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TITLE: Do Carcinoma-Associated Fibroblasts Adjacent to Breast Cancer Cells
Originate from Circulating Mesenchymal Stem Cells?

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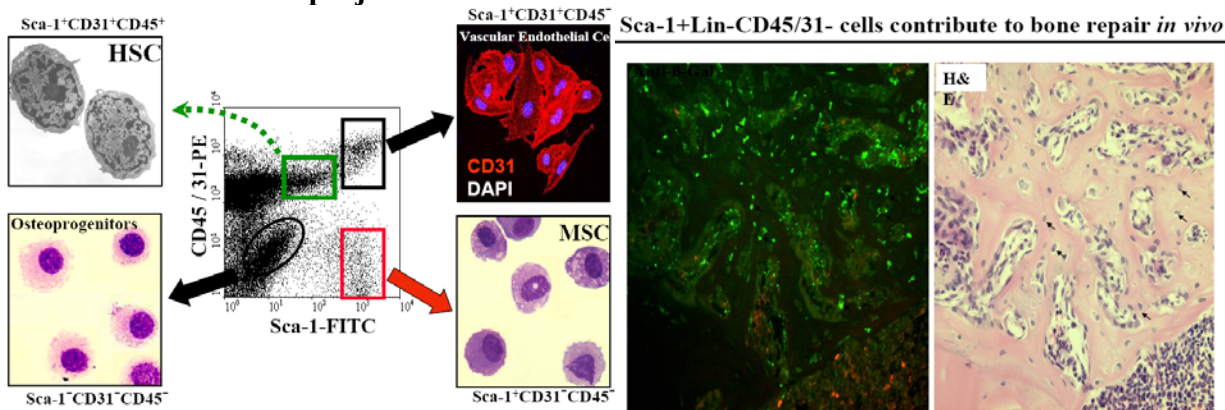
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14. ABSTRACT An understanding of the factors outside the cancer cell that promote invasion and metastasis or keep the cell contained could provide an entirely new avenue of therapy that tumour cells may find difficult to evade, and could prove relevant to patients who are resistant to current therapy. The overall aim of this proposal is to determine whether cancer associated fibroblasts (CAFs), which promote tumor progression (Olumi et al. 1999; Orimo et al. 2005), arise locally in the tumour bearing tissue or whether they are recruited from other organs such as bone, bone marrow or other precursor pools (Sangai et al. 2005). Aims: 1) To determine whether bone marrow derived fibroblast precursor cells migrate to the site of primary tumors and become CAF; 2) To isolate bone marrow derived fibroblast precursors from primary tumors and test their ability to promote tumor growth and distant metastasis. EO771 cells, a C57Bl/6 derived mammary tumor line will be used as the breast cancer model. Mesenchymal precursor cells (MPC) will be isolated from Bones (Short et al. 2003) either from ROSA/lacZ mice (expressing _Gal) or from Granzyme M KO mice (expressing the neoR selectable marker). These MPCs will be introduced via an intrafemoral injection into 6 week old female C57BL/6 mice, resulting in mice populated with tagged mesenchymal precursor cells. The potential of these cells to promote cancer were compared.					
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Are Mesenchymal Stem Cells the Origin of Carcinoma-Associated Fibroblasts?

Rational behind the project:



Left panel: MPC purification scheme. Right Panel: Immunohistochemistry with α - β Gal antibody, demonstrating the incorporation of the MPCs into the bone healing process following intra-tibial injection

During the past five years, we have been investigating the biology behind the nature and mechanism of formation of carcinoma associated fibroblasts (CAFs), as partial drivers of the disease progression. We first observed a signature of genes shared among gastric, lung, breast and ovarian cancer that is differentially expressed between cancer and normal tissues (Boussiotas et al., 2003), and predicts unfavorable outcome (Chang et al., 2005; Tothill et al., 2008). Surprisingly, the genes in this signature are expressed by fibroblasts in the tumor. Using co-culture of epithelial cells with fibroblasts, we recorded robust induction of gene expression (DoD grant DAMD17-03-1-0720), yet very little of it recapitulated the signature observed *in vivo*. To our surprise, xenografts, which were generated by co-inoculation of fibroblasts with the cancer cells and exhibited phenotypic effects of the fibroblasts, did not contain detectable human fibroblasts in the xenograft tissue sections, as reflected by immunohistochemistry with Anti-human mitochondria antibodies, but did contain infiltrating fibroblasts from the mouse. This suggested that distant tissues, such as bone marrow precursor pools, substantially contribute to tumor infiltrating fibroblasts. Direct evidence that the robust interaction with epithelial cells is involved in the generation of CAFs was obtained while pursuing the SOW of this grant. By generating primary normal-tissue associated fibroblasts from a breast reduction operation (NAFs) that are Puromycin resistant, we could recover these cells from xenograft tumors that were generated by co-inoculation of MDA-MB-231 cells with the fibroblasts. If interactions with cancer cell were capable of generating CAFs, the primary tumor derived fibroblasts should have assumed the xenograft promoting phenotype of CAFs. They did not. Furthermore, we recently overruled the possibility that the CAFs are products of somatic genomic aberrations in the fibroblast cells (Qiu et al., 2008). Apparently, a dynamic event in the progression of cancer, involved the immergence of CAFs, but not through somatic mutations, nor through dynamic interaction with the local fibroblast population in the breast. Since our epithelial-NAF co-culture expression profiles include a substantial component of inflammatory response, including many chemokines, we proposed that the CAFs are progeny of mesenchymal precursor cells that are recruited to early lesions of the breast, potentially DCIS, and then ameliorate the pathology with what these cells pursue as a wound healing program. To demonstrate that model, we generated chimeric mice, where the mesenchymal precursor cells (MPCs) are labeled with a transgene, β Gal or G418 resistance, and the rest of the mouse is not. In such chimeric mice, we induced breast cancer via inoculation with mammary tumor cell lines, and measured the selective enrichment of MPCs in the tumor mass, and the contribution of viable cells of this fibroblast sub-population to the CAF xenograft promotion assays/phenotype. Unfortunately for us, this work was recently published by a competing group, who has done an exquisite job at pursuing a similar hypothesis, i.e. that MPCs are responsible for metastatic dissemination of breast cancer cells (Karnoub et al., 2007). Due to this publication, we have focused our effort on pursuing task 3, which still has some added message to the Karnoub et

al paper. Unfortunately, at the time of writing this report, we are still in the middle of a large experiment which would hopefully provide experimental evidence, on which to base the conclusion of whether the null hypothesis (CAFs are progeny of local breast NAFs) or test hypothesis (CAFs are progeny of MPCs) is correct for this model system. Typically, MPCs are produced from Collagenase I treated bone (White material, bone marrow flushed away), via the FACS sorting shown in the figure below (on the left). When these cells are injected to mice intratibially, they form a bone healing tissue, as shown in the figure (on the right)(Semerad et al., 2005; Short et al., 2003). While this data was with us when we prepared the application for this grant, the Simmons lab moved to MD Anderson hospital in Houston Texas, and we had serious difficulties repeating these results in the absence of their day to day support, thus the delay in the final report.

Task 1. Generation of Chimeric C57Bl/6 mice bearing tagged MPC, and EO771 BrCa tumours:

a. Isolate MPCs from 15 ROSA 26 (Lin⁻/CD45⁺/βGal tagged).

Briefly, MPC cell fractionation are accessed from femora, tibiae, and iliac crests after thorough removal of associated muscle tissue and then crushed and disaggregated mechanically and enzymatically. The resulting population of bone-derived cells was then depleted of residual hematopoietic cells by incubation with a cocktail of rat antimouse antibodies (B220, Mac-1, Gr-1, CD4, CD8, CD3, CD5, and Ter119) using a FACSDiva high-speed cell sorter (BD Biosciences). Following lineage depletion, the cells are stained with a PE-conjugated anti-CD45, FITC-conjugated anti-CD31, biotinylated anti-CD51, and streptavidin-coupled allophycocyanin (all from PharMingen) and separated to three fractions: endothelial cells (Lin⁻ CD45⁻ CD31⁺), osteoblasts (Lin⁻ CD45⁻ CD31⁻ CD51⁺), and MPC cells (Lin⁻ CD45⁺). The purity of is determined via RT-PCR on cell lineage specific markers.

A substantial duration of the award was spent on the hurdle of isolating the mesenchymal stem cells according to the proposed method. The method repeatedly resulted in miserably minute yields, which substantially slowed the proposed downstream experiments. In addition, the little amount of cells that we got from this protocol, we needed to use to confirm that the cells are indeed pluripotent mesenchymal precursors. We also tried a number of alternative protocols, and eventually found that the protocol described in (Beyer Nardi and da Silva Meirelles, 2006; Hall et al., 2007; Karnoub et al., 2007; Nakamizo et al., 2005; Wolf et al., 2005) and (Sangai et al., 2005). The latter was far from representing a pure population of mesenchymal precursors, and represented a largely indistinct cell population of cells to the Lin⁻ CD45⁻ CD31⁺ we isolated initially. I am adding a bone repopulation figure of the bone-derived MPCs we sought, as was observed by the laboratory, this work was proposed to be performed at, i.e. Paul Simmons lab.

b. Isolate MPCs from 30 Granzyme M KO mice as in 1a (Lin⁻/CD45⁺/neo^R tagged).

For this aim too, we ran into difficulties. Unfortunately, the four different knock out mice we tried to use as source for Neo/G418 resistant mesenchymal precursors and fibroblasts, fibroblasts were not effectively G418 resistant. We are currently exploring a mouse strain, MTK Neo2, which expresses Neo^r gene in all his cells. Unfortunately, this mouse, which we thought was on Balb/c background, immunologically rejected our mouse model cell line, 4T1. It must have been back-crossed from undefined background. Since the Neo^r mice were in neither the C57Bl/6 nor the Balb/c backgrounds, in which we have our syngeneic mouse models, we re-designed the experiment to overcome the strain compatibility the following way: We are producing MPCs from bone marrow of Nzeg-EGFP mice and mammary gland fibroblasts from Balb/c strain, followed by transfection with the Cherry-red protein plasmid. We are using these isolates of resident and bone marrow derived fibroblasts to address our experiment in immune compromised mice. The experiment is underway, and is the main reason why we haven't submitted the report as yet.

c. Inject MPC cells intra-femoral, into lethally irradiated, six weeks old, C57Bl/6 female mice

(one donor mouse per one transplant host mouse, i.e. 45 mice).

This step was performed ~6 times. Considering the low incorporation of MPCs in the tumors in Task2, we decided to add control of irradiated and not reconstituted mice, and realized that 10G radiation was not enough for C57/B6 to fully lose their bone marrow regeneration, and consequently, the β Gal/Neo MPCs were not the only MPCs in these mice. Therefore, the experiment is now performed with the control of non-reconstituted mice, to confirm ablation of bone marrow stem cells of the host mice. We also changed our strategy to introduce fluorescent-protein labeled cells, rather than Neo^r, to avoid the strain compatibility issue.

- d. Four weeks later, transplant (10^5 /mice) EO771 spontaneous BrCa cell line into the fourth mammary gland of these mice.

In the current setting, this experiment is underway.

Task 2. Assess the distribution of the MPC through different mouse organs.

- a. Collect the tissues to be analyzed and split them in two, one half for qPCR (Task 2b-2c) and half for the IHC (Task 2d-2e).
- b. Isolate genomic DNA from the primary tumor and several organs, including lung, lymph nodes and liver (from 15 ROSA 26 and 15 Granzyme M KO, chimeric C57Bl/6 mice).
- c. Screen genomic DNA for the presence of the marker (β Gal or neo^R) by quantitative PCR as a rapid screen for tissues positive for the transplanted MPC.

MPC dissemination within a tissue was measured using a novel multiplexed q-RT-PCR assay. This assay quantitates the relative amount of neomycin (tumor tissue) compared with vimentin (endogenous mouse tissue) from the genomic DNA extracted from the tissue of interest. Average MPC burden scores from an experiment using five mice per tumor line. PCR measurement of the β Gal transgene reflected enrichment in the tumor mass, however, the overall intake of bone marrow derived cells was very poor, with at best 1:10,000 abundance difference from the Vim gene. We hope that increasing the radiation dose would improve on this weakness of our experiment.

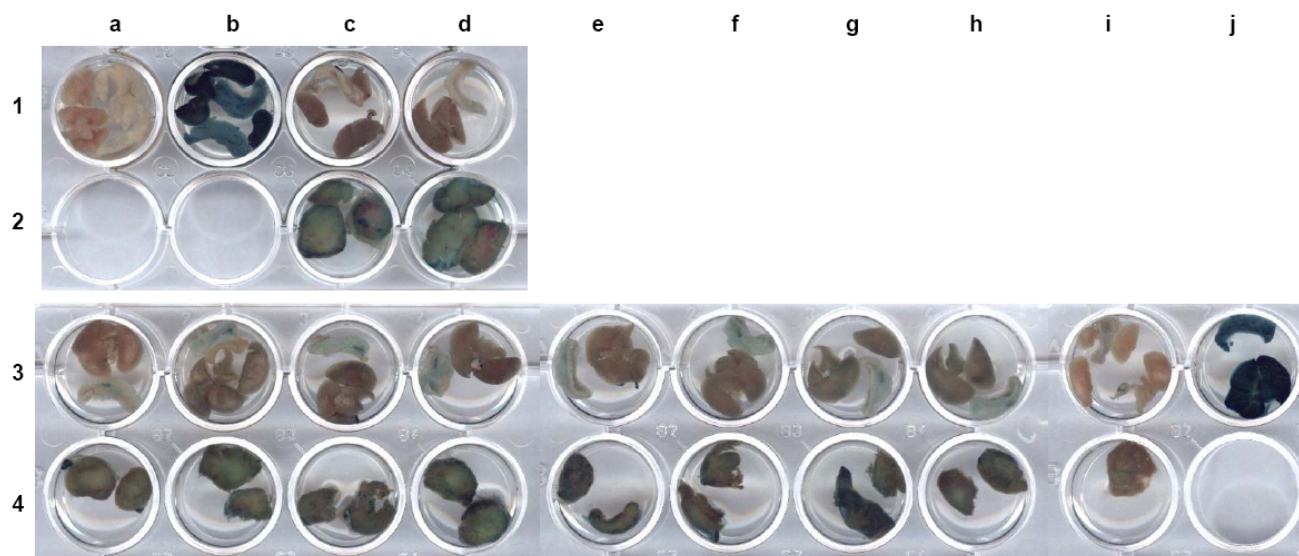
- d. Formalin fix and paraffin embed the tumor and positive organ tissues into immunohistochemistry (IHC) compatible sections.

The image in the next page demonstrates on the macroscopic level, that β Gal+ MPCs from RPSA 26 bone marrow populate the tumors more effectively than corresponding organs in non-tumor bearing mice. There is also a substantial

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1 and 3: lungs and fat pads

2 and 4: Primary Tumours

1(a): Neg Control (C57Bl/6), 1(b): Pos Control (Rosa26), 1 and 2 (c): Chimera 1, 1 and 2 (d): Chimera 2

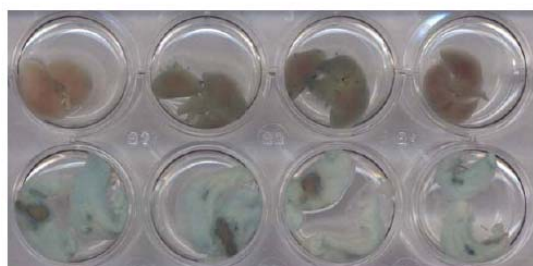
3 and 4 (a-h): Chimeras 3-10

3 and 4 (i): Neg Control (C57Bl/6), 3 (j): Pos Control (Rosa26)

Further results from a second experiment are shown below: Lungs and primary tumors are populated with elevated levels of blue cells (β Gal⁺ bone marrow derived mesenchymal precursors).



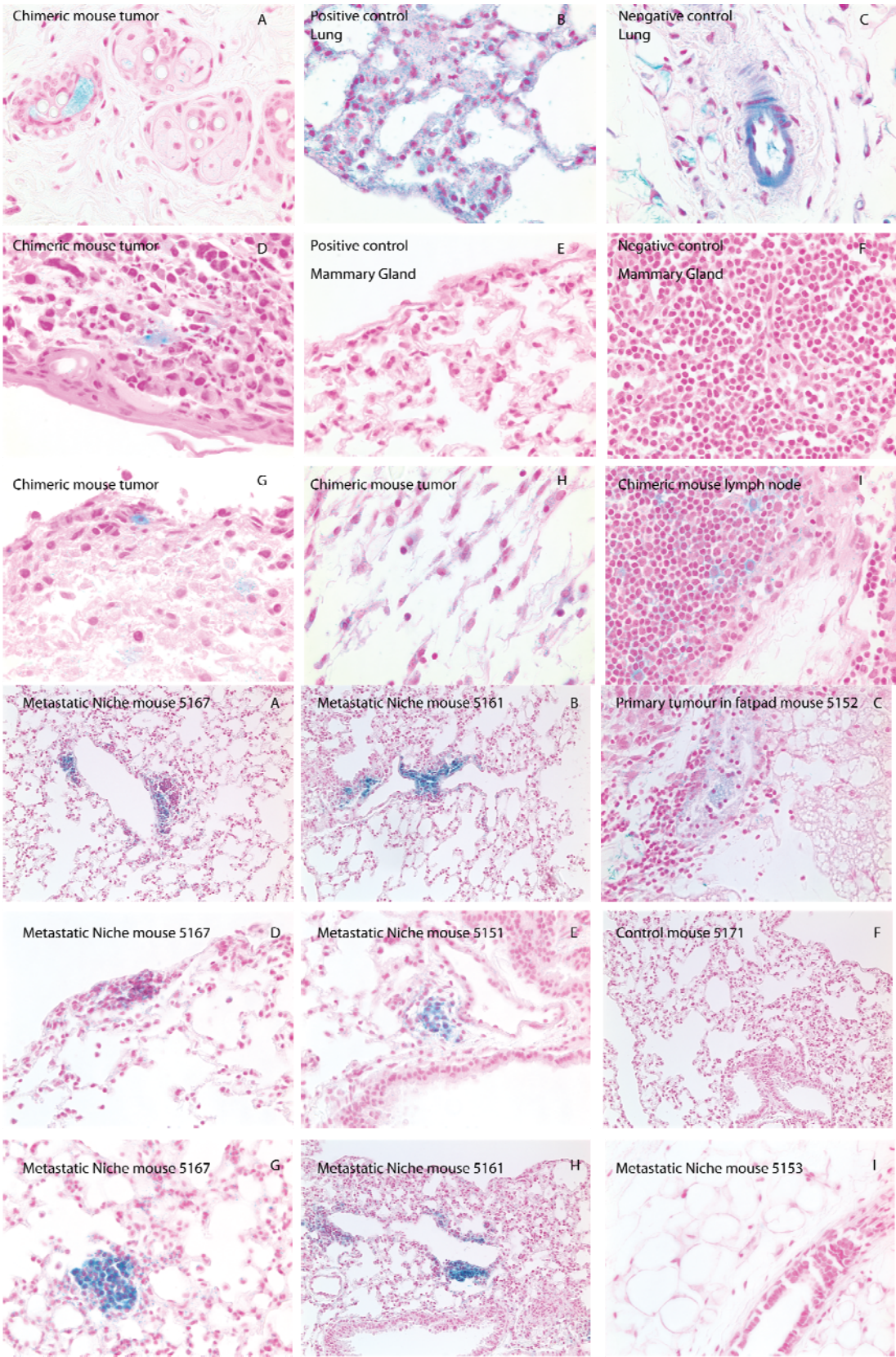
m1-4 Lungs (top), Fat Pads (bottom)



m5-8 Lungs (top), Fat Pads (bottom)



m9-10, neg control (Bl/6), Pos control (Rosa 26): Lungs (top), Fat Pads (bottom), and primary tumour in neg control.



Hematoxiniln & Eosin staining of different tissues of mice, with β Gal activity staining with XGal (bright blue). Top panel (experiment 14): B and C. ROSA 26 mouse tissue (positive control, E and F. C57Bl/6 mouse tissue (negative control), A, D, G-I. Chimeric mouse tissue (β Gal⁺ cells occasionally infiltrating the tumor tissue). Bottom panel (Experiment 15): F. Tumor tissue in C57Bl/6 mouse (negative control), A-E, G-I, chimeric mouse lung with clear β Gal⁺ colonies in peri-vascular regions, some with metastatic tumor cells infiltrating them.

- e. Perform IHC against the tag protein (anti- β Gal) and typical mesenchymal/fibroblast markers, to assess the local incorporation of MPC into connective tissue, in the tumor, and other tissues.

Most β Gal⁺ cells could be discerned by the pathologist, however, we intend to perform this aim with chimeric mouse tissue sections, once the β Gal⁺ cell infiltrates improve in frequency. At the observed frequency, it is unlikely that the CAFs are substantially contributed to by the MPCs.

- f. Digest freshly collected bone, otherwise positive organs (as determined by the qPCR in 2b) and Breast Cancer tissues, from the 15 remaining Granzyme M KO chimeric C57Bl/6 mice.

The rest of this Task is ongoing with GFP and Cherry red proteins marking the mammary gland-derived fibroblasts, and the MPCs respectively. In this case, re-deriving these cells will involve FACS sorting, rather than antibiotic selection (which may loose variant cells due to in vitro culturing potential).

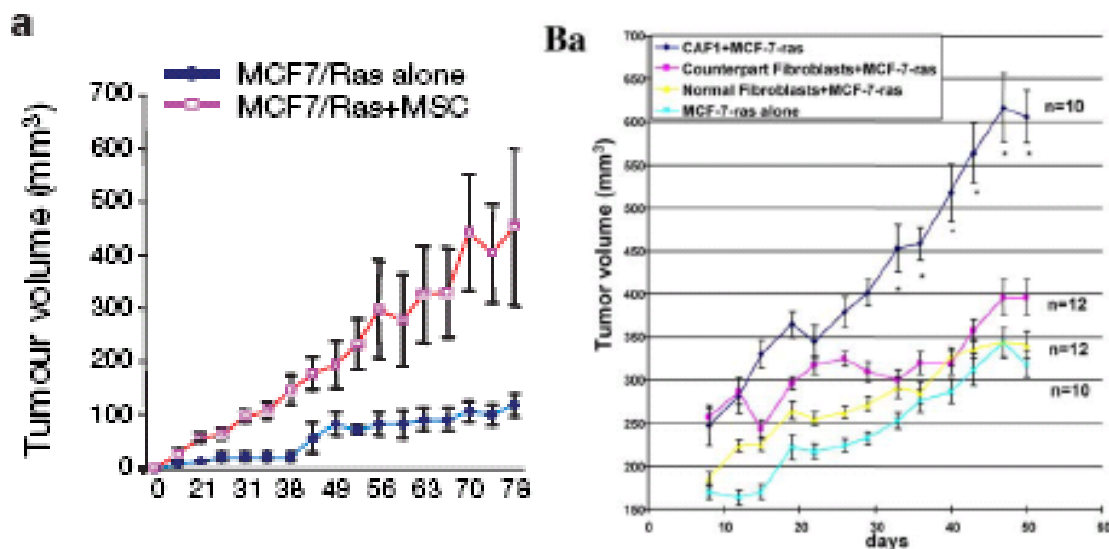
- g. Isolate cells from the freshly collected tissues as described at 1a.
- h. 2.0×10^4 Lin⁻ CD45⁺ nucleated cells in 2.5 mL methylcellulose media supplemented with a cocktail of recombinant cytokines (MethoCult 3434; StemCell Technologies, Vancouver, BC, Canada), and 200 μ g/ml Geneticin (G418). Cultures were plated in duplicate and placed in a humidified chamber with 6% CO₂ at 37°C. Colonies containing at least 50 cells were counted on day 7 of culture.
- i. Compare the enrichment of MPCs in the breast tumor over other tissues in the mice, as measured by the qPCR (1b-1c), IHC (1d-1e), and fibroblast colony forming units (CFU-F, 1f-1h). Assess concordance between the β Gal MPCs and the neo^R MPCs, and between the different methods.

Task 3. Assess the CAF activity of the neo^R fibroblasts, derived from the emerging breast EO771 tumors.

- a. Generate 15 more Granzyme M KO chimeric C57Bl/6 mice carrying EO771 tumors as described in Task 1.
- b. Digest the tumors (10 mm size, approximately four weeks tumors), and isolate the MPC-derived, G418/neo^R fibroblasts, from within the tumors.
- c. Isolate MPC-derived, G418/neo^R fibroblasts from non-tumor tissue of the chimeric mice as in Task 3b
- d. Isolate normal mammary fibroblasts as in Task 3b (from 15 C57Bl/6 females).
- e. Introduce the three fibroblastic cell fractions (10^5 cell per mice) into naïve C57Bl/6 females, together with EO771 cells (10^5 cell per mice), into the fourth mammary gland.
- f. Generate a control group of 15 C57Bl/6 mice, transplanted with EO771 as in Task 3e, yet without fibroblasts.
- g. Follow cancer growth rate of the resulting four mouse groups (total of 60 mice; EO771 alone, and together with either normal mammary fibroblast, neo^R/G418^R from tumors, and neo^R/G418^R from normal tissue).
- h. Compare groups for cancer growth rate, and histopathology of the resulting tumors, to assess

the CAF activity exhibited by the different mesenchymal isolates.

This experiment has not been initiated yet. Hopefully, by the Era of Hope meeting, we would have the definitive results. I am adding Figure 1 from Karnoub et al (Karnoub et al., 2007), where in panel A, MCF7+RAS cell primary tumor is accelerated by the co-injection of normal mesenchymal precursors, derived from human knee aspirate (non-cancer patient), much like the original observation with CAFs (Orimo et al., 2005), both producing approximately double the tumor size by 50 days of xenograft. While we failed to produce these results in time for first publication, our original hypothesis has been vindicated by the Bob Weinberg lab.



Key Research Accomplishments: We have used chimeric mice to monitor the migration of bone marrow derived cells into the tumor microenvironment. While we detect occasional bone-marrow derived cells in the tumors, they are far rarer an event than in published results. We believe that much of this bias was due to incomplete bone marrow ablation of the acceptor in the chimera. We are looking to improve on this point. Outside the aims of this grant we have validated published results, reporting migration of bone-marrow derived cells to lungs of tumor bearing mice, prior to detectable tumor metastases (Kaplan et al., 2005).

Reportable Outcomes: This work is ~90% on its way to publication. We hope to submit soon, upon completion of task 2 and 3, to Nature Cell Biology, or Journal of Cell Biology.

Conclusion: *A complete understanding of the molecular basis of these changes and how these influence epithelial cell growth and behavior could lead to novel strategies for the prevention and treatment of OvCa. While a few mechanisms for the CAF activity have been reported, somewhat of a biochemical approach to this cell biology issue is required, with increased purification of the xenograft promoting activity, we would gain more insight to the underlying biological process.*

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