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14. ABSTRACT
Tumor metastasis is a complex and often fatal complication of most 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:**

Despite successful treatment of the primary tumor and years of disease free survival in breast cancer patients, recurrent metastatic disease is a major cause of morbidity and mortality. 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. In addition, this study also seeks to identify interactions between dormant tumor cells and stromal cells that contribute to the dormant-to-proliferative switch. Identifying these mechanisms that regulate dormancy or the dormant-to-proliferative switch will potentially provide molecular targets that could be exploited to prevent the proliferative outbreak of dormant tumor cells or perhaps enhance this proliferative switch to more effectively kill these disseminated tumor cells in combination with current treatment options, thus preventing disease progression.

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 the tumor microenvironment, and may recruit MSCs and HCs to the dormant cell niche to enhance proliferation of the dormant cells; 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; and 3) To target cytokines/ chemokines based on a candidate approach and those identified in Goals 1 and 2 to prevent the proliferative switch in dormant tumor cells.

What was accomplished under these goals?

The Kaplan and Green laboratories have been working to accomplish the stated tasks in the submitted SOW. We meet regularly to review data and to plan experiments. We have made considerable progress on this 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.**

Tumor-secreted cytokines during dormant to proliferative switch using an in vitro 3D culture model.

We have previously demonstrated that D2.0R breast cancer cells cultured in Matrigel ("3D culture") alone remain dormant ("dormant D2.0R") and proliferate when cultured in Matrigel supplemented with collagen type-1 ("proliferative D2.0R") (Barkan, Cancer Research, *2008*, 68(15)). In order to determine what cytokines are secreted by D2.0R cells during this dormant to proliferative switch, we collected the culture supernatants from our 3D culture model for use on a protein-based cytokine array. We identified a panel of cytokines that were differentially secreted by proliferative D2.0R cells (Matrigel + collagen) compared to dormant D2.0R cells (Matrigel alone). The most differentially secreted cytokine in this assay was IL6. We next confirmed that IL6 is elevated under these *in vitro* conditions using an ELISA-based system (Fig 1). We are now investigating the potential functional role of IL6 in the dormant-to-proliferative switch with the use of neutralizing antibodies to IL6 *in vitro*, and plan to investigate the role of IL6 in mobilization or maturation of MSCs to enhance the dormant-to-proliferative switch.

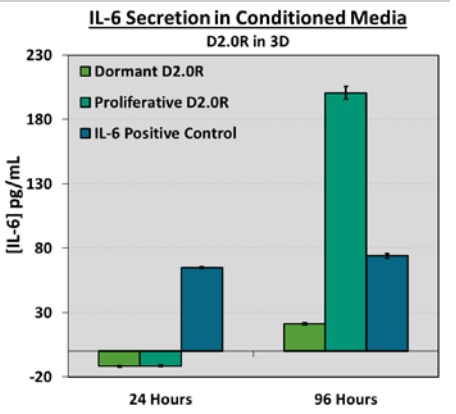

Figure 1. *ELISA validation of IL-6 Expression.* D2.0R cells in the proliferative state secrete 10-fold more IL-6 compared to the dormant state.

Gene expression signature of dormant to proliferative switch.

We previously characterized the gene expression profile of dormant and proliferative D2.0R cells in 3D cultures and identified genes that were increased in expression within proliferative D2.0Rs, including FoxM1, STAT3, DNMT1, and TK1. We further validated these genes by quantitative real-time PCR and narrowed our focus on DNMT1 which encodes for a DNA methyltransferase that is key in regulating global epigenetic methylation

patterns. We are now assessing the functional contribution of DNMT1 in tumor break from dormancy using small molecule inhibitors in our 3D culture system, where our work thus far demonstrates that DMNT1 indeed plays a role in the proliferation of D2.0R cells grown in 2D (Fig 2B).

While our gene expression data was collected at 24 hours post-plating of D2.0R cells in either Matrigel or collagen-containing Matrigel, we have now determined that dramatic morphological changes take place as early as 3.5 hours post-plating (Fig 2B). Thus, we hypothesize that early reprogramming of D2.0R cells may drastically alter their proliferative behavior within our culture system. As such, we will next determine the contribution of DMNT1 to early break from dormancy by conducting time-course proliferation and gene expression experiments. Future experiments will assess the functional role of DNMT1 in our *in vivo* dormant-to-proliferative switch model, and determine whether elevated DNMT1 results in altered STAT3 transcriptional regulation, as has been observed in other breast cancer studies including those that examine breast cancer stem cells.

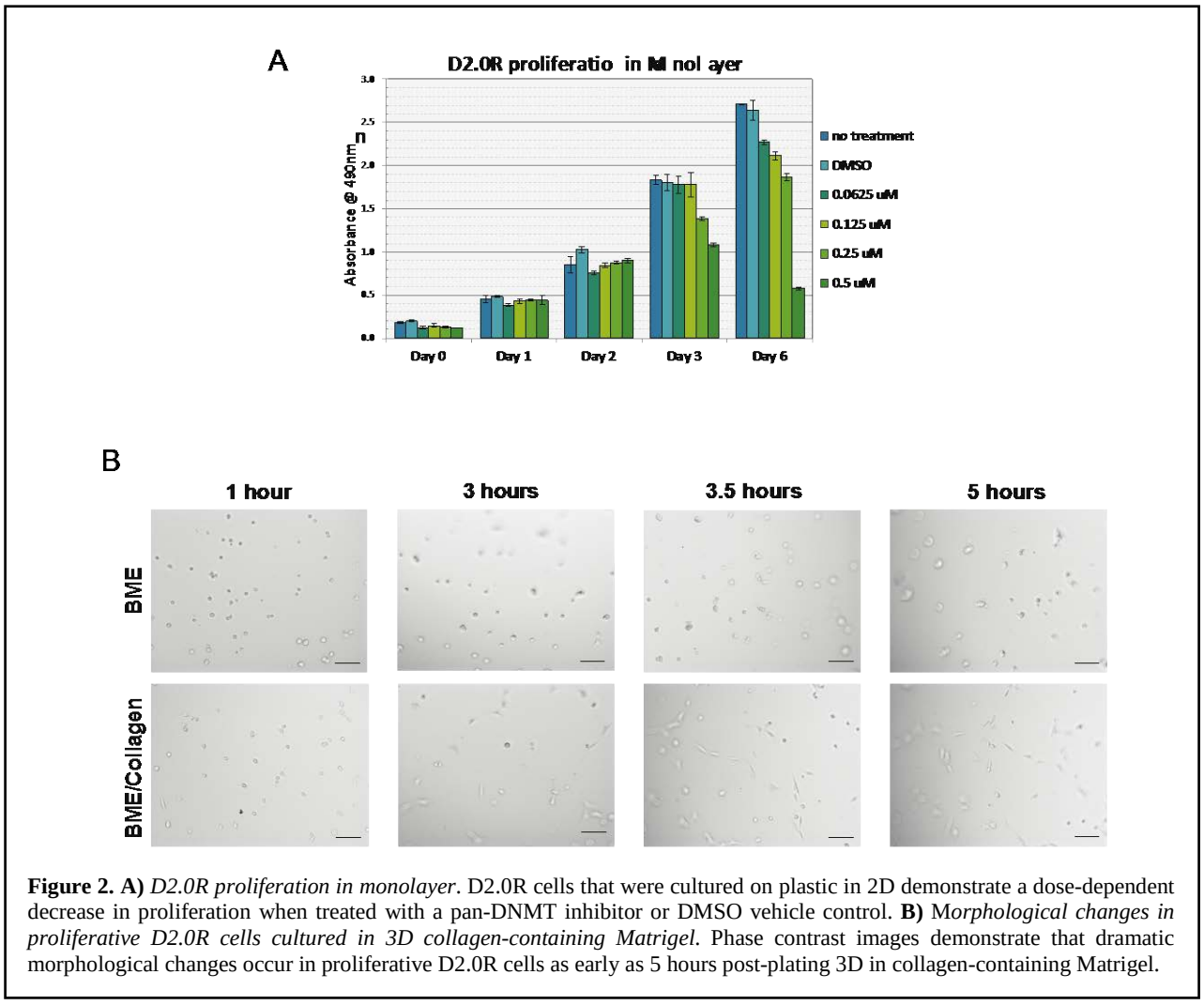

Figure 2. A) *D2.0R proliferation in monolayer*. D2.0R cells that were cultured on plastic in 2D demonstrate a dose-dependent decrease in proliferation when treated with a pan-DNMT inhibitor or DMSO vehicle control. **B)** M*orphological changes in proliferative D2.0R cells cultured in 3D collagen-containing Matrigel.* Phase contrast images demonstrate that dramatic morphological changes occur in proliferative D2.0R cells as early as 5 hours post-plating 3D in collagen-containing Matrigel.

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.**

Characterizing the HC, MSC and resident stromal cell populations during the dormant to proliferative switch in vivo.

Previously, we have demonstrated that D2.0R breast cancer cells that are intravenously (IV) injected into naïve mice arrive in the lungs, yet remain dormant as single cells. In contrast, D2.0R cells proliferate when IV injected into mice that have TGFß-induced fibrosis in the lungs (Barkan, Cancer Research, *2008*, 68(15)). In order to characterize the cellular players within the microenvironment that may contribute to the dormant-to-proliferative switch, we performed *in vivo* experiments and performed extensive immunophenotyping in the lungs of mice with or without TGFβ induced fibrosis and with or without D2.0R tumor cells. We determined that MSC levels are decreased in fibrotic lungs prior to the introduction of tumor cells, potentially due to MSC differentiation into activated fibroblasts (Fig 3a). Interestingly, we observed expansion of the MSC population in fibrotic lungs after tumor cell injection.

To validate our new findings that MSC levels are altered with fibrosis and the introduction of tumor cells, we have also performed a Colony Forming Unit-Fibroblast (CFU-F) assay to functionally assess the number of MSCs within the lungs of fibrotic and non-fibrotic tumor bearing and non tumor bearing mice (Fig 3b). We saw a significant increase in type-1, fast replicating colonies from fibrotic, non-tumor-bearing mice compared to non-fibrotic, non-tumor-bearing mice. These type-1 colonies, defined by morphological characteristics, are differentiating MSCs and may recapitulate the decreasing MSC population shown at weeks 3 and 6 by flow cytometry (Fig 3a) that may be differentiating into activated fibroblasts. Although we did not see any change in type-2, slow replicating colonies, which are the most stem-like MSCs we did observe a significant increase in type-1D, dense fast replicating colonies from tumor-bearing mice with or without fibrosis. We hypothesize that these colonies may represent activated fibroblasts, suggesting that tumor specific factors potentiate or activate MSCs to become myofibroblasts. Given the limited data on mesenchymal stem cell differentiation into different lineages including fibroblasts these investigations into the role of organ and bone marrow-derived mesenchymal stem cells and fibroblasts during fibrosis and disseminated tumor cell growth are fruitful and much need of further investigation. These studies can potentially provide new insights into this critical stromal cell biology and new therapeutic strategies for fibrosis and cancer progression. We are currently performing flow cytometric analysis of both our *in vitro* MSC cells and our *in vivo* MSC populations for fibroblast markers to better define and functionally characterize these different stromal cell populations in our dormancy and proliferative settings. We anticipate these investigations will allow us to better drill down on the signaling cross talk in specific stromal cell populations and/or phenotypes that may vary in the dormant and proliferative settings and promote critical changes in the D2.0R cells in the dormant to proliferative state. Furthermore, we plan to perform more detailed gene expression profiling to examine IL6 and STAT3 pathway signaling within these stromal cell populations.

In addition to mesenchymal cell investigations we did extensive analysis of hematopoietic stem cells and their progeny including myeloid cell populations and were unable to characterize these populations due to the mixed background of CD1 nude mice that can alter the baseline levels of these immune cells (Fig 3c). We therefore are using the alternate approach of colony forming assays to assess hematopoietic stem and progenitor cell functionally within the fibrotic and non-fibrotic lung.

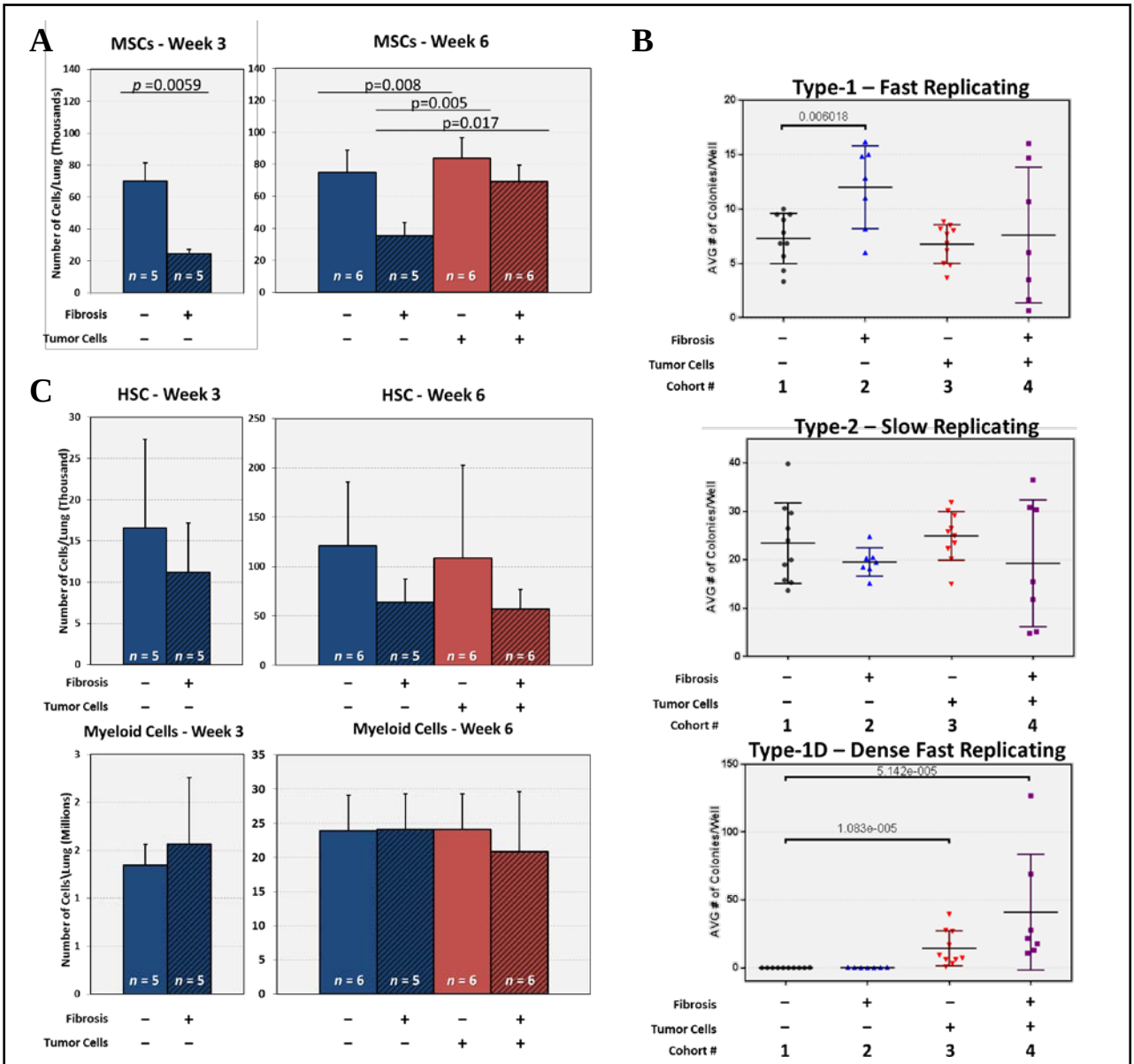

Figure 3. A) *Mesenchymal stem cell (MSC) population numbers by flow cytometry.* MSC populations decreased in response to fibrosis and absence of tumor cells, compared to non-fibrotic, tumor-free mice at weeks 3 and 6. The presence of tumor cells in the context of fibrosis ameliorated this decrease in MSCs. **B)** *Characterization of MSCs by Colony Forming Unit – Fibroblast assay*. Fibrotic, non-tumor bearing mice show a modest increase in Type-1 colonies as compared to non-fibrotic, non-tumor bearing mice. There were no significant differences in type-2 colonies. There was a significant increase in Type-1D colonies in tumor bearing mice compared to non-tumor bearing, which was further increased in the presence of fibrosis. **C)** *Hematopoietic Stem cells (HSCs) and Myeloid Cell population numbers by flow cytometry*. No statistical difference amongst groups due to intra-group variability.

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 proposed to acquire a breeding pair of B6.Cg-Tg(CAG-mRFP1)1F1Hadj/J mice from Jackson labs. Mice were to be crossed with CD1nu/nu mice for 5 – 10 generations to generate CD1nu/nu-Tg(CAG-mRFP1) mice for use in bone marrow transplant studies. Our flow cytometric studies using the *in vivo* tumor dormancy fibrosis model revealed that the mice are of different background (Fig 3c), thus 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 these mice given the mixed background. Consequently, we will use colony forming unit assays 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, tumor-bearing mice compared to non-fibrotic, non-tumor-bearing mice. Similarly, we will postpone experiments using AMD3100 to mobilize bone marrow derived cells in the context of our *in vivo* tumor dormancy fibrosis model. These experiments will be performed in the future as outlined in the SOW after we have performed more detailed studies as outlined above regarding changes in MSC infiltration in fibrotic, tumor-bearing lungs compared to non-fibrotic, non-tumor bearing lungs.

Aim 3: To target cytokines/ chemokines based on a candidate approach and those identified in Goals 1 and 2 to prevent the proliferative switch in dormant tumor cells.

Given our findings that MSC levels are decreased in the lungs of non-tumor-bearing mice in response to fibrosis, but are elevated when tumor is introduced, we are focusing our functional *in vitro* studies on the MSC population. We have previously demonstrated that MSCs secrete increased IL6 when cultured in TGFß-containing medium (Fig 4A), like D2.0R cells that are cultured in Matrigel supplemented by collagen (Fig 1). This TGFß-stimulated MSC-conditioned medium (MSC-TGFß-CM) and corresponding vehicle control stimulated MSC conditioned medium (MSC-Vehicle-CM) have now been used in functional studies of D2.0R dormancy and proliferation. We have now determined that D2.0R cells cultured in MSC-TGFß-CM exhibit decreased break from dormancy when compared to those cultured in MSC-Vehicle-CM (Fig 4B). Conversely, proliferative D2.A1 cells that are cultured in MSC-Vehicle-CM exhibit decreased proliferation that is restored in those cultured in MSC-TGFß-CM (Fig 4C). D2.0R cells cultured with recombinant IL6 also exhibited decreased break from dormancy (Fig 4D), suggesting that MSCs secrete increased IL6 in response to TGFß stimulation, which in turn inhibits D2.0R break from dormancy in response to a collagen-containing microenvironment. Maintenance of IL-6 mediated dormancy was not cell-autonomous in that neutralizing IL6 antibody treatment of D2.0Rs did not affect tumor break from dormancy (Fig 4E), despite elevated levels of IL6 secreted by D2.0Rs when cultured in collagen-containing Matrigel (Fig 1).

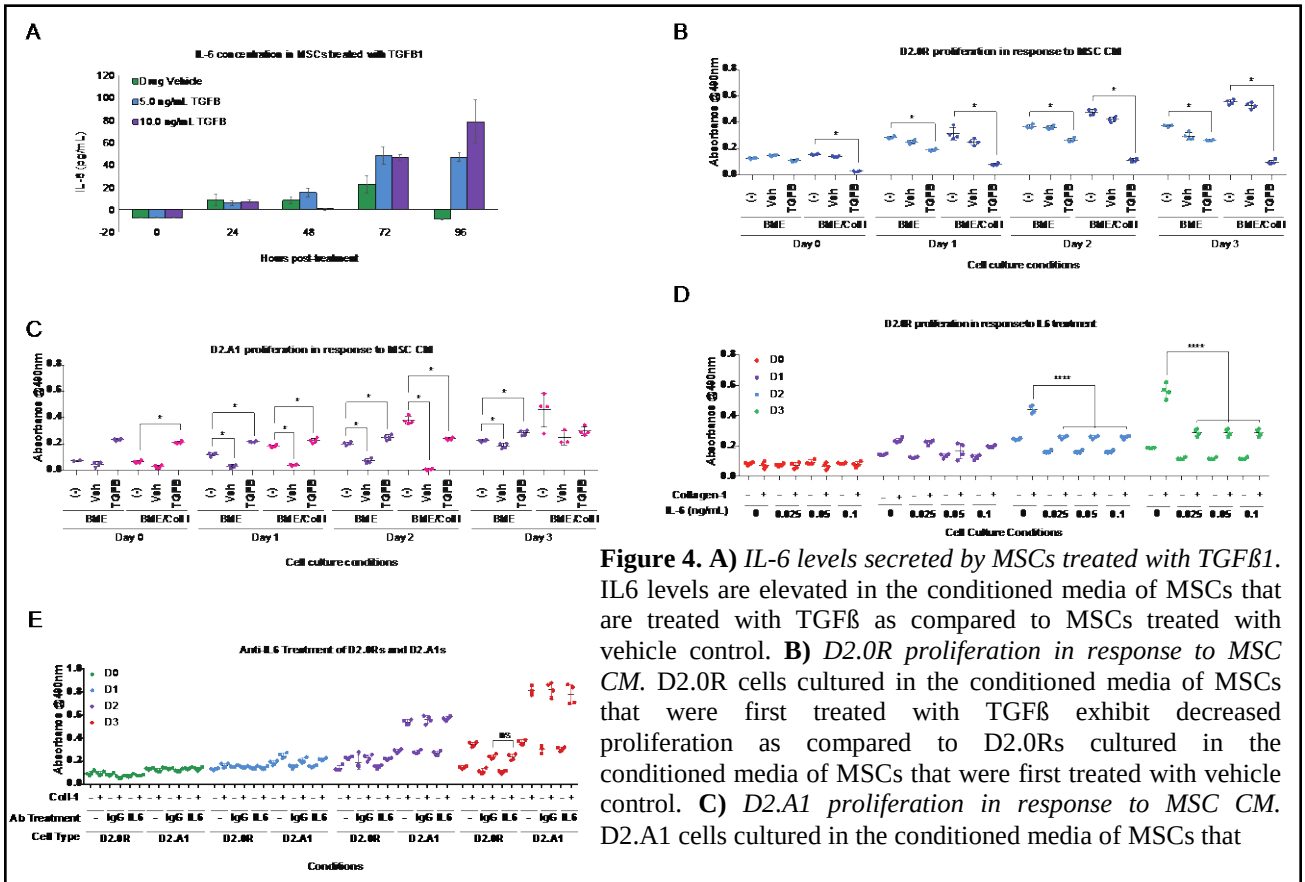

Figure 4. A) *IL-6 levels secreted by MSCs treated with TGFß1.* IL6 levels are elevated in the conditioned media of MSCs that are treated with TGFß as compared to MSCs treated with vehicle control. **B)** *D2.0R proliferation in response to MSC CM.* D2.0R cells cultured in the conditioned media of MSCs that were first treated with TGFß exhibit decreased proliferation as compared to D2.0Rs cultured in the conditioned media of MSCs that were first treated with vehicle control. **C)** *D2.A1 proliferation in response to MSC CM.* D2.A1 cells cultured in the conditioned media of MSCs that were first treated with vehicle control exhibit decreased proliferation as compared to D2.0Rs cultured in either normal media or the conditioned media of MSCs that were first treated with TGFß. **D)** *D2.0R proliferation in response to IL6 treatment.* D2.0R cells exhibit decreased proliferation when treated with recombinant IL 6 as compared to vehicle control. E) D2.0R and D2.A1 proliferation in response to Anti-IL6 treatment. Neither D2.0R nor D2.A1 cells exhibited a change in proliferation in response to neutralizing anti-IL6 antibodies.

Following these surprising results, we are now investigating the dual role of MSC-mediated dormancy maintenance and proliferation. We will investigate whether IL6 induced dormancy maintenance is required at early time-points of D2.0R culture and the response of D2.A1 proliferation to recombinant IL6 treatment. As well, we will employ siRNA and neutralizing antibody approaches to confirm the role of MSC secreted factors including IL6 in

mediating dormancy and proliferation. Finally, we will examine the gene expression profile of D2.0R and D2.A1 cultured with MSC-Vehicle-CM and MSC-TGFß-CM to further delineate the mechanisms by which this dual response to MSC secreted factors occurs in dormant and proliferative tumor cells.

What opportunities for training and professional development did the project provide?
This work supports the training of one post-baccalaureate student in the Kaplan lab, one post-baccalaureate student in the Green lab as well as training of the labs in the continued growing 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 Kaplan and Green labs 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?
IL6 targeting experiments in vitro and in vivo along with DNMT1 inhibition with a small molecular inhibitor will provide functional insight into these discoveries made regarding the biology of MSC contribution to myofibroblasts and the dormant-to-proliferative switch. These investigations will be part of the larger body of work that we are making into a manuscript, which we plan to publish in the coming year.

4. IMPACT:

- **the development of the principal discipline(s) of the project;**
  Nothing to report generating data
- **other disciplines;**
  Nothing to report
- **technology transfer; or**
  Nothing to report, generating data regarding the inhibitory role of IL6 in the dormant tumor cell to proliferative switch.
- **society beyond science and technology.**
  Nothing to report – working on a disclosure related to the role of the tumor microenvironment in quiescent disseminated tumor cells.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change.**
  Nothing to report. We are accumulating new data regarding the earliest changes in D2.0Rs during transition from dormant to the proliferative state and therefore plan to focus our gene expression studies on these earliest timepoints as likely most influenced by local changes in the microenvironment.
- **Actual or anticipated problems or delays and actions or plans to resolve them.**
  Nothing to report
- **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;**
  Jennifer Zhu submitted an abstract and will present this work at the Annual Biomedical Research Conference for Minority Students in November 2017? We also plan to submit an abstract to an upcoming first AACR meeting focused on tumor dormancy in June 2018.
- **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:

Name:	Jeffrey E. Green, M.D.
Project Role:	Initiating P.I.
Nearest person month worked:	4
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:	4
Contribution to Project:	Designs and oversees experimental progress; interprets data.
Funding Support:	The Center for Cancer Research, NCI, Bethesda, MD.

Name:	Ryan Nini, B.Sc.
Project Role:	Post-baccalaureate student
Nearest person month worked:	24
Contribution to Project:	Performed *in vitro* and *in vivo* experiments; Performed cytokine analyses *in vitro*; analyzed gene expression profiling data; performed FACS analyses of *in vitro* experiments designs experiments; interpreted data.

Name:	Meera Murgai, Ph.D.
Project Role:	Post-doctoral fellow
Nearest person month worked:	17
Contribution to Project:	Designs and oversees experimental progress; conducts *in vitro* and *in vivo* studies; interprets data.

Name:	Jennifer Zhu, B.Sc.
Project Role:	Post-baccalaureate student
Nearest person month worked:	13
Contribution to Project:	Performs in vitro and in vivo experiments.

Name:	Kush V Bhatt, B.Sc.
Project Role:	Post-baccalaureate student
Nearest person month worked:	12
Contribution to Project:	Performed *in vitro* and *in vivo* experiments.

Name:	Lara El Touny, Ph.D.
Project Role:	Post-doctoral Fellow
Nearest person month worked:	2
Contribution to Project:	Designed and oversaw experimental progress; interpreted data.

Name:	Shil Patel, B.Sc.
Project Role:	Post-baccalaureate student
Nearest person month worked:	1
Contribution to Project:	Performs *in vitro* and *in vivo* experiments.

- **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