. Brief outline of methods. De-identified human ascites cells were thawed from liquid nitrogen storage, pre-incubated with appropriate blocking proteins/sera and incubated with the appropriate fluorescently labeled monoclonal antibodies and isotype controls (all commercially obtained) using dilutions recommended by the manufacturers. Labeled FSHR antibody of sufficient quality was not available commercially so monoclonal antibody provided by Dr. Jim Dias was labeled in our laboratory using an Alexa 647 labeling kit. The labeling procedure presented problems, which were successfully overcome. The primary issue, among others, was the low yield of conjugated antibody (FSHR-Alexa 647) recovered when the column elution procedure was carried out according to the manufacturer's instructions. This eluted material led to several results showing a very low percentage of FSHR<sup>+</sup> cells in the IVF positive-control granulosa/luteal cell samples used to validate each labeling procedure. We solved the problem by cutting off the pipet tip, ejecting the gel material plus elution buffer into a microfuge tube and letting the elution buffer and gel interact for a longer time

(empirically determined, four min.). Neutralization buffer was then added and the phases separated by brief high-speed centrifugation. Labeled antibody obtained in this manner has consistently yielded superior results both in IVF and cancer cell samples. The best explanation I can offer is that the affinity of this particular A647-conjugated FSHR monoclonal antibody type (IgG2b kappa) for the gel resin is so high that a longer exposure to the elution buffer is required. FACS analyses were carried out by the Flow Cytometry Core Facilities of the medical center. RT-PCR of FSHR<sup>+</sup> cells (and others) was accomplished after RNA isolation (InVitrogen's RNeasy RNA isolation kit) followed by first-strand cDNA synthesis. RNA samples were DNase treated. In certain instances with very small numbers of FSHR<sup>+</sup> cells (~ 3K) we used InVitrogen's PicoPure RNA extraction procedure (including DNase treatment) followed by the Ovation Pico WTA system<sup>®</sup> to amplify the RNA into a double stranded cDNA product. Real-time PCR using intron-spanning primers was carried out using a BioRad iCycler. Expression levels of FSHR and the candidate markers were normalized to that of GAPDH. Validation criteria included water blanks, no RT, and melt curve analyses. The PCR results provided independent confirmation of the FACS data and also served as a screen for active gene expression of the non-membrane-associated candidate markers that are not amenable to live-cell FACS analysis. These markers will be analyzed by immunostaining methods. Techniques are in the development and validation stage for FSHR to date. Studies *in vivo* with athymic nude mice have been initiated. Twenty-four mice are used for each FSHR/CSC analysis: three each (10-20K, 1-2K, and 100-200 cells) for each "arm" of FACS-separated cell preps (4 arms: FSHR+/CSC+, FSHR+/CSC-, CSC+/FSHR-, and FSHR-/CSC-) and two groups: cycling, and ovariectomized. Cells were sorted by FACS and immediately diluted for IP injection of the various cell groups within an hour of sorting. The mice are then observed over time for solid tumor and/or ascites formation. Recovered ascites cells will be analyzed by FACS and re-injected into a second set of animals to repeat the findings. Additional ascites cells will be processed and frozen in liquid nitrogen and solid tumor tissue stored/frozen in OCT embedding medium for immunohistochemical analysis.

## 2. FSHR and co-expression analyses.

*FACS analysis.* As mentioned above, FSHR was expressed by the ascites cells of 18 of 21 patients (85.7%). Even though the patient number is very small, this result confirms and extends the literature findings showing that expression of the FSHR in ovarian cancer patients is 60% or greater (1). By FACS and/or mRNA analyses of 13 candidate CSC markers (Notch 1-4, CD44, CD117, CD133, CD24, Nanog, Oct4, ALDH1, LGR5, and ABCG2), six of the 13 (46.2%) were co-expressed with the FSHR (Notch 2 and 3, CD24, CD44, CD133, and Nanog). These results may be providing evidence that FSHR action ranges across and facilitates several stem/progenitor cell activity pathways. They may also imply that FSHR is co-expressed on multiple types of CSC populations – suggesting it might demarcate an as yet unknown common progenitor. The existence of multiple types of ovarian cancer stem cells has been proposed (2).

Appendix 1 Figure 1 illustrates FACS scans of the typical cellular distribution in two patients and that we have noted in virtually all the patients: all the possible combinations are usually but not always present -- double positives, single positives, and double negatives -- although to differing degrees. Taken together with the previous results, this presumably indicates that different markers and marker combinations are present at any given time of cancer cell development and differentiation.

*mRNA analysis.* Messenger RNA analysis was used as an independent confirmation of FSHR and CSC membrane protein co-expression demonstrated by FACS analysis. Examples for FSHR, Notch 2, and GAPDH are shown in Figures 2a -2c, respectively.

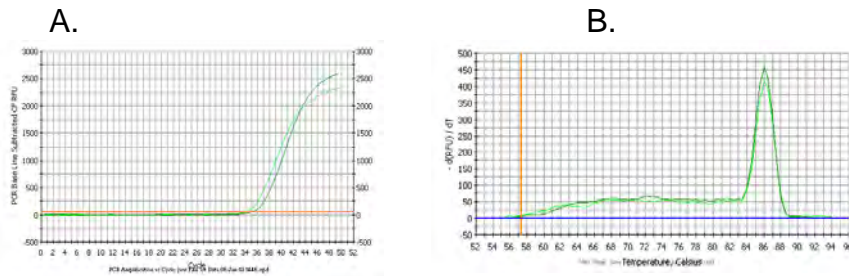


Figure 2a. Real-time analysis of FSHR expression in FSHR/Notch2 double-positive cells determined by FACS analysis. A. PCR quantitation (duplicate samples), B. Melt curves (duplicate samples).

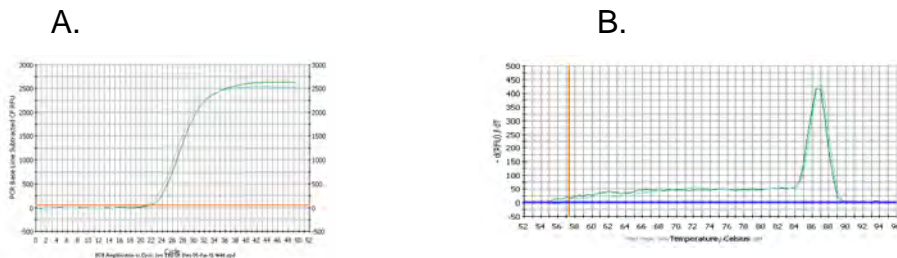


Figure 2b. Real-time analysis of Notch 2 expression in FSHR/Notch 2 double-positive cells determined by FACS analysis. A. PCR quantitation (duplicate samples), B. Melt curves (duplicate samples).



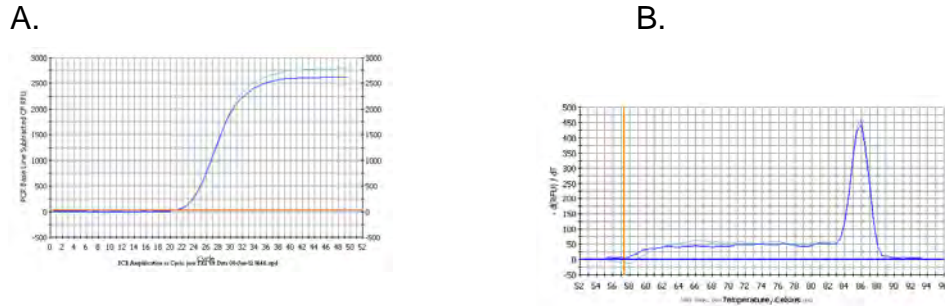


Figure 2c. Real-time analysis of GAPDH expression in FSHR/Notch2 double-positive cells determined by FACS analysis. A. PCR quantitation (duplicate samples), B. Melt curves (duplicate samples).

By these criteria, Nanog mRNA was also demonstrated in FSHR-positive (FACS) cells.

Real-time expression and melt curves were also validated for CD24, CD44, CD133, and Notch 3 but specific co-expression each with FSHR mRNA has not yet been accomplished because of limiting cell numbers. Also, Oct4, ALDH1, and LGR5 may qualify as additional markers co-expressed with the FSHR; however, in the first set of analyses the melt curves of their PCR products were not of sufficient quality. Different primer sets may yield acceptable curves and these are being currently tested. Co-expression of some markers were not detected by either FACS or PCR methodology (Notch 1 and 4, CD117) or PCR (ABCG2). For purposes of this investigation these candidates likely will not be examined further.

*FSHR expression by cultured cells.* The objective of this study was to determine whether FSHR mRNA expression would be maintained over long-term primary culture. FSHR mRNA expression by non-cloned cells positive for FSHR and Notch 2 (FACS) was maintained over several generations as the cell grew to confluence during two weeks of primary culture. This result is very encouraging because FSHR expression typically is not easily maintained in primary culture of granulosa cells of all species beyond a few days. We will still attempt to demonstrate co-expression following monoclonal expansion of co-expressing cells +/- FSH versus non-FSHR-expressing cells +/- FSH.

*Stem cell criteria: Tumor growth in vivo.* FACS analysis was performed and FSHR/Notch 2 co-expressing cells (+/+, “double positives”), FSHR or Notch 2 “single positives” (+/-) and “double negatives” (-/-) were injected in three graded amounts (100-150, 1,000-1,500, 10,000-15,000 cells, IP) into cycling and castrated female (high FSH) nude mice. At approximately three months after injection solid tumors surrounded by a significant degree of fluid formed from two of the three cell inocula in the Notch-2-single-positive arm of the ovariectomized group. Ascites was not evident in any of the animals at this point (Figure 3).

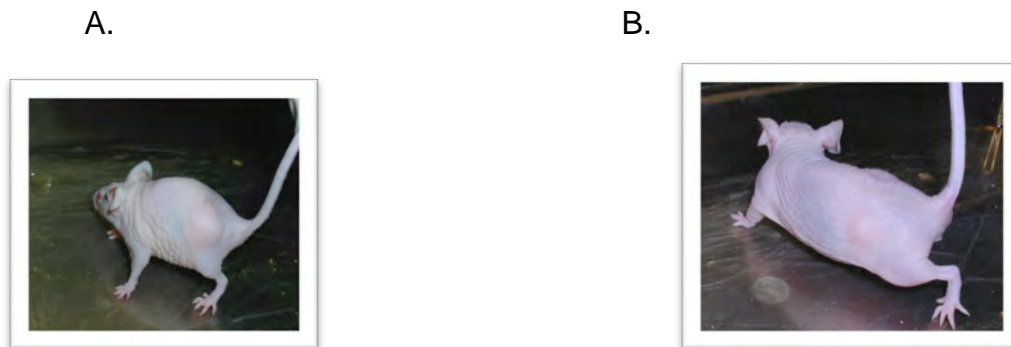


Figure 3. Visible subcutaneous tumors developed from injection of Notch 2 single-positive cells: A. 10-,000 – 15,000 cells; B. 100 – 150 cells. They are characterized by a palpable solid mass surrounded by considerable fluid.

According to our hypothesis, the FSHR/Notch 2 double positives should have also formed tumors, and at an even earlier age. However, tumor growth is very variable and we intend to run this experiment for at least six months to allow development of slow-growing tumors. This experiment or parts of it will also be replicated.

*Stem cell criteria: monoclonal expansion of co-expressing cells in vitro.*

We attempted to demonstrate monoclonal growth and expansion of FACS-separated cells the above cell groups but without success to date. We will continue these efforts. Spheroid formation will not be used as evidence of stem cell maintenance/function. Our collaborators have shown that aggregation rather than monoclonal expansion explains ovarian cancer cell spheroid formation *in vitro* to a large extent and that spheroid formation should only be used very cautiously as a functional end point characteristic of ovarian cancer stem cells (Appendix 2 ).

#### Year 2: experimental summaries, both subtasks.

1. Our veterinary staff determined that the tumors mentioned above and shown in Figure 3 were in fact lipomas and not solid tissue tumors. Macroscopically, swollen lymph glands but no ovarian-cancer-like solid tumors or ascites were noted in this group.
2. In the next group of animals our *in vivo* protocol did produce solid tumors with classical ovarian cancer-like gross morphology after 5 months in an ovariectomized animal (high circulating FSH) that received Notch 2 single-positive cells (see Figure 4 below and the comparison to human ovarian cancer).
3. After nine months the remaining animals in this group were sacrificed. Gross morphological nodules and lymphoid swellings that looked ovarian-

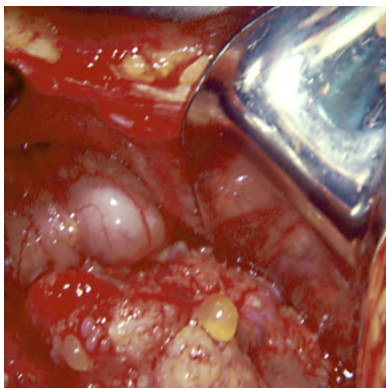
cancer-like were harvested (from liver, kidney, pancreas, mesentery -- noted principally in the ovariectomized group) and analyzed by RT-PCR for FSHR mRNA expression. At the level of sensitivity obtained without cDNA amplification, FSHR mRNA expression was not detected.

4. New technical procedures for FACS analysis were developed and characterized after the supply of the initial ant-FSHR monoclonal antibody (mab 106.105) was exhausted. This is a secondary antibody detection method and it appears to be at least as good as the initial procedure because: 1) it is more stable over time, and 2) more procedures can be performed with a given quantity of this lot of 106.105 mab.

#### Year 2: Detailed experimental results:

Tumor formation by FACS-isolated co-expressing cells *in vivo*. The tumors shown in Figure 3 (year 1, above) were analyzed in more detail in year 2 by our veterinary staff and determined to be lipomas, a condition not uncommon in this particular strain of nude mice. In the repeat of this study no lipomas were noted. However, in this study a mouse in the ovariectomized (presumably high circulating FSH; blood serum was collected for later analysis) group lost weight and died suddenly approximately five months after injection with 1,000 – 2,000 Notch 2 single-positive cells (see Figure 4 below and the comparison to human ovarian cancer). The morphology of the mouse ovarian cancer-like nodules very closely resembles that of the human. The vascular nature of the mouse tumors appears much less pronounced but that is because the animal had been previously exsanguinated to provide blood serum for possible FSH assay. Tissues have been stored in OCT embedding medium for frozen sections for upcoming immunocytochemical analysis.

A.



B.



Figure 4. Ovarian cancer nodules in human (A) and in a mouse (B) that received Notch 2 single-positive cells

After nine months the remaining animals in this group showed gross morphological nodules and lymphoid swellings that looked ovarian-cancer-like and were harvested (from liver, kidney, pancreas, mesentery -- noted principally in the ovariectomized group) and analyzed by RT-PCR for FSHR mRNA expression. At the level of sensitivity obtained without cDNA amplification, FSHR mRNA expression was not detected. In a repeat of this experiment the same classical ovarian cancer-like morphology was not observed.

*Year 2, development and characterization of a second antibody detection procedure.* As mentioned above, the labeling of our primary 106.105 mab was problematic but the issue has been resolved. (Note: with HEK cells; OVCA cells had not yet been tested) Table 1 exemplifies that mab amounts and ratios can be empirically determined that accomplish labeling of essentially all the FSHR-positive cells.

Year 2 Table 1. FACS analysis, characterization of secondary antibody procedure: percent HEK cells expressing FSHR.

<u>ug/ml goat anti-Mouse A-647</u>	<u>% FSHR<sup>+</sup> cells</u>
Isotype control (10.0)	0.25
4.0	73.9
8.0	83.1
16.0	89.0
32.0	88.0
<u>replicate, ug/ml 106.105</u>	
isotype control (10.0)	0.28
2.5	88.1
5.0	92.3
10.0	93.3

Conditions: live, single HEK cells, previously transfected with FSHR including a portion of the extracellular domain; approximately 70,000 FSHR<sup>+</sup> cells/tube; HEK cell stock provided by Dr. Jim Dias. SUNY

### Year 3 and following

Candidate OCSCM messenger RNA analysis. Messenger RNA expression for 13 candidate stem cell markers was performed on the various FACS-isolated fractions (FSHR/marker double positives, FSHR single positives, marker single positives, double negatives) to analyze whether marker candidate mRNA levels were /were not very preferentially clustered and associated with mRNA levels of the FSHR and/or a particular marker. Marker distribution was not clustered.

mRNA co-expression analysis. In a pilot study using cells from one patient we were able to generate cDNA from all 4 FACS expression types and analyze mRNA

expression for 13 candidate ovarian cancer stem cell markers (Notch 1-4, CD44, CD117, CD133, CD24, Nanog, Oct4, ALDH1, LGR5, and ABCG2). The objective was to ascertain whether any particular marker or cluster of markers associated strongly (positively or negatively) with expression of FSHR mRNA. This would be very interesting to know if the FSHR proves to be a valid stem cell marker target. Although FSHR mRNA expression in this patient was marginally detectable, the results shown in Table 1 do not indicate any particular association or clustering.

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Year 3 Table 1. Ct values of candidate SC markers co-expressed with FSHR in FACS-isolated cells (normalized to GAPDH @ 15.0)

	Marker ID													
	<u>FSHR</u>	<u>CD133</u>	<u>CD44</u>	<u>CD24</u>	<u>Lgr5-3</u>	<u>N1</u>	<u>N2</u>	<u>N3</u>	<u>N4</u>	<u>ALDH1</u>	<u>Nanog</u>	<u>ABCG2</u>	<u>CD117</u>	<u>4-Oct</u>
<u>Cell type</u>														
Double pos	39	24	19.5	17	27	25	17	29	40	31	25	43	40	22
FRSP	33.5	37	25	18	26	27	20	26	34	35	23	34	39	24
CD44SP	41	41	21	17	26	24	18	26	30	32	23	27	29	22
Double Neg	34	35	23	16	24	21	17	19	31	28	23	29	27	19

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Development of a new monoclonal antibody. Additional characterization of the second antibody procedure initiated in year 2 showed that more extensive characterization was necessary. More work is needed on this issue. See *problems encountered in Accomplishing certain tasks, #2, below*.

Data relating latest studies to previous findings: 1. Additional FACS analyses and summary.

FACS analysis of cells from additional patients showed that FSHR was expressed in 81.6% of the patients (31/38). This is very close to the percentage we observed in year 1 (85.7% [18 /21]). These results indicated a high percentage of ascites ovarian cancer cells express the FSHR. Additional results showing the variation of distribution of FSHR marker expression with respect to the given markers is interesting.

The percentage of double-positive cells (i.e., co-expression of FSHR and marker) for the various markers was: 1) CD24, 7/7 patients, 100%; 2) CD44, 8/10 patients, 80%; 3) CD133, 6/28 patients, 21.4%; Notch 2, 4/9 patients, 44.4%.



Data relating latest studies to previous findings: 2. *In vivo* tumor initiation experiments.

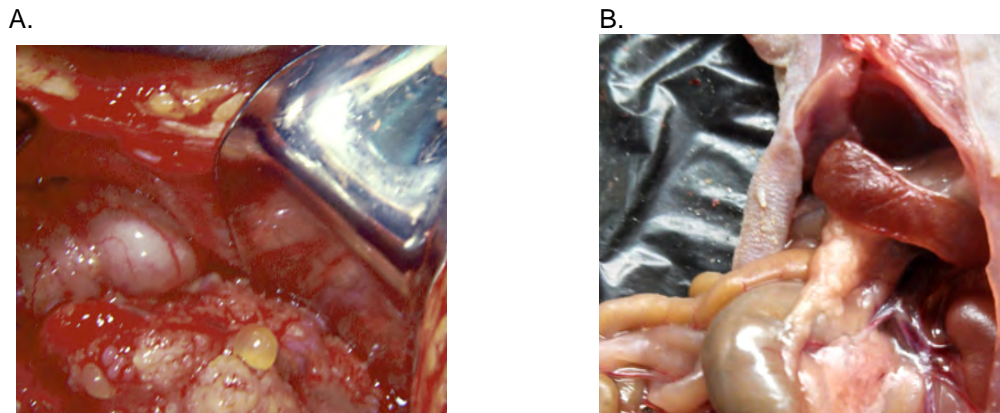
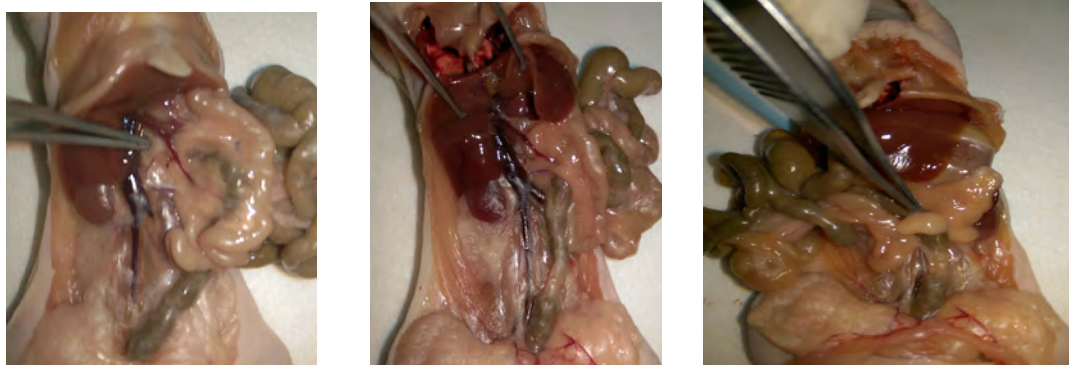


Figure 1 (copied from above and the annual report of 9/25/13). Ovarian cancer nodules in human (A) and in a mouse (B) that received Notch 2 single-positive cells.

The morphology of the mouse ovarian cancer-like nodules very closely resembles that of the human (Figure 1). The vascular nature of the mouse tumors appears much less pronounced but that is because the animals are previously exsanguinated to provide blood serum for possible FSH assay.

Because of the difficulty of low cell yield after FACS analysis (see Section C,4) we were only able to perform one additional study *in vivo* at one cell number (5,000 cells in all groups plus 500 cells in the Double Positive group) instead of a graded series of three cell numbers in all groups as originally designed. Both ovariectomized and normal animals (data not shown) were used. The results at the macroscopic level showed ovarian cancer-like nodule/blister development, possibly in all groups. Histological analysis is pending. We are presently interpreting these results as proof-of-principle rather than definitive.



A. CD24 SP

B. DP 500 cells

C. DP 5,000 cells



Figure 2. Gross morphology showing intraperitoneal ovarian cancer-like nodule development in ovariectomized animals 10 months after injection of FSHR/CD24 double-positive (DP), CD24 single-positive (CD24 SP), and double negative (DN) cells. FSHR single-positive cells were not detected in the FACS analysis and so were not able to be tested.

D. DN

#### KEY RESEARCH ACCOMPLISHMENTS:

- By FACS analysis, FSHR was expressed in 81.6% of the patients (31/38). This demonstrates that a very large proportion of ovarian cancer patients with ascites cells expressed the FSHR.
- Definitive co-expression of FSHR and OCSCs at the membrane level by FACS analysis and mRNA expression was shown for Notch 2, CD24, CD44, and CD133. This is four of 13 of the stem/progenitor cell candidate markers analyzed or 30.7%.
- Marginal co-expression at the mRNA level was observed for Notch 3, Nanog, Oct4, ALDH1, and LGR5.
- Candidates not showing co-expression at the membrane protein and/or mRNA level were Notch 1, Notch 4, CD117, and ABCG2. .
- FSHR mRNA expression by non-cloned cells co-expressing FSHR and Notch 2 (FACS) was maintained over several generations in primary culture.
- Macroscopic ovarian-cancer-like nodules developed from Notch-2-single-positive cells in one ovariectomized animal but not in a normal ovary-intact match after approximately 9 months. (The complete design included FSHR/Notch 2 co-expressing cells (+/+, “double positives”), FSHR or Notch 2 “single positives” (+/-) and “double negatives” (-/-) injected in graded amounts into normally cycling and ovariectomized animals.)
- Macroscopic ovarian-cancer-like nodules also developed *in vivo* in ovariectomized nude mice from 500 and 5,000 FSHR/CD24 co-expressing cells, from 5,000 CD24 single positive cells and 5,000 double negative cells. Histological analysis is pending. We are presently interpreting these results as proof-of-principle rather than definitive.

- Milligram amounts of new, purified mab have been generated.

## REPORTABLE OUTCOMES:

The demonstration by FACS analysis that the FSHR is co-expressed with several candidate ovarian cancer stem cell markers is a reportable outcome. The results we will have when the histological results of the cancer-like nodules are examined could be included in the co-expression study but likely will not be a stand-alone report.

## CONCLUSION:

*Accomplishments.* The results obtained indicate that several candidate ovarian cancer stem cell markers are co-expressed with the FSHR. This is very encouraging and provides the first step to suggest that targeting the FSHR with cytotoxic effectors could simultaneously destroy multiple stem/progenitor cell types and their lineages. The more difficult tasks of demonstrating whether or not the FSHR maintains co-expression with candidate markers following monoclonal expansion *in vitro* and repeat tumor cell inoculation *in vivo* were attempted but were not able to be completed to a definitive degree.

*Recommended changes for future work to better address the problem.* If the field continues with the current criteria for defining stem cells in this area (cloning co-expressing cells from a single ascites tumor cell; tumors resulting from the first injection of co-expressing cells; repeat development of co-expressing tumors from the injection of very low cell numbers from the first tumor), then we will have to be very patient and analyze a very large number of ascites samples to obtain those with sufficient numbers of single viable cells.

Another approach would be to loosen the stem cell criteria: 1) reason that tumors developing from the injection of low cell numbers and maintain the expression of the appropriate markers after several generations *in vivo* likely resulted from stem cells, 2) analyze solid tumor morphology and marker expression by immunofluorescent/immunochemical methods rather than FACS, (this option would still require FACS isolation of initial cells) or 3) attempt to perfect procedures that would yield single cells from the digestion of solid tumor tissue without destroying membrane markers in the process (FACS analysis could again be the end point of choice here).

*Significance.* To our knowledge, the successful demonstration of co-expression of even a single stem/progenitor cell marker and a specific membrane hormone receptor is novel and would be the first in the field of ovarian cancer. As mentioned above, successful results initiated in this project could provide justification for the designing of appropriate immunotoxin-based and/or laser-activated conjugates targeting the FSHR.



What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

IMPACT: There is nothing to report on any aspects in this category

CHANGES/PROBLEMS:

Negative as well as positive findings:

1. Cloning of FACS-separated single cells. We were not able to clone successfully FACS-separated single cells because of poor survival in culture. This is probably most closely related to the fact that cell viability was usually very low, most likely because of the time and stress involved in preparation for and execution of the FACS procedures.

C. Problems encountered in accomplishing certain tasks:

1. Need to prepare a new monoclonal antibody. We exhausted the supply of mouse anti-human FSHR mab 105.106 from our collaborator Dr. J. Dias during the second year of the study. This was not unexpected. We therefore thawed a vial of the original cells and successfully harvested and purified new mab.
2. Dissociation of mab-A647 label from the ascites cancer cells but not hamster embryonic (HEK) cells – a totally unexpected result. Continued characterization of the secondary antibody approach and of our new primary mab showed that the stability of labeled cells designated as FSHR double- or single- positives in the initial FACS sort was not as expected. The cells in these groups should be >90% positive if they are analyzed again within minutes. This was true for transfected HEK cells expressing the FSHR but not for the ascites cells of four patients which averaged 51% FSHR+; (range 33-65%). Interestingly, a second sort and post-second-sort purity analysis routinely resulted in an approximate 90% proportion of FSHR-positive cells, seemingly correcting the problem. Unfortunately, due to continued cell death during these analyses the final yield of viable cancer cells was usually very low – sometimes in the region of 10%. This issue needs to be pursued to determine whether it results from a mix of higher affinity and lower affinity FSH receptors on the cancer cells and/or a slight heterogeneity in the mab preparation.
3. New clinical management of ascites fluid accumulation. Many patients in our oncology clinic are now undergoing implantation of an indwelling abdominal catheter allowing them to drain their ascites at home rather than a visit to the clinic to undergo paracentesis. This reduces significantly the number of ascites samples available for research and was beyond our control.

4. Limiting number of total ascites cells available and/or in sub-optimal proportions.  
Our designed experimental objective for a given *in vivo* study required at least 450,000 single, live pre-sort OVCA cells that would yield sufficient cell numbers in each of the four FACS-isolated sub-populations. This rarely was the case in that a given vial of frozen cells usually contained too few viable single cells and/or an inadequate number of cells in one of the sub-groups (double positive, single positive FSHR, single positive candidate marker, double negative). To address this problem we were forced to reduce the scope of the experiments and compare fewer cell numbers across the sub-groups (e.g., original design, 10,000, 1,000 and 100 cells/sub-group; actual available, 5,000/sub-group, 5). In the case of the animals shown in Figure 2, no FSHR single positive cells were available.
- D. Recommendations for a better future approach(es): Suggestions are included as part of the conclusion.

There were no changes that had a significant impact on expenditures, patients, experimental animals or chemical agents.

PRODUCTS: Nothing to Report

#### PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

David W. Schomberg, PI

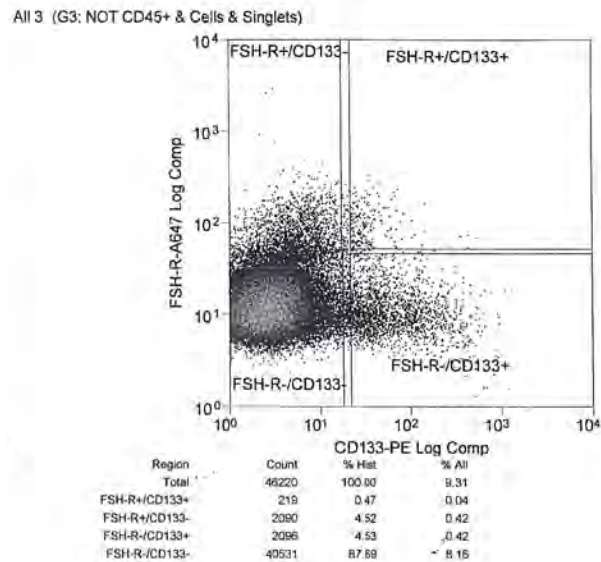
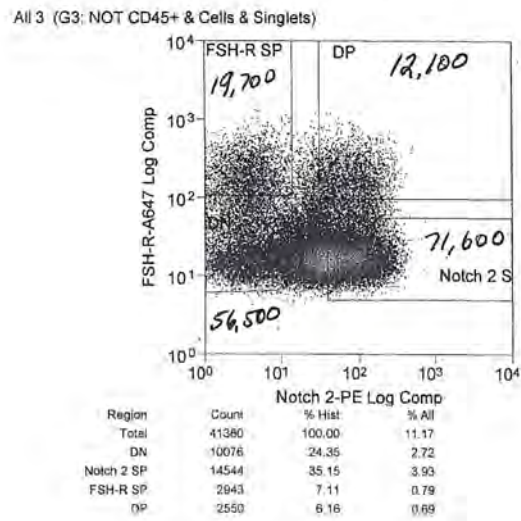
Jane Wrenn, Technician – Ms Wrenn provided technical expertise mainly in cell handling and FACS analysis

#### SPECIAL REPORTING REQUIREMENTS:

Nothing to Report

## APPENDICES:

### Appendix 1.



Appendix 1 Figure 1. FACS analysis (raw data) of ascites cells from two patients showing different degrees of FSHR expression and co-expression with Notch 2 (top) or CD133 (bottom).

## **Aggregation rather than monoclonal expansion explains ovarian cancer spheroid formation**

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**Purpose:** It is thought that a subpopulation of ovarian cancer cells possess stem cell like properties and are responsible for both the emergence of cancer and for subsequent chemoresistance and recurrence. A common approach used to enrich for cancer stem cells *in vitro* involves culture in selective media on low attachment plates resulting in non-attached multicellular spheres. Spheroids are present in ascites fluid of women with ovarian cancer, and may contain stem cells responsible for metastasis and recurrence. Our objective was to address whether spheroids are truly monoclonal in origin and arise from expansion of a single common progenitor cell.

**Procedures:** We cultivated 5 ovarian cancer cell lines (DOV13, HEYC2, OVCAR2, OVCAR3, and PEO4) to confluence in monolayer cultures. The cell lines were chosen for their ability to form spheroids as well as variation in expression of the stem cell marker CD133 (OVCAR2, OVCAR3 CD133+, others negative). The cells were trypsinized to form single cell suspensions and plated at densities ranging from  $1 \times 10^2$  to  $5 \times 10^3$  cells per plate in stem cell-selective media in ultra-low attachment culture dishes. A Zeiss Axio Observer microscope was used for time-lapse photography of the plated cells at 5-minute intervals over a 24-hour period. In addition, single cell suspensions were subjected to flow activated cell sorting to plate one cell in each well of 96-well Costar ultra-low cluster plates to monitor clonal formation of spheroids.

**Results:** Cells were dissociated and plated under stem cell-selective conditions and 288 images of each cell line were recorded over 24 hours. The cells were found to begin to aggregate within two to four hours, regardless of cell numbers plated. By 24 hours post-plating, there was pronounced aggregation in 5/5 (100%) cell lines with formation of tightly compacted spheroid structures in 4/5 (80%) cell lines. Single cells in the 96-well plates showed no evidence of spheroid formation over a 14-day observation period.

**Conclusion:** We observed spheroid formation in both CD133 positive and negative ovarian cancer cell lines. Formation occurred due to cellular aggregation rather than clonal expansion from a single progenitor with stem cell properties. Although cellular aggregation may be relevant to ovarian cancer biology, these data suggest that spheroid formation should be viewed with caution if used as a proxy for monoclonal expansion of ovarian cancer stem cells.