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TITLE: Role of Mesenchymal-Derived Stem Cells in Stimulating Dormant Tumor Cells to Proliferate and Form Clinical Metastases

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14. ABSTRACT
Tumor metastasis is a complex and often fatal complication of many different cancers. One of the biggest challenges to treatment is that prior to diagnosis or during treatment tumor cells can disseminate and remain dormant in distant tissue sites. These cells can become proliferative and lead to metastatic disease late after completion of therapy. The biology of this outbreak of dormant tumor cells that leads to relapsed metastatic disease is the major focus of this grant. Using a fibrosis model of tumor dormancy we have determined the break in dormancy is dependent on collagen and other fibrotic extracellular matrix components for the induction of a proliferative state in these dormant D2.0R breast cancer cell lines. Performing gene expression array on these dormant D2.0R cells exposed to collagen to induce a break from dormancy compared to dormant D2.0R cells revealed a set of genes that overlap with published dormancy gene sets. We also have performed immunophenotyping of the microenvironment of proliferating D2.0R cells in the fibrosis model of tumor dormancy and have identified an expansion of mesenchymal stem cells coincident with this metastatic outgrowth. We are now performing studies to analyze the key chemokine/cytokines released from the tumor cells transitioning from a dormant to proliferative state that may recruit these mesenchymal cells. We then plan to delve deeper into the crosstalk between these mesenchymal cell populations and the tumor cells to delineate the molecular pathways, which inform this complex biology. We plan to use both our *in vivo* and *in vitro* models with conditional gene deletion in the specific cell populations to determine the functional role of each key molecular component in the break from tumor dormancy. We anticipate these findings can identify potential therapeutic approaches to inhibit metastatic progression.

15. SUBJECT TERMS Breast cancer; dormancy; tumor recurrence; stroma; cytokines; chemokines; mesenchymal stem cells; hematologic stem cells; metastasis; quiescence; animal models; fibrosis; basement membrane extract; 3D culture

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1. INTRODUCTION:

Recurrence of breast cancer as metastatic disease many years after successful treatment of the primary tumor is a major cause of morbidity and mortality for breast cancer patients. Accumulating evidence strongly suggests that the extended period of tumor latency is due to the survival of disseminated tumor cells that exist in a dormant state. The purpose of this research is to identify mechanisms of tumor cell dormancy using novel *in vitro* and *in vivo* models of mammary cancer dormancy that we have developed. This work seeks to identify chemokines/cytokines that are involved in regulating the switch of dormant cells into a proliferate state. This study also seeks to identify interactions between dormant tumor cells and stromal cells that contribute to the dormant-to-proliferative switch. Identification of these mechanisms that regulate dormancy or the transition to proliferative, metastatic outgrowth will potentially provide molecular targets that could be exploited to prevent the proliferative outbreak of dormant tumor cells or perhaps to novel approaches to kill dormant tumor cells, thus preventing disease recurrence.

2. KEYWORDS:

Breast cancer; dormancy; tumor recurrence; stroma; cytokines; chemokines; mesenchymal stem cells; hematologic stem cells; metastasis; quiescence; animal models; fibrosis; basement membrane extract; 3D culture

3. ACCOMPLISHMENTS:

- **What were the major goals and objectives of the project?**
 - The major goals of this project are 1) to identify chemokines/cytokines that are involved in cross-talk between dormant tumor cells and stromal cells that influence the dormant-to-proliferative switch and that influence the tumor microenvironment, and may recruit MSCs and HCs to the dormant cell niche to enhance proliferation of the dormant cells; and 2) To determine the contribution of HCs, MSCs and resident stromal cells in activating the dormant-to-proliferative switch and metastasis using established *in vivo* models of mammary tumor cell dormancy.
- **What was accomplished under these goals?**
 - The Green and Kaplan laboratories have been working collaboratively with the work divided based on each lab's expertise to accomplish the stated tasks in the submitted SOW. We meet regularly to review data and to plan experiments. We have been very pleased with the progress of the work.
Aim 1: To identify cytokines/chemokines produced by dormant tumor cells triggered to proliferate *in vitro* and *in vivo* that influence the tumor microenvironment and may recruit MSCs and HCs.
 - Isolation of D2.0R cells and supernatants from 3D cultures +/- Col I.
We are working to optimize the conditions to obtain the supernatants for the chemokine/cytokine array. This has been a challenging endeavor since our 3D system requires the use of a relatively small number of dormant cells which limits the amount of secreted protein that can be assayed using the cytokine array. We are optimizing growth conditions to utilize the lowest possible FBS to maintain cell survival and limit exogenous protein content. Once completed collaboratively between the Green and Kaplan labs, we will compare the results of the cytokine protein array with our data from our recently analyzed gene expression microarray data and select high priority candidates for further functional testing of their roles in dormancy and mobilization of MSCs and HSCs to enhance the dormant-to-proliferative switch.
 - Preparation of RNA from cell isolates and Affy array hybridizations. The Green lab has performed Affy microarray on cell isolates from D2.0R dormant tumor cells grown with and without collagen in 3D matrix. We are currently in the process of analyzing the microarray data and comparing to published data sets of tumor dormancy. The Green lab identified a set of genes whose expression change during the dormant-to-proliferative switch as well as a set of genes whose expression is associated with a dormant state (proliferative D2.0R cells on BME+Col1 vs. dormant D2.0R cells on BME). This work was performed using the Affymetrix Exon Array ST 1.0 with ~190,000 probes covering ~ 23 K transcript IDs, and 16 K gene symbol annotations. ANOVA was used with FDR p value < 0.05% and fold-change +/- 2 to identify genes whose expression was most significantly changed. We have found that 14 of the genes we identified potentially related to dormancy through our microarray analyses have also been identified in a previous study that reported a dormancy signature derived from *in vitro* analyses and patient tumor samples (Kim et al, PlosOne, 2012;7(4):e35569). We believe that since these genes have been identified in two different experimental systems for dormancy, that they are high priority candidates for further evaluation in future studies.
 - Validation of Affy array data by Q-PCR/western blot – We are actively validating the genes we identified to be highly expressed in the D2.0R cells by qPCR and western blot.
 - Analysis of protein extracted from cells and culture supernatants for cytokines/chemokines by ELISA and protein arrays. This will be performed in the coming month after the chemokine array is completed based on outcome as discussed in isolation of D2.0R cells and supernatants from our 3D culture model system.

- Comparative analysis of Affy and protein experimental data to prioritize candidate cytokine/chemokines. We already have candidate gene list based on the Green lab's microarray data and will compare this list the cytokine/chemokine array we perform on the D2.0R 3D model of dormancy.
 - Identification of cytokines/chemokines generated by D2.0R cells during the dormant to proliferative switch *in vitro*. As described above we will perform the chemokine/cytokine array shortly after optimizing the culture conditions for this assay.
 - Generation of *in vivo* models undergoing the dormant to proliferative switch and controls using our established protocols. The Green and Kaplan labs have performed two large experiments using the *in vivo* model of dormant to proliferative switch activated by TGF β induced fibrosis. The Kaplan laboratory has performed extensive immunophenotyping in the lung of mice from TGF β induced fibrosis, non fibrosis with and without D2.0R tumor cells. We determined that mesenchymal stem cell levels decrease in response to fibrosis likely due to expansion into activated fibroblasts. Interestingly, we observed expansion of the MSC population after tumor injection and in association with the break from dormancy to tumor outgrowth within the lung. We did do extensive analysis of hematopoietic stem cells and their progeny including myeloid cell populations and have determined that the CD1 Nude mice are challenging due to mixed background that can alter the baseline levels of these immune cells. We therefore are using an alternate approach with colony forming assay to assess hematopoietic stem and progenitor cell functionally within the fibrotic and non fibrotic lung. We also plan to perform a fibroblast colony assay that functionally assess number of mesenchymal stem cells within the lung of fibrotic and nonfibrotic tumor bearing and non tumor bearing mice to validate the immunophenotyping for MSCs in these different groups. We anticipate will be able to enumerate the MSC Colony forming unit fibroblasts colonies to count and then use for further downstream analysis of the impact of these mesenchymal cells on the D2.0R cells directly *in vitro*.
 - Analyses of cytokine/chemokine production *in vivo* by expression profiling and proteomics. We are repeating the *in vivo* tumor dormancy fibrosis model experiments to obtain tissue for IF and expression profiling and proteomics to determine if the changes we observed in mesenchymal cell populations results in gene expression and protein changes that can help delineate the key molecules promoting this dormant to proliferative switch.
 - Identification of cytokines/chemokines generated during the dormant to proliferative switch *in vivo*. We plan to perform cytokine/chemokine array potentially on mesenchymal cells isolated from the lungs of the *in vivo* tumor dormancy fibrosis model.
- Aim 2: To determine the contribution of HCs, MSCs and resident stromal cells in activating the dormant-to-proliferative switch and metastasis using established *in vivo* models of mammary tumor cell dormancy.
- Breeding RFP-nu/nu mice to provide sufficient cohorts of mice for experimentation
Breeding pair of [B6.Cg-Tg\(CAG-mRFP1\)1F1Hadj/J](#) mice from Jackson labs. Mice to be crossed with CD1nu/nu mice for 5 – 10 generations to generate CD1nu/nu-Tg(CAG-mRFP1) mice. As our pilot experiment with the *in vivo* tumor dormancy fibrosis model revealed that the mice are of different background we have postponed the transplantation experiments until after we obtain further data as to which populations of cells are altered in this setting and could be contributing to the dormant to proliferative switch. We cannot rely on immunophenotyping of these mice given the mixed background and will use colony forming unit assay to help answer the question of hematopoietic cells. The transplant studies will also be used to understand better the origins of the mesenchymal cell populations we found altered in the lung of fibrotic and tumor bearing fibrotic mice compared to nonfibrotic and non tumor bearing non fibrotic mice.
 - Bone marrow transplantation of RFP- labeled bone marrow cells into recipient nude mice, induction of fibrosis and injection of dormant D20R cells and imaging/flow cytometry of lungs to characterize MSC and HC infiltration. We have performed these experiments without the transplantation model and have found differences in MSC infiltration in tumor bearing fibrotic lung compared to nonfibrotic and nontumor bearing lung. We will consider repeating these experiments in BMT mice to answer the question of origin of the MSCs that are elevated in tumor bearing mice.
 - Bone marrow transplantation of RFP- bone marrow cells into recipient nude mice, induction of fibrosis and mobilization of BMDCs with AMD3100 treatment followed by injection of dormant D20R cells and imaging/flow cytometry of lungs to characterize MSC and HC. These experiments will be performed in the future as outlined in the SOW after we have validated our preliminary results regarding changes in MSC infiltration in tumor bearing fibrotic lung compared to nonfibrotic and nontumor bearing lung.
- Aim 3: To target cytokines/ chemokines based on a candidate approach and those identified in Aim I/II to prevent the proliferative switch in dormant tumor cells.
- Determination of effects of inhibiting MSC and HC functions in initiating dormant-to- proliferative switch. We are actively designing experiments to determine if MSCs expand *in vitro* in response to fibrosis or D2.0R co-culture. We anticipate more in depth studies over the next two years of the grant as outlined in the SOW.

- Inhibition of IL6, FGF, and CCR2 using the *in vivo* model of fibrosis-induced dormant-to-proliferative switch. We currently have an experiment underway targeting FGF in the *in vivo* model of fibrosis induced dormant to proliferative switch. We anticipate more in depth studies over the next two years of the grant as outlined in the SOW.
- Generation of shRNA lentiviral expression vectors to knock-down candidate genes identified in Aims I and II. We have not initiated these studies yet.
- Determine the effects of the knock-down of candidate genes using *in vitro* D2.0R cells on the dormant-to-proliferative switch. We have not initiated these studies yet.

- **What opportunities for training and professional development did the project provide?**

This work supports the training of two post-baccalaureate students in the Green and Kaplan labs as well as training of post-doctoral fellows in the area of tumor cell dormancy, cytokine/chemokine analyses, gene expression profiling and characterization of stromal cell components that may play critical roles in the dormant-to-proliferative switch.

- **How were the results disseminated to communities of interest?**

We will publish our findings once these studies have been completed. The Green and Kaplan groups meet regularly to share data and discuss experimental designs of future experiments.

- **What do you plan to do during the next reporting period to accomplish the goals and objectives?**

We expect to complete the cytokine/chemokine array analyses in the next few months. We are repeating our initial *in vivo* experiments to confirm the changes in MDSC populations in the *in vivo* dormancy model. We will also initiate experiments to determine the roles of candidate cytokines in regulating the dormant-to-proliferative switch *in vivo* as described in the grant proposal. Ongoing studies will functionally validate the role of the selected genes we identified in the microarray analyses of dormancy. This may provide support for inhibition of selected genes or pathways in either killing dormant cells or preventing the dormant cells from transitioning into a proliferative state.

4. IMPACT:

- **the development of the principal discipline(s) of the project;**
Nothing to report
- **other disciplines;**
Nothing to report
- **technology transfer; or**
Nothing to report
- **society beyond science and technology.**
Nothing to report

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change.**
Nothing to report.
- **Actual or anticipated problems or delays and actions or plans to resolve them.**
We have been optimizing methods to improve the expression and concentration of proteins in the *in vitro* culture system we use for performing the cytokine arrays. The system that we developed requires a very low density of tumor cells in culture. This limits that amount of protein that gets secreted into the media. We have performed several preliminary experiments to try to optimize culture conditions to maximize protein content in the media. We are also determining whether we can perform the experiments in very low (0.1%) FBS or with no FBS. We expect to overcome these concerns very soon and perform the cytokine arrays in the near future. As discussed above we have determined that the model of fibrosis and tumor dormancy using the CD1 Nude mice are a mixed background and this makes immune cell profiling a challenge. We have planned experiments performing a colony forming unit assay to assess functional stem/progenitor cells which may reveal differences in the nonfibrotic versus fibrotic group and the nontumor bearing and tumor bearing groups but delineating myeloid populations given the heterogeneity proves challenging and may preclude using this model to assess the contribution of hematopoietic cells in the dormancy process.
- **Changes that have a significant impact on expenditures.**
Nothing to report
- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.**
Nothing to report.

6. PRODUCTS:

- **publications, conference papers, and presentations;**
Nothing to report
- **website(s) or other Internet site(s);**
Nothing to report
- **technologies or techniques;**
Nothing to report
- **inventions, patent applications, and/or licenses; and**
Nothing to report
- **other products.**
Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Provide the following information on participants:

- what individuals have worked on the project?

Name: Jeffrey E. Green, M.D.
Project Role: Initiating P.I.
Nearest person month worked: 2
Contribution to Project: Designs and oversees experimental progress; interprets data.
Funding Support: The Center for Cancer Research, NCI, Bethesda, MD.

Name: Rosandra Kaplan, M.D.
Project Role: Co-P.I.
Nearest person month worked: 2
Contribution to Project: Designs and oversees experimental progress; interprets data.
Funding Support: The Center for Cancer Research, NCI, Bethesda, MD.

Name: Ryan Nini
Project Role: Post-baccalaureate student
Nearest person month worked: 12
Contribution to Project: Performs *in vitro* and *in vivo* experiments related to dormancy; analyses gene expression profiling data

Name: Lara El Touny, Ph.D.
Project Role: Post-doctoral Fellow
Nearest person month worked: 2
Contribution to Project: Designs and oversees experimental progress; interprets data.
Funding Support: The Center for Cancer Research, NCI, Bethesda, MD.

Name: Caitin Reid
Project Role: Post-baccalaureate student
Nearest person month worked: 12
Contribution to Project: Performs FACS analyses of *in vivo* experiments.

Name: Amber Giles, Ph.D.
Project Role: Post-doctoral Fellow
Nearest person month worked: 2
Contribution to Project: Performs FACS analyses of *in vivo* experiments.

Name: Meera Murgai
Project Role: Post-doctoral fellow
Nearest person month worked: 2
Contribution to Project: Performs cytokine analyses *in vitro* and *in vivo*.

- **has there been a change in the other active support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report

- **what other organizations have been involved as partners?**

Nothing to report