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14. ABSTRACT Screens using RNAi have identified genes required for specific cellular processes including apoptosis, proliferation and tumorigenesis. Stable expression of a vector based short-hairpin RNA (shRNA) library allows one to screen human genes for their individual roles in specific biologic processes. Specifically we will use an shRNA library screen to identify known and novel metastasis suppressor genes in ovarian carcinoma. Our model will identify both metastasis suppressor genes as well as genes involved in suppression of intraperitoneal attachment and tumor growth. Using this approach we generated several ovarian cancer cell sub clones that exhibited metastatic intraperitoneal tumorigenesis compared to vector or untransfected cell lines. These lines exhibited in vitro selectable marker resistance and rederived intraperitoneal tumorigenesis in second pass validation studies.					
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INTRODUCTION: At the time of diagnosis ovarian carcinoma often presents at an advanced stage with disseminated intraperitoneal disease. The mechanisms that control ovarian carcinoma progression to a locally advanced and ultimately a metastatic phenotype remain incompletely understood. Given the lethality of advanced metastatic ovarian carcinoma, identifying specific mechanisms of ovarian carcinoma progression to a metastatic phenotype are critical to develop novel strategies for therapeutic intervention. Recently, a number of genes have been identified that affect metastasis without affecting tumorigenicity, such as NM23, integrin interacting factors (KAI1), GPCRs (KiSS1), MKK4 and the Rho/Rac regulator (RHOGDI2) among others . Given the pattern of local spread and subsequent metastasis of ovarian carcinoma, new approaches for identification of genes controlling tumor progression are required. RNA interference (RNAi) is a powerful approach to identify specific genes as targets for therapeutic intervention. Screens using RNAi have identified genes required for specific cellular processes including apoptosis, proliferation and tumorigenesis. Stable expression of a vector based short-hairpin RNA (shRNA) library allows one to screen human genes for their individual roles in specific biologic processes. The purpose of this study is to use an shRNA library screen to identify known and novel metastasis suppressor genes in ovarian carcinoma, Our model will identify both metastasis suppressor genes as well as genes involved in suppression of intraperitoneal attachment and tumor growth..

BODY:

Task 1: Generate shRNA library retrovirus and stable transduce non-intraperitoneal studding, non-metastatic ovarian carcinoma cells (BG-1 , OC4) with shRNA expressing retrovirus.

Cells will be transduced in vitro with retrovirus and stable selection with puromycin will be initiated the following day. Growth of cells in selectable media will only be performed for 48 hours to eliminate non-transduced cells. Following this cells will be harvested for intraperitoneal injection. For control purposes some flasks of cells will be transfected with scrambled or empty shRNA containing retrovirus. For positive controls flasks of cells will be transduced with retrovirus shRNAs for KiSS1, nm23 and MKK4. These are known metastasis suppressors and are expected to function by enhancing i.p. implantation and metastasis of BG-1 and OC4 cell lines. (month 1)

ACCOMPLISHED: Initial studies confirmed the transduction and stable selection condition for our library. We generated stable pools for BG-1 and OC4 shRNA library overexpressing cells.

Task 2: Analyze shRNA library for effects in vivo on i.p. tumorigenesis and metastasis.

Female Nod/SCID mice (50 mice total), will be injected i.p. with cell lines as follows:

negative control cell lines (no i.p. tumors, no metastasis)

BG-1/(shRNA-scrambled) [5 mice], OC4/(shRNA-scrambled) [5 mice]

Positive control groups (i.p. tumors, positive metastasis)

OC4/(shRNA-MKK4) [5 mice], OC4/(shRNA-KiSS1) [5 mice], OC4/(shRNA-nm23) [5 mice]

BG1/(shRNA-MKK4) [5 mice], BG1/(shRNA-KiSS1) [5 mice], BG1/(shRNA-nm23) [5 mice]

Test group for shRNA library

BG-1/(shRNA library) [10 mice], OC4/(shRNA library) [10mice]

Animal will be followed three times a week and harvested in groups of 2 weekly starting at first signs of disease or morbidity. Tumors, metastases and organs will be harvested as indicated. (months 2-4)

ACCOMPLISHED: The screens from this study yielded 20 isolatable i.p. tumors from the BG-1-ShRNA group. No tumors were observed or isolated from the BG-1-shRNA-scrambled (vector) group. Two of the three expected positive control shRNA did not yield tumors (MKK4, KiSS1). Some small tumors were detected in the shRNA-nm23 group. The parallel screen with OC4 cells did not yield any detectable tumors in any groups. This may be a limitation of the time involved in the initial screen or a lack of one-hit metastasis suppressor activity in these cells.

Task 3: Animal model harvesting of tumors, metastasis and organs Sequencing of shRNA and frequency mapping

Cell lines will be established from individual i.p. tumors and macroscopic metastasis. Remaining 1/ of individual tumors will be frozen for sequencing. Liver lungs and i.p organs will be harvests and split. With a part for H&E staining and the remainder flash frozen for RNA isolation. RNA from frozen tumors will be PCR sequenced using flanking sequence primers specific for the shRNA region of the retroviral library. RNA from frozen organs will be analyzed by affymetrix arrays specific for the shRNA regions to determine shRNA frequency. (Months 4-6)

PARTIALLY ACCOMPLISHED: from the 20 tumors derived for the i.p. screen in BG-1 were able to establish 10 cell lines under stable marker (puro) selection. Initial survey of distant organs – lung

liver, did not reveal gross metastasis. Given the limited number of i.p. clones and lack of distant Mets, the follow-up AFX screen was not implemented.

Task 4: rederive specific shRNA clones in OC4 and BG1 cells

Analysis of the above data will yield specific metastasis and i.p. tumor growth suppressors genes based upon shRNA targeting from the retroviral pool library. Those sequences identified or our lead candidates based upon frequency will be used to rederive individual clones stably expressing a single shRNA of interest. In this way we expect to be able to validate that knockdown of a specific gene will produce an individual BG1 or OC4 cell lines capable of i.p tumor growth or metastasis from an i.p. site. Cell lines will be analyzed by RT-PCR and western blot to confirm knock-down. (months 5-8)

PARTILLY ACCOMPLISHED: WE have established the cell lines from our original screen. Sequencing for genomic DNA incorporation of shRNA plasmids is still pending.

Task 5: validation of shRNA clone effects on i.p. tumorigenesis and metastasis in vivo.

Those cell lines generated from the above identified shRNAs will be injected into mice (5 animal per shRNA clone) compared to shRNA scrambled. This task will test the ability of individual clone knockdown to produce an in vivo tumorigenic and metastatic phenotype. (months 7-12)

PARTIALLY ACCOMPLISHED: Using the 10 cell lines from the above screen we tested their ability to form tumors. Of the 10 we were able to successfully demonstrate that 5 exhibited stable metastatic phenotype and generated i.p. tumor in vivo.

Task 6: Analysis of specific metastasis suppressor genes mechanisms of action in vitro.

Those clones with the greatest promise of knocking out a specific metastasis suppressor gene will be pursued in vitro to identify mechanism. The mechanism tested will include migration invasion assays and survival proliferation assays. We will focus on cell signaling pathways as well. The specific of each pathways or assay used will be based upo gene information and informatics from the above studies. (months 7-12)

NOT ACCOMPLISHED: given the time put into the original screen and cell line generation we were not able to reach the point in this study that follow up mechanisms were able to be investigated.

KEY RESEARCH ACCOMPLISHMENTS

- 1- Demonstrated the utility of a genetic shRNA in vivo screen to test for knco-down induced tumor progression and metastasis.**
- 2- Established 5 ovarian cell lines with more aggressive tumorigenesis from non-tumorigenic parental lines.**

REPORTABLE OUTCOMES:

WE have generated 5 metastatic ovarian cancer cell lines derived from an original non-metastatic parent cell line.

BG-1(shRNA-SC1)

BG-1(shRNA-SC2)

BG-1(shRNA-SC3)

BG-1(shRNA-SC4)

BG-1(shRNA-SC5)

And the respective non-metastatic vector line.

BG-1(Vec)

CONCLUSIONS:

Overall the research here has demonstrated the feasibility of using transfection/transduction based shRNA screen for analyzing in vivo phenotypic changes. This offers advantages to typical in vitro screen based approaches in that it retains a greater degree of host-cancer (stromal-matrix, tissue architecture) components that might otherwise not be observed in an in vitro cell culture system. Using this in vivo screen approach we have demonstrated the generation of 5 independent metastatic ovarian cancer cell lines derived from a parental non-metastatic non tumorigenic (i.p.) line.

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APPENDICIES:

none

RESEARCH TECHNICAL REPORTING REQUIREMENTS

Format Requirements for Annual/Midterm/Final Reports

a. Annual and Midterm reports must provide a complete summary of the research accomplishments to date with respect to the **approved** Statement of Work. Journal articles **can be** substituted for detailed descriptions of specific aspects of the research, but the original articles **must** be attached to the report as an appendix and appropriately referenced in the text. The importance of the report to decisions relating to continued support of the research can not be over-emphasized. A report shall be submitted within 30 calendar days of the anniversary date of the award. A final report will be submitted upon completion of the research (last year of the award).

b. A final report summarizing the entire research effort, citing data in the annual/midterm reports and appended publications shall be submitted at the end of the award performance period. The final report will provide a complete reporting of the research findings. Journal publications can be substituted for detailed descriptions of specific aspects of the research, but an original copy of each publication must be attached as an appendix and appropriately referenced in the text. All final reports must include a bibliography of all publications and meeting abstracts and a list of personnel (not salaries) receiving pay from the research effort.

c. Although there is no page limitation for the reports, each report shall be of sufficient length to provide a thorough description of the accomplishments with respect to the approved Statement of Work. Submission of an original and two copies of the report are required. Reports shall be forwarded to:

Commander
U.S. Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
504 Scott Street
Fort Detrick, Maryland 21702-5012

d. All reports **shall** have the following elements in this order: front cover, Standard Form (SF 298), table of contents, introduction, body, key research accomplishments, reportable outcomes, conclusions, references, and appendices. Pages shall be consecutively numbered throughout the report. **DO NOT RENUMBER PAGES IN THE APPENDICES BUT DO INCLUDE THE APPENDICES IN THE PAGE COUNT IN BLOCK 15 ON THE SF 298.** Mark all pages of the report which contain proprietary or unpublished data that should be protected. **DO NOT USE THE WORD "CONFIDENTIAL" WHEN MARKING DOCUMENTS.** Indicate in your letter accompanying the report that the report contains proprietary or unpublished data, and that the distribution statement should indicate the limitations of the report.

FRONT COVER: Sample front cover provided above. The Accession Document (AD) Number should remain blank.

STANDARD FORM 298: Sample SF 298 provided above. The abstract in Block 13 **must** state the purpose, scope, major findings and be an **up-to-date** report of the progress in terms of results and significance. Subject terms are keywords that may have previously assigned to the proposal abstract or are keywords that may be significant to the research. The number of pages shall include all pages that have printed data (including the front cover, SF 298, table of contents, and all appendices). Please count pages carefully to ensure legibility and that there are no missing pages as this delays processing of reports. **Page numbers should be typed: please do not hand number pages.**

TABLE OF CONTENTS: Sample of table of contents provided above.

INTRODUCTION: Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

BODY: This section of the report shall describe the research accomplishments associated with each task outlined in the **approved** Statement of Work. Data presentation shall be comprehensive in providing a complete record of the research findings for the period of the report. Appended publications and/or presentations **may** be substituted for detailed descriptions but **must** be referenced in the body of the report. If applicable, for each task outlined in the Statement of Work, reference appended publications and/or presentations for details of result findings and tables and/or figures. The report shall include negative as well as positive findings. Include problems in accomplishing any of the tasks. Statistical tests of significance shall be applied to all data whenever possible. Figures and graphs referenced in the text may be embedded in the text or appended. Figures and graphs can also be referenced in the text and appended to a publication. Recommended changes or future work to better address the research topic may also be included, although changes to the original Statement of Work **must** be approved by the Grants Officer. This approval must be obtained prior to initiating any change to the original Statement of Work. **Spell out all acronyms the first time they appear in the report.**

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

- manuscripts, abstracts, presentations;
- patents and licenses applied for and/or issued;
- degrees obtained that are supported by this award;
- development of cell lines, tissue or serum repositories;
- informatics such as databases and animal models, etc.;
- funding applied for based on work supported by this award;
- employment or research opportunities applied for and/or received based on experience/training supported by this award.

CONCLUSIONS: Summarize the results to include the Importance and/or implications of the completed research and when necessary, recommend changes on future work to better address the problem. A "so what section" which evaluates the knowledge as a scientific or medical product shall also be included in the conclusion of the report.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science*, *Military Medicine*, etc.).

APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

BINDING: Because all reports are entered into the Department of Defense Technical Reports database collection and are microfiched, it is recommended that all reports be bound by stapling the pages together in the upper left hand corner. All original reports shall be legible and contain original photos/illustrations. Figures shall include figure legends and be clearly marked with figure numbers.