AWARD NUMBER: W81XWH-14-1-0099

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

PRINCIPAL INVESTIGATOR: Rosandra Kaplan

CONTRACTING ORGANIZATION:
The Geneva Foundation

Tacoma, WA 98402

REPORT DATE: SEPT 2018

TYPE OF REPORT: Final Report

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

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

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
SEPT 2018	Final Report	15JUN2014 - 14JUN2017

4. TITLE AND SUBTITLE
Role of Mesenchymal-Derived Stem Cells in Stimulating Dormant Tumor Cells to Proliferate and Form Clinical Metastases

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0099

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
PIs: Jeffrey Green and Rosandra Kaplan

EMAIL: fmyers@genevausa.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Geneva Foundation
917 Pacific Ave. #600
Tacoma, WA 98402

8. PERFORMING ORGANIZATION REPORT NUMBER

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

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

10. SPONSOR/MONITOR'S ACRONYM(S)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
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 and alteration of mesenchymal stem cells coincident with this metastatic outgrowth. We have performed 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 have delved into the crosstalk between these mesenchymal cell populations and the tumor cells to delineate the molecular pathways, which inform this complex biology. We 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. These findings are aiding in the identification of potential new 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

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

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Page

1. **Introduction……………………………………………………………...1**
2. **Keywords……………………………………………………………....1**
3. **Accomplishments………..……………………………………………....1**
4. **Impact…………………………...…………………………………….12**
5. **Changes/Problems...….………………………………………………..13**
6. **Products…………………………………….……….….……………...13**
7. **Participants & Other Collaborating Organizations………………...14**
8. **Special Reporting Requirements……………………………………NA**
9. **Appendices…………………………………………………………NA**

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. The goal of this work focused on identifying chemokines/cytokines that are involved in regulating the switch of dormant cells into a proliferate state. In addition, this research is leading to elucidation of 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 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, 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:

<u>What were the major goals and objectives of the project?</u>

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. 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.

<u>What was accomplished under these goals?</u>

The Green and Kaplan laboratories have been working collaboratively with the work divided based on each labs' expertise to accomplish the stated tasks in the submitted SOW. We met regularly to review data and to plan experiments. We have made strong gains in our understanding of pathways involved in microenvironmental regulation of the quiescent and proliferative state of breast cancer cells. These insights have lead to development of new approaches to limit break in tumor dormancy and metastatic progression. In addition to contributing to a publication this body of work is resulting in development of a clinical trial for patients with high solid tumors.

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 have been investigating the potential functional role of IL6 in the dormant-to-proliferative switch with the use of neutralizing antibodies to IL6 *in vitro*, and *in vivo* to better understand the role of IL6 and other secreted factors in mobilization or maturation of MSCs to enhance the dormant-to-proliferative switch. We have performed multiple experiments using both IL6 treatment of D2.OR cells (Figure 2) as well as IL6 neutralizing antibodies and GP130 treatment (Figures 3 and 4) which targets IL6 signaling with cells that both do not express IL6 receptor. The goal of these studies were used to inhibit the proliferative switch the D2.OR cells undergo in response to collagen. The results of these investigations suggest that IL6 plays a complex role in regulating breast cancer cell dormancy as both recombinant IL6 and IL6 targeting can limit D2.OR break in dormancy in response to collagen.

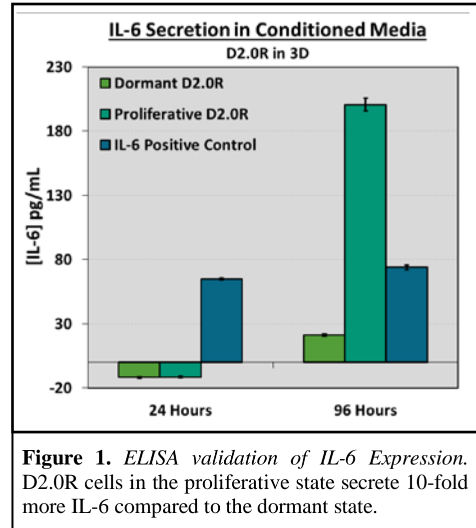


Figure 1. *ELISA validation of IL-6 Expression.* D2.0R cells in the proliferative state secrete 10-fold more IL-6 compared to the dormant state.

IL-6 TREATMENT OF D2.0R CELLS SHOW SUPPRESSED PROLIFERATION COMPARED TO D2.0R CELLS THAT WERE NOT TREATED WITH RECOMBINANT IL-6

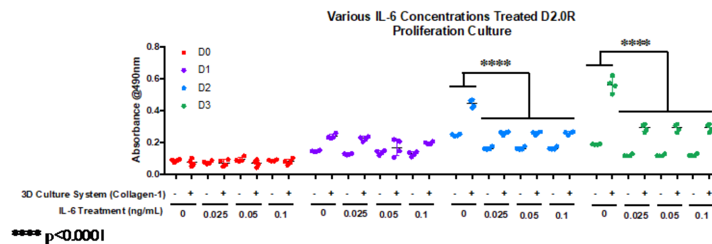


Figure 2 Recombinant IL6 treatment of D2.OR cells that are placed in Matrigel 3D culture condition alone (-) or with Collagen (+) and with increasing concentration of IL6 treatment.

40ng/mL GP130 TREATMENT OF D2.0R CELLS SUPPRESSES PROLIFERATION COMPARED TO LOWER CONCENTRATIONS OF GP130

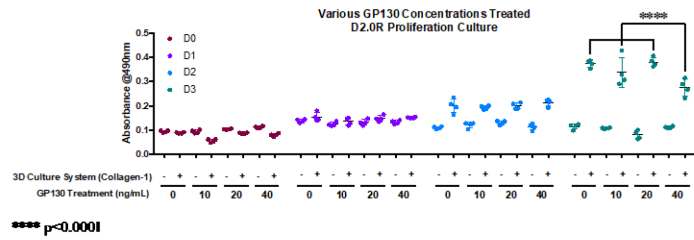


Figure 3 IL6 Targeting treatment of D2.OR cells that are placed in Matrigel 3D culture conditions alone (-) or with Collagen (+) and with increasing concentrations of GP130.

IL-6 NEUTRALIZING ANTIBODY AND IgG CONTROL SUPPRESS D2.0R PROLIFERATION IN MATRIGEL + COLLAGEN

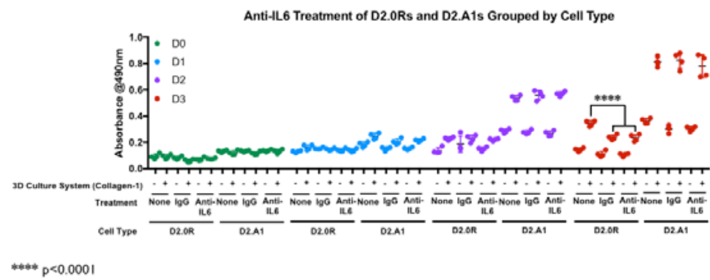


Figure 4 IL6 Neutralizing Antibody treatment of D2.OR cells (dormant) and D2.A1 cells (proliferative) that are placed in Matrigel 3D culture conditions alone (-) or with Collagen (+) and with neutralizing antibody to IL6 or IgG control.

Gene expression signature of dormant to proliferative switch.

We have characterized gene expression of proliferative (collagen + matrigel) vs. dormant (matrigel alone) D2.0R cells in 3D culture. This work was performed using the Affymatrix 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 analyzed the microarray data and compared our differentially expressed gene sets to a published data set of tumor cell dormancy. We identified a set of 14 genes associated with the dormant-to-proliferative switch which corresponds to a previous study that described a dormancy signature derived from analyses of *in vitro* and patient tumor samples (Kim et al, PlosOne, 2012;7(4):e35569). Given that since these genes have been identified in two different experimental systems for dormancy, these genes are high priority candidates for further evaluation in future studies.

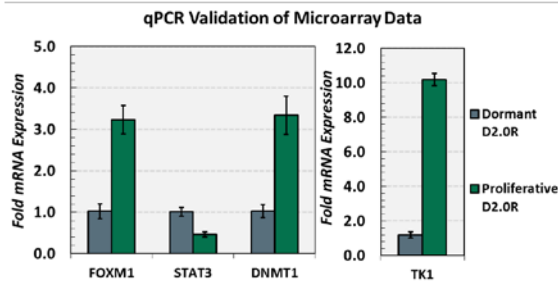
Candidate genes from our microarray analysis (Fig 5a) were further validated by quantitative real-time PCR, including FoxM1, STAT3, DNMT1, and TK1 (Fig 5b). To determine if the dormant-to-proliferative switch in our *in vitro* 3D system is dependent on FOXM1, DNMT1 or TK1, we have established D2.0R cell lines with shRNA-mediated stable knock-down of each of these three genes. Their knock-down efficiency was validated via q-rtPCR (Fig 5c) prior to performing functional assays. Experiments utilizing these stable cell lines in our 3D model system demonstrated decreased proliferative growth of these transduced cell lines. In parallel, we utilized a DNMT1-specific small

molecule inhibitor in our 3D system to complement our shRNA-mediated stable knock-down approach.

A *D2.0R in Proliferative-Outbreak vs. Dormant States (24 Hours)*

Gene Name	Abbr.	Fold Change	P-Value	Function
Budding Uninhibited by Benzimidazoles 1 homolog	*BUB1*	3.27	0.0029	*Centromere assembly*
Budding Uninhibited by Benzimidazoles 1 homolog, beta	*BUB1B*	2.63	0.0069	*Cell Cycle, spindle assembly*
Cyclin-Dependent Kinase Inhibitor 3	*CDKN3*	2.42	0.0135	*Cell cycle*
DNA Methyltransferase (cytosine-5)	*DNMT1*	1.84	0.0001	*DNA methylation*
Fos-like Antigen 1	*FOSL1*	1.29	0.0053	*Differentiation, proliferation*
Forkhead Box M1	*FOXM1*	2.96	0.0009	*Cell Cycle, DNA damage*
Phosphatidylinositol 3-Kinase, Catalytic, beta polypeptide	*PIK3CB*	1.19	0.0234	*Motility, proliferation, and cell survival*
Thymidine Kinase 1	*TK1*	3.45	0.0009	*Biomarker for recurrence*
Cathespsin D	*CTSD*	-1.48	0.0044	*Intracellular breakdown of proteins*
Signal Transducer and Activator of Transcription 3	*STAT3*	-1.5	0.0005	*Cell growth, apoptosis*
Actavin A receptor, type 1	*ACVR1*	-1.28	0.027	*Cell growth?*
Basic Helix-Loop-Helix Family, member e41	*BHLHE41*	-1.32	0.0518	*Cell differentiation*
Collagen, type 5, alpha 5	*COL4A5*	-1.39	0.0046	*Basement Membrane Component*
Tropomyosin 1, alpha	*TPM1*	-1.14	0.0436	*Cytoskeleton stability, muscle contraction*

B



C

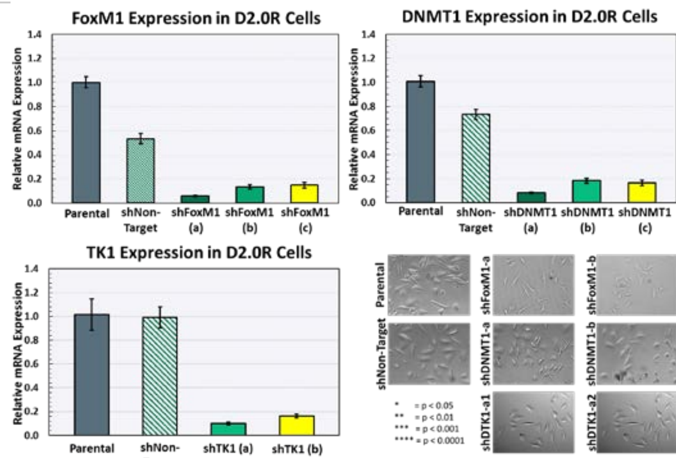


Figure 5. A) *Summary of genes implicated in tumor cell dormancy*. D2.0R cells were cultured in BME (dormant) or BME+Col-1 (proliferative) conditions for 24 hours, and gene expression was characterized by microarray. **B)** *Validation of tumor cell dormancy genes by RT-qPCR*. Genes FoxM1, STAT3, DNMT1, and TK1 showed differential gene expression by qPCR, which matches microarray data. **C)** *Expression of shRNA targeting dormancy genes shows stable knockdown of genes in D2.0R cells*. We see a stable decrease in expression of tumor cell dormancy genes by 80-90% of parental expression levels. There are no appreciable morphological differences amongst cell lines.

The DNMT1 inhibitor used in our 3D in vitro system inhibited the growth of D2.OR cells (Figure 6). We are currently focused on combining DNMT1 inhibitor therapy with other drug combinations to improve the efficacy and current in vivo studies with DNMTI inhibition in the setting of TGFb induced fibrosis are underway.

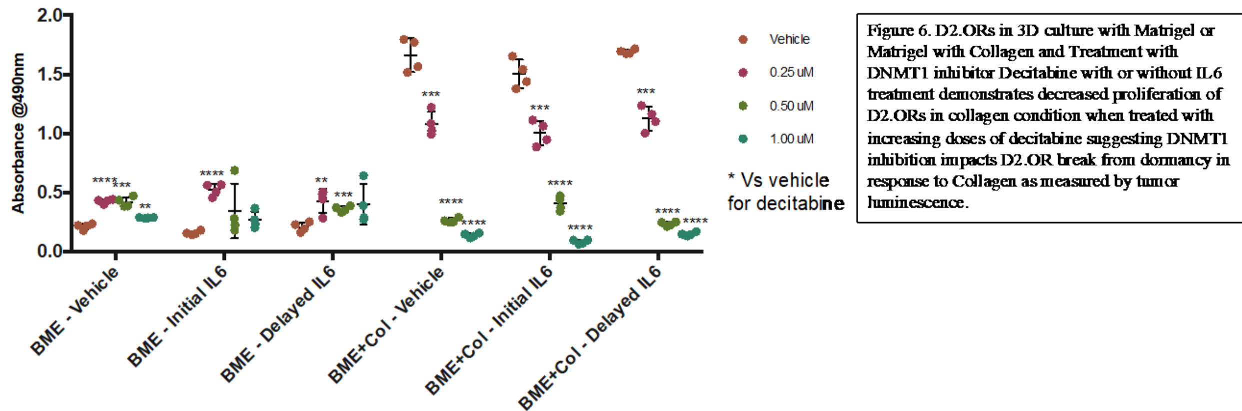


Figure 6. D2.ORs in 3D culture with Matrigel or Matrigel with Collagen and Treatment with DNMT1 inhibitor Decitabine with or without IL6 treatment demonstrates decreased proliferation of D2.ORs in collagen condition when treated with increasing doses of decitabine suggesting DNMT1 inhibition impacts D2.OR break from dormancy in response to Collagen as measured by tumor luminescence.

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 D2.ORswitch 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 have performed two large *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 or perivascular cells (Fig 7a+ update). 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 7b). 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 characterize differentiating MSCs and may recapitulate the decreasing MSC population shown at weeks 3 and 6 by flow cytometry (Fig 7a) that may be differentiating into activated myofibroblasts or pericytes. Although we did not see any change in type-2, slow replicating colonies, which characterize 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 pericytes or myofibroblasts, suggesting that tumor specific factors potentiate or activate MSCs to become perivascular cells or myofibroblasts. Based on these findings in vivo we tested this hypothesis by treating mesenchymal stem cells in 3D culture in Matrigel with TGFB. After 24 hours we then added D2.0Rs which were observed and cell proliferation was assessed for each condition of MSCs with only the TGFB treated MSCs induced D2.0Rs break dormancy to become proliferative unlike the mesenchymal

stem cells that do not induce D2.0R proliferation (Figure 9). 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 have been in much need of further investigation. These studies are beginning to provide new insights into this critical stromal cell biology and opening new therapeutic strategies for fibrosis and cancer progression.

In addition to mesenchymal cell investigations we performed 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 (Figure 7c). 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.

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 7c), 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 were to 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 defered experiments using AMD3100 to mobilize bone marrow derived cells in the context of our *in vivo* tumor dormancy fibrosis model. These experiments were not performed as outlined in the SOW as we have validated and focused on our results regarding changes in MSC infiltration in fibrotic, tumor-bearing lungs compared to non-fibrotic, non-tumor bearing lungs.

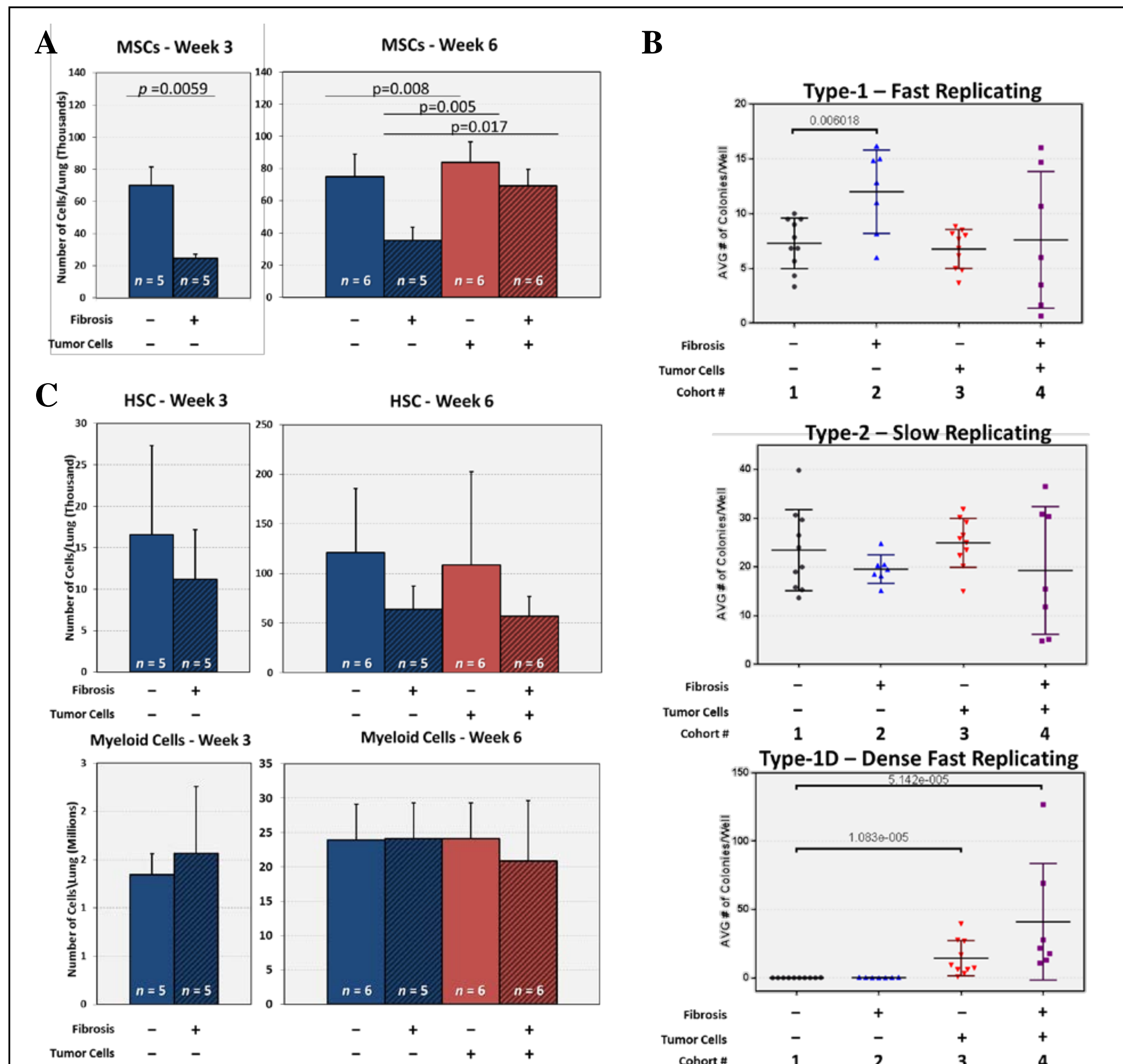


Figure 7. A) *Mesenchymal stem cell (MSC) population numbers by flow cytometry.* MSC populations decreased in response to fibrosis and absence of tumor cells, compared to non-fibrotic, non-tumor-bearing mice at weeks 3 and 6. The presence of tumor cells in the context of fibrosis ameliorated this decrease 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.

MORPHOLOGICAL CHANGE FROM SPHERICAL TO SPINDLY (DORMANT TO PROLIFERATIVE) OCCURS AS EARLY AS 3 HOURS

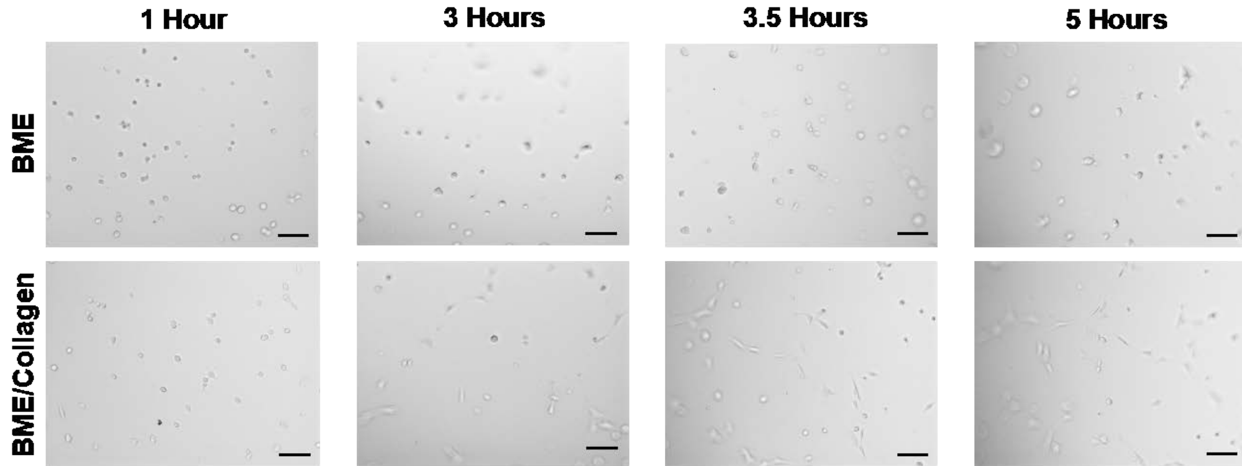


Figure 8 M*orphological changes in proliferative D2.0R cells cultured in 3D collagen-containing Matrigel*. Phase contrast images demonstrate that dramatic morphological changes occur in proliferative D2.0R cells as early as 3 hours post-plating 3D in collagen-containing Matrigel.

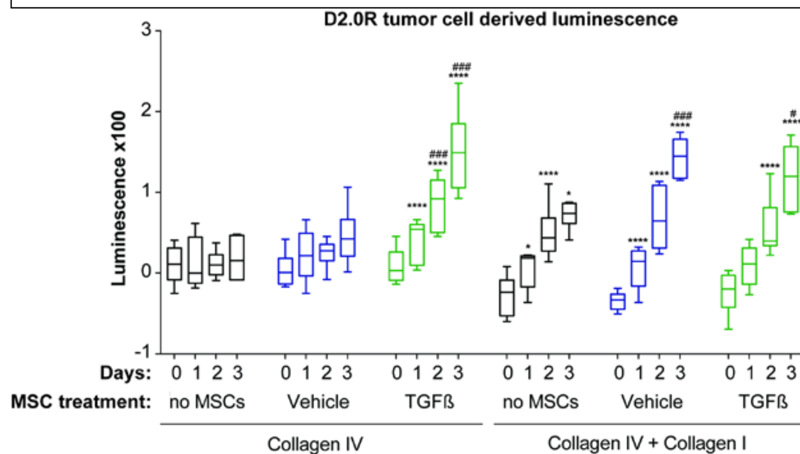
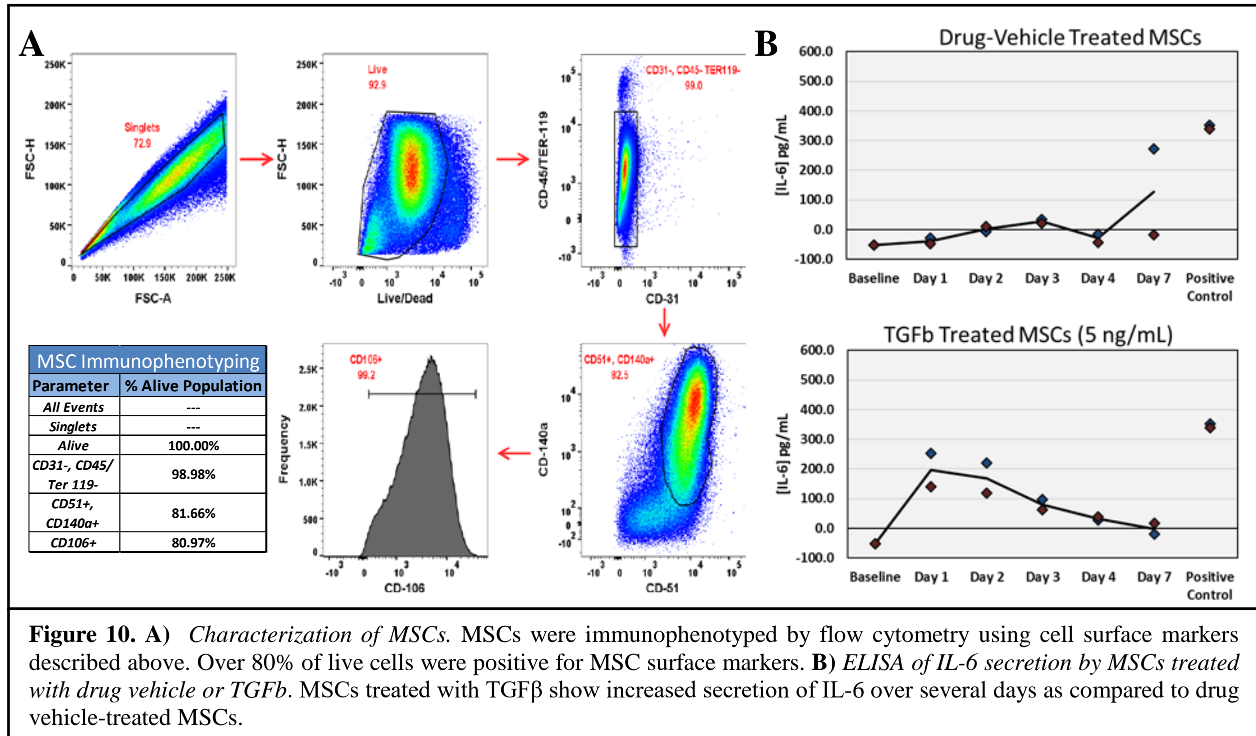


Figure 9 *D2.0R proliferation in response to co-culture with MSCs.* D2.0R cells cultured with MSCs that were first treated with TGFß exhibit increased proliferation as compared to D2.0Rs cultured with MSCs that were first treated with vehicle control. These findings suggest that MSCs are not inherently activating D2.ORs to break dormancy but when conditioned with TGFb they change to induce D2.OR proliferation.

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.

Determination of inhibiting MSC and HC functions in initiating dormant-to-proliferative switch. 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 acquired an MSC cell line from Cyagen and immunophenotyped these cells (Fig. 10a). We have demonstrated that, like D2.0R cells that are cultured in matrigel supplemented by collagen (Fig. 1), MSCs secrete IL6 when cultured in TGFß-containing medium (Fig. 10b). This finding re-enforces that IL-6 might be a key signaling axis in the dormant-to-proliferative switch, and is a key target for our proposed inhibition studies *in vivo*. To determine the functional role of IL6 signaling, we are currently utilizing recombinant IL6 protein and siRNA targeting IL6 in our *in vitro* culture systems for MSCs, D2.0R breast cancer cells, and in combination. Concurrently, we are using culture supernatants from MSCs +/- TGFß, and D2.0Rs in matrigel +/- collagen to assess whether IL6 is a key signaling axis for cross-talk between these

two populations to promote the dormant-to-proliferative switch by D2.0R cells. Consequently, we hypothesize that IL-6 mediates signaling between these two populations to promote the dormant-to-proliferative switch of D2.0R cells.



MSC Immunophenotyping	
Parameter	% Alive Population
All Events	---
Singlets	---
Alive	100.00%
CD31-, CD45/ Ter 119-	98.98%
CD51+, CD140a+	81.66%
CD106+	80.97%

Figure 10. A) *Characterization of MSCs.* MSCs were immunophenotyped by flow cytometry using cell surface markers described above. Over 80% of live cells were positive for MSC surface markers. **B)** *ELISA of IL-6 secretion by MSCs treated with drug vehicle or TGFb*. MSCs treated with TGFβ show increased secretion of IL-6 over several days as compared to drug vehicle-treated MSCs.

Inhibition of IL6 using the in vivo model of fibrosis-induced dormant-to- proliferative switch.

We targeted IL6 in our *in vivo* fibrosis model of the dormant-to-proliferative switch to assess proliferative outgrowth of D2.0R cells in the context of neutralizing antibody-based IL6 inhibition (Figure 11). Subsequent experiments will focus on the contribution of MSCs to the dormant-to-proliferative switch under the context of IL6 inhibition.

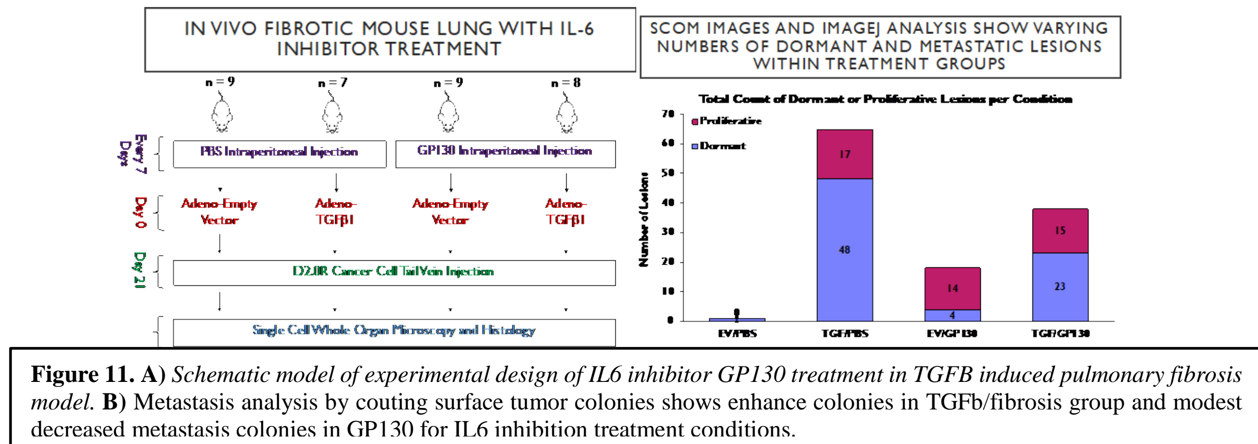


Figure 11. A) *Schematic model of experimental design of IL6 inhibitor GP130 treatment in TGFB induced pulmonary fibrosis model.* **B)** Metastasis analysis by couting surface tumor colonies shows enhance colonies in TGFb/fibrosis group and modest decreased metastasis colonies in GP130 for IL6 inhibition treatment conditions.

In addition to work discussed above we have determined that mesenchymal cells that are treated with TGFb and cultured with D2.0Rs in Matrigel induces proliferation similar to D2.0Rs placed in Matrigel and collagen. In order to better understand the role these mesenchymal cells play in promoting break in dormancy for breast cancer cells we performed RNA sequencing of MSCs treated with vehicle co-cultured with D2.0Rs in Matrigel condition, MSCs treated with TGFb co-cultured with D2.0Rs in Matrigel condition, MSCs treated with vehicle co-cultured with D2.0Rs in Matrigel and collagen condition and MSCs treated with TGFB co-cultured with D2.0Rs in Matrigel and collagen. These studies revealed that mesenchymal stem cells change their phenotype in response to TGFb. We determined that based on the expression profile of mesenchymal cells exposed to TGFb it suggests that the mesenchymal stem cells undergo a transition to a more differentiated state and begin to express genes associated with pericyte/vascular smooth muscle cell/myofibroblast type cell (Figure 12). This transition we hypothesizes occurs in response to fibrosis and may explain the apparent decrease in MSCs in vivo in lungs of TGFB adenovirus treated mice. These perivascular cells we have shown support metastatic seeding of disseminated tumor cells.

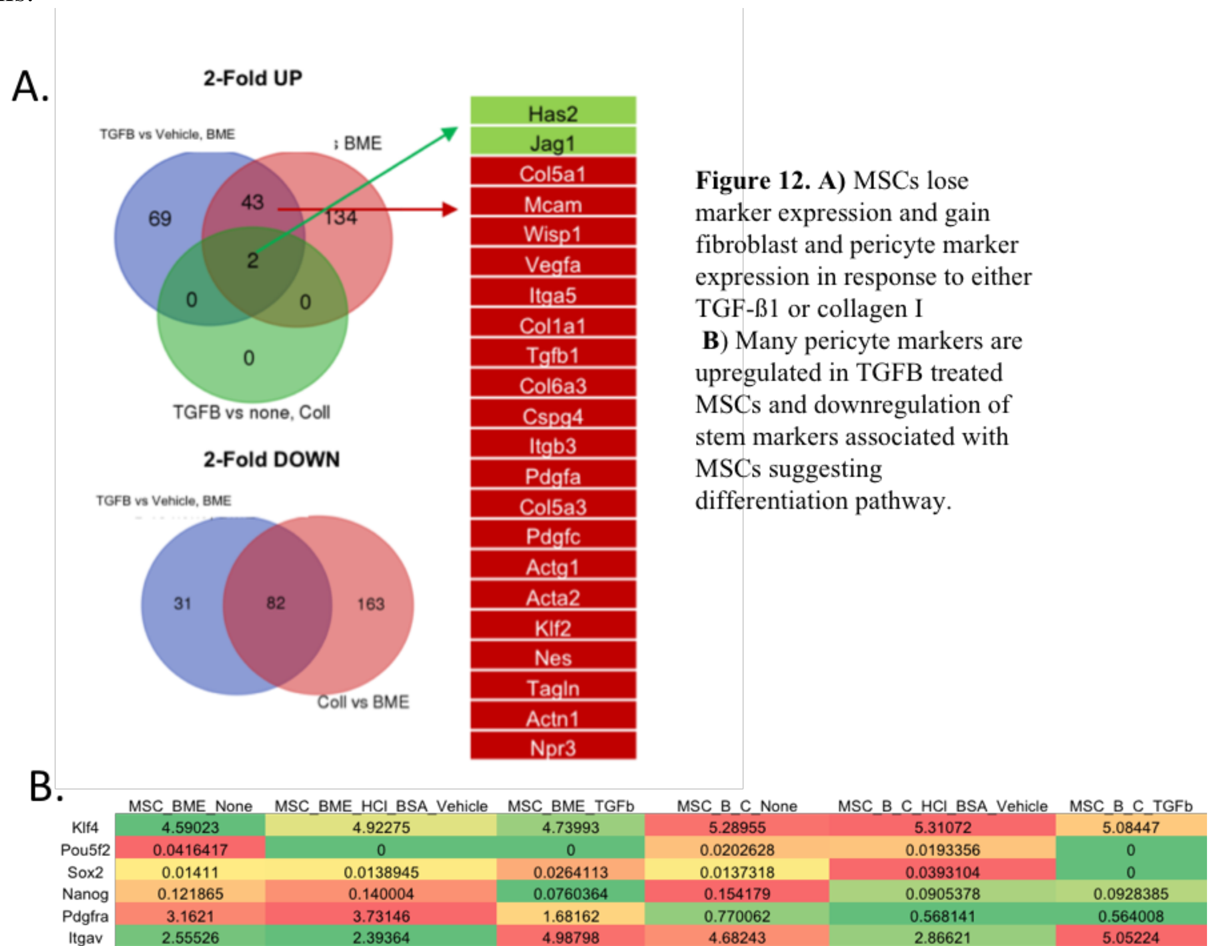


Figure 12. A) MSCs lose marker expression and gain fibroblast and pericyte marker expression in response to either TGF-ß1 or collagen I
B) Many pericyte markers are upregulated in TGFB treated MSCs and downregulation of stem markers associated with MSCs suggesting differentiation pathway.

	MSC_BME_None	MSC_BME_HCl_BSA_Vehicle	MSC_BME_TGFb	MSC_B_C_None	MSC_B_C_HCl_BSA_Vehicle	MSC_B_C_TGFb
Klf4	4.59023	4.92275	4.73993	5.28955	5.31072	5.08447
Pou5f2	0.0416417	0	0	0.0202628	0.0193356	0
Sox2	0.01411	0.0138945	0.0264113	0.0137318	0.0393104	0
Nanog	0.121865	0.140004	0.0760364	0.154179	0.0905378	0.0928385
Pdgfra	3.1621	3.73146	1.68162	0.770062	0.568141	0.564008
Itgav	2.55526	2.39364	4.98798	4.68243	2.86621	5.05224

In order to determine what secreted factors that mesenchymal cells treated with TGFb are producing/secreting compared to mesenchymal stem cells without TGFb we performed a chemokine array on the supernatant from cells either treated with TGFb or cells expsosed to vehicle alone. Although several factors were differentially expressed in these two groups the matrix metalloprotease MMP2 was upregulated by MSCs in response to TGFb treatment compared to

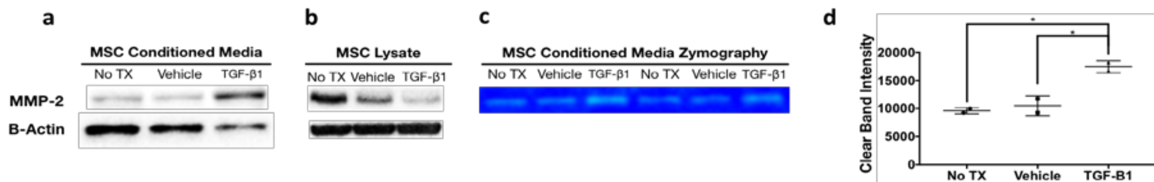


Figure 13. Matrix remodeling protein secreted by TGFB1 activated MSCs. MSCs were either untreated, or treated with either 5 ng/mL recombinant TGF-β1 or the vehicle control (4mM HCL + 0.1% BSA) for 72 hours. To assess intracellular proteins, MSCs were lysed in RIPA buffer. To assess secreted molecules, treatment containing media was replaced with serum free media. After an additional 72 hours, the resultant media was collected as conditioned media. **(a)** Western blotting on conditioned media confirms increased MMP-2 secretion with TGF-β1 treatment. **(b)** Correspondingly, western blotting on MSC lysates shows a decrease in intracellular MMP-2. **(c,d)** Further, gelatin zymography shows increased MMP-2 activity in conditioned media from MSCs treated with TGF-β1

MSCs given vehicle media (Figure 13). MMP2 specifically allowed for D2.0R break in dormancy when MMP2 was given in place of TGFb activated MSCs.

Furthermore, MMP2 inhibitor prevented the break in dormancy seen with TGFb treatment of MSCs (Figure 14). These findings suggest that MMP2 mediated matrix remodeling may be essential for TGFB conditioned MSCs promotion of D2.OR break in dormancy and initiation of proliferation.

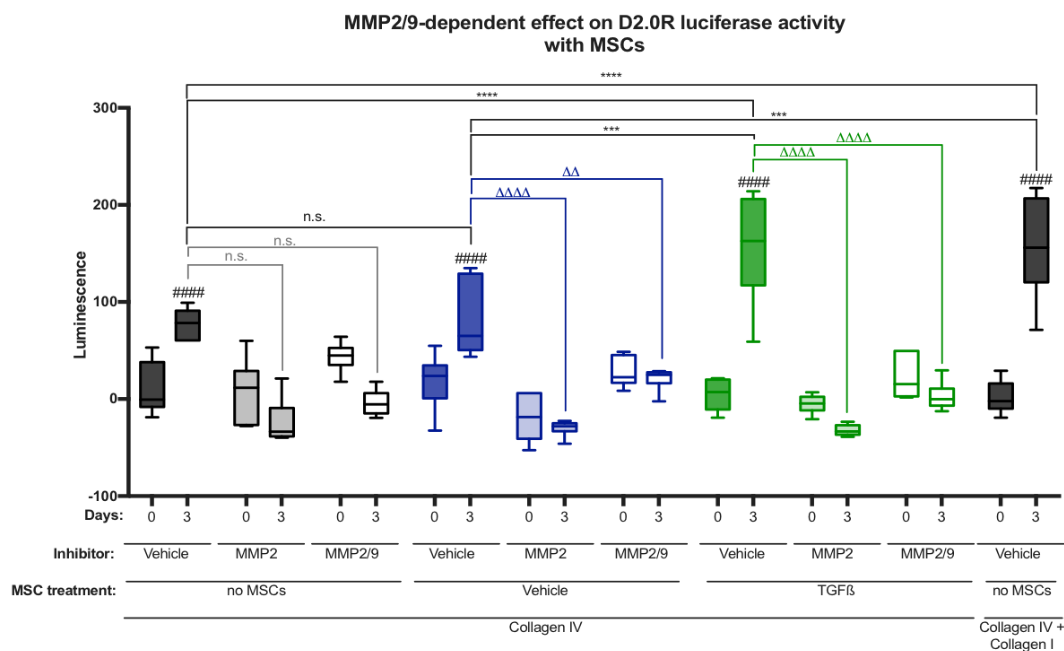


Figure 14. D2.ORs in 3D culture with MSCs either pretreated with TGFb or vehicle in Matrigel or MSCs in Matrigel with Collagen and Treatment with MMP2 or MMP2/9 inhibitors. MMP2 and MMP2/9 inhibition prevents the TGFb conditioned MSC break in dormancy of D2.OR cells. MMP2 and MMP2/9 Inhibition decreases proliferation of D2.OR cells cultured alone in Matrigel but most notably decreased proliferation when cultured in Matrigel and TGFB conditioned MSCs.

We are now performing a deeper dive into the biology of MMP2 and hypothesize that D2.ORs may have defective matrix remodeling abilites that may explain their propensity to remain dormant when faced with a new environment such as the lung. However, these cells when exposed to collagen may activate matrix remodeling proteins that allow for effective break in dormancy and restore metastatic efficiency. We have studies underway to inhibit matrix remodeling in D2.A1 cells in

order to assess if this creates a dormancy phenotype in these cells similar to D2.OR cells. These findings can lead to a new avenue of treatment for both fibrosis by limiting MSC differentiation as well as metastasis by targeting matrix remodeling proteins.

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

This work has supported the training of two post-baccalaureate students for three years in the Green and Kaplan labs as well as training of the lab 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. Ryan Nini presented a the early work on DNMT1 inhibition at the annual AACR meeting in New Orleans in 2016. Shil Patel, Jennifer Zhu, Meera Murgai and PI Rosandra Kaplan presented this work at the 2018 AACR Special Conference on Cancer Dormancy and Residual Disease where Dr. Kaplan chaired a session on microenvironmental regulation of tumor cell dormancy.

How were the results disseminated to communities of interest?

We have published an abstract at AACR Annual Meeting in 2016 as well as at the 2018 AACR Special Conference on Cancer Dormancy and we presented our findings at these meetings as well. We will publish our findings shortly as we are completing manuscript preparation. The Green and Kaplan groups met regularly to share data and discuss experimental designs of all experiments.

4. IMPACT:

- **The development of the principal discipline(s) of the project;**
  These investigations has lead to a much deeper understanding of the role of microenvironment and in particular mesenchymal stem cell biology in response to TGFb/Fibrosis to transition their phenotype to allow for a break in breast cancer disseminated tumor cell dormancy. These findings are particularly important to the field because it highlights that no one particular microenvironmental cell is a pro-tumorigenic or anti-tumorigenic but rather is context depenedent on how these cells support or limit disseminated tumor cell development into metastasis. Understanding the basic biology of mesenchymal stem cells in response to fibrosis and other injuries is very important for better understanding human diseases and allows for novel approaches to target dormancy to limit metastatic progression. We also have developed new insights into the role of matrix remodeling in cell proliferation. The connection between these two processes that are criticial to metastasis have not been well linked previously and will be a fruitful area of future investigation in the coming years that will likely shed important light on the connection to many disease states and wound healing processes.
- **Other disciplines;**
  As discussed above this work not only provides important information for the field of tumor metastasis but also provokes much interest and adds new perspective on the fields of tissue fibrosis, tissue regeneration in response to injury and wound healing mechanisms in place in response to injury such as radiation pneumonitis and idiopathic pulmonary fibrosis and other inflammatory states.
- **Technology transfer; or**

The new approach to limit metastatic progression by eliminating dormant tumor cells by their activation with matrix remodeling proteins in combination with chemotherapy to prevent relapsed metastatic disease will be submitted for a provision patent. The targets we are developing to limit or enhance matrix remodeling have therapeutic potential in a myriad of disease processes and will likely be of interest to biotechnology and pharma companies.

- **Society beyond science and technology.**
  Cancer and Metastatic disease impacts every aspect of society. It is the leading cause of death in the US and growing worldwide. These investigations that lead to new approaches to treating and preventing metastatic progression is critical to have more well members of society who can pursue their dreams and ambitions without illness or death limiting their quality life years.

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change.**
  As discussed above we redirected our initial studies on hematopoietic cells and bone marrow transplant studies based on the challenges in interpreting the results in our variable background mouse model and we pursued findings that had strong phenotype and reboust reproducibility as all scientific endevaeours are pursued.
- **Actual or anticipated problems or delays and actions or plans to resolve them. None**
- **Changes that have a significant impact on expenditures.**
  Expenditures were as anticipated based on studies planned. Timing was a bit longer than anticipated due to transition of postbac personnel but overall expenditures were on target for as projected in intial grant SOW.
- **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;**
  Mansucript is in preparation for submission in the next month and this work was presented at two prestigious scientific meetings and were published in conference paper abstracts. Dr. Kaplan was part of the session chairs of a Tumor Dormancy meeting based on the work from this grant and a manuscript from that conference will be forthcoming in the next few months.
- **Website(s) or other internet site(s);**
  Nothing to report
- **Technologies or techniques;**
  A new technology to study mesenchymal stem cell differentiation has been developed based on these investigations of MSCs and TGFB treatement of MSCs. Furthermore, the new techniques developed to co-culture tumor cells with specific cells of the tumor microenvironment has been expanded and are being implemented as 3D priniting and being tested as a potential approach to allow for the discuovery of new therapies for fibrosis and metastasis.
- **Inventions, patent applications, and/or licenses; and**

As described above given the determination of new therapeutic approaches to cancer metastasis based on matrix remodeling we are working to submit an invention disclosure to have NIH investigation submission of a provisional patent.

- **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:	Performs *in vitro* and *in vivo* experiments related to dormancy; Performs cytokine analyses *in vitro and in vivo;* analyzes gene expression profiling data; performs FACS analyses of *in vitro* experiments designs experiments; interprets 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.

Name:	Caitlin Reid, B.Sc.
Project Role:	Post-baccalaureate student
Nearest person month worked:	15

Contribution to Project:	Performs FACS analyses of *in vivo* experiments.

Name:	Amber Giles, Ph.D.
Project Role:	Post-doctoral Fellow
Nearest person month worked:	3
Contribution to Project:	Performs FACS analyses of *in vivo* experiments.

Name:	Meera Murgai, Ph.D.
Project Role:	Post-doctoral fellow
Nearest person month worked:	4
Contribution to Project:	Performs cytokine analyses, flow cytometry, co-culture *in vitro and in vivo* studies. Performed and analyzed MSC RNA Seq gene expression studies. Designs and oversees experimental progress; interprets data.

Name:	Kush V Bhatt, B.Sc.
Project Role:	Post-baccalaureate student
Nearest person month worked:	1
Contribution to Project:	Continuing previously conducted FACs analyses of *in vivo* experiments

Name:	Jennifer Zhu, B.Sc.
Project Role:	Post-baccalaureate student
Nearest person month worked:	2
Contribution to Project:	Perform In vitro culture and co-culture studies and in vitro and in vivo IL6 studies as well as helping to develop the co-cutlure studies. Performed and analyzed MSC RNA Seq gene expression studies

Name:	Shil Patel, B.Sc.
Project Role:	Post-baccalaureate student
Nearest person month worked:	2
Contribution to Project:	Perform In vitro culture and co-culture studies and in vitro and in vivo MMP2/MMP9 studies as well as helping to develop the co-cutlure studies. Performed and analyzed MSC RNA Seq gene expression studies and co-culture RNA and flow anaylsis. Also helped to design platform for MSC differentiation technique and development of drug discovery platform based on the 3D co-culture system.

- **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