FROM NAÏVE TO CANCER - ASSOCIATED FIBROBLASTS

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THE CHARACTERIZATION OF THE TRANSFORMATION/ TRANSITION OF NAÏVE FIBROBLASTS TO CANCER ASSOCIATED FIBROBLASTS USING 3D CO-CULTURES.

Abstract

The tumor stoma is an important component of the tumor that plays an increasingly significant role as the tumor grows and progresses, and encompasses a basement membrane, resident fibroblasts, extracellular matrix, immune cells, and endothelial cells lining the vasculature. Fibroblasts are a common and major stromal cell type. Although typical naive fibroblasts display suppressive functions against cancer-initiating and metastatic cells through a variety of mechanisms. This anti-inflammatory role is lost when fibroblasts evolve into cancer-associated fibroblasts (CAFs), a potential new cancer targets.

The main aim of the study is to evaluate the reprogramming of the naïve fibroblast when cultured in proximity to tumor cells using the 3D spheroid model and to further analyze the effect of activated fibroblast on tumor cells within spheroids by assessing their rates of proliferation. To accomplish our goal, various approaches were employed, including Flow cytometry and immunofluorescence staining using histological tissues.

1 Introduction

Fibroblasts are a prominent cell population in the tumor stroma, where they secrete soluble factors and cytokines as well as extracellular matrix components, including collagen (1). Typical naïve fibroblasts have been shown to exert suppressive functions against cancer-initiating and metastatic cells, this suppressive role is lost when fibroblasts evolve into cancer-associated fibroblasts (CAFs). CAF are spindle-shaped cells that construct and remodel the extracellular matrix (ECM) (3). The presence of CAFs in the tumor microenvironment is linked to tumor progression.
CAFs were found to enhance tumor cell proliferation, trigger angiogenesis, change the architecture of the extracellular matrix by increased collagen deposition, and mediate increased cross-linking of collagen fibers, which stiffens the stroma, all of which may facilitate tumor cell migration (2). Immunohistochemistry is regularly used on all breast cancers to collect valuable knowledge regarding the prognosis and to predict exposure to particular anticancer therapies. Keratin and Vimentin are among the most significant markers for studying breast cancers, collagen type 1, and platelet-derived growth factor receptor a and b (PDGFRa/b) are considered general markers of fibroblasts, including both naïve and CAFs subpopulations, while Alpha-smooth muscle actin (aSMA), and fibroblast activation protein alpha (FAP) are used to specifically identify CAFs in the tumor stroma.

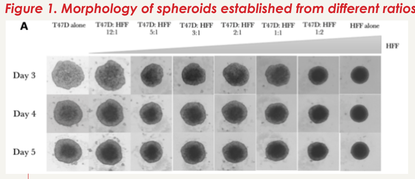
2 Intro. Continued

More studies are required to further elaborate on the reciprocal interactions between the tumor cells and surrounding naïve fibroblasts, resulting in reprogramming of fibroblasts from a naïve phenotype into a CAF one. Our main aim is to characterize the transformation of naïve fibroblast to CAFs is to:

- Determine the effect of tumors and fibroblasts on each other, using 3D spheroid models to mimic in vivo solid tumors where fibroblasts and tumor cells in proximity to each other.
- Evaluate the reprogramming of CAFs using markers such as αSMA and FAP.
- Study whether the addition of the fibroblasts to tumor spheroids would halt or enhance proliferation of the tumor cells, reflecting their anti-versus pro-inflammatory phenotype.

1 Data Analysis

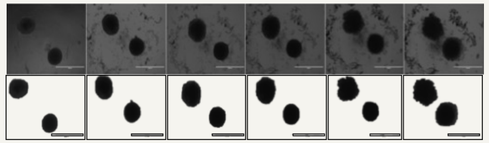
Figure 1. Morphology of spheroids established from different ratios of T47D and HFF cells.



A total of 15,000 cells consisting of T47D cells, HFF cells and different ratios of both cell types were seeded in ultra-low attachment 96 well plate containing 200 ml of DMEM media, nonessential amino acids, pyruvate and 5%FBS. Imaging was done using EVOS FL Life Technologies Cell imager using normal light at 4x (scale bar: 1000 µm).

Result:
Increasing the amount of HFF cells in co-culture spheroids resulted in a noticeable decrease in the spheroid size, which was accompanied with an increase in the darkness of the spheroid. Over the days, spheroids of each condition became more uniform. Notably, increasing the ratio of fibroblasts to tumor cells in spheroids resulted in a better/ more circular spheroids.

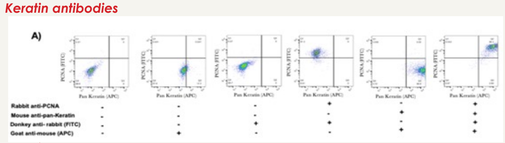
Figure 2. 3D migration assay of T47D and HFF spheroids



T47D and HFF-established spheroids were transferred to a new well in an ultra-low attachment 96 well plate and were observed overtime. Images were taken using EVOS FL Life Technologies Cell imager using normal light at 4x (scale bar: 1000 µm).

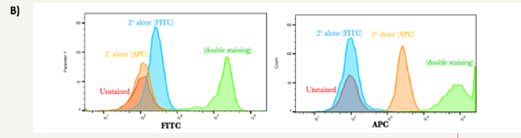
Result:
The distance between the spheroids decreased overtime while the size of each spheroid increased.

Figure 3. Optimization of Flow cytometry conditions using anti-PCNA and anti-pan-Keratin antibodies



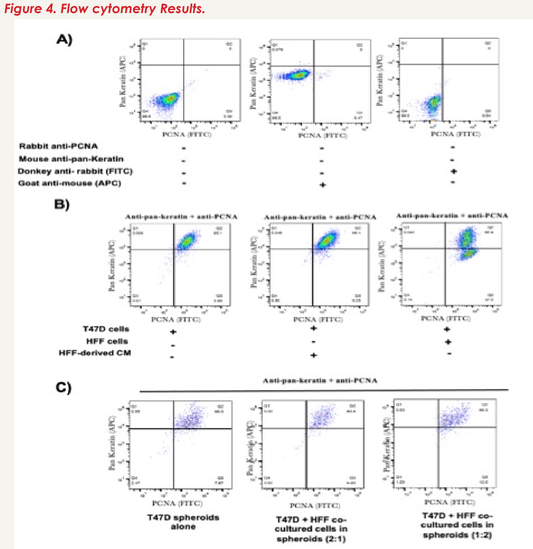
T47D cells were labeled using mouse anti-Pan-keratin (1:100) and visualized using anti-mouse secondary antibody that was conjugated with APC (1:300). Pan-Keratin+ T47D cells were assessed for their proliferative capacity using rabbit anti-PCNA antibody (1:250) and visualized using anti-mouse secondary antibody that was conjugated with FITC (1:50). The samples were acquired using the BD ISRFortessa Flow Cytometer and analyzed using FlowJo. Scatter plots.

2 Data Analysis Continued



Histograms were derived from scatter plots showing the fluorescence of live cells.

Figure 4. Flow cytometry Results.



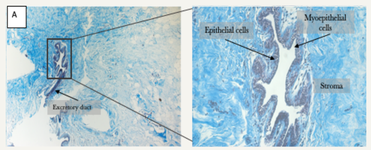
(A) T47D cells unlabeled, labeled with APC (1:300) or FITC (1:50) secondary antibodies.

(B) T47D cells alone, cocultured with HFF cells, or with HFF derived conditioned media, were labeled using mouse anti-Pan-keratin (1:100) and visualized using anti-mouse secondary antibody that was conjugated with APC (1:300). Pan-Keratin+ T47D cells were assessed for their proliferative capacity using rabbit anti-PCNA antibody (1:250) and visualized using anti-mouse secondary antibody that was conjugated with FITC (1:50).

(C) 8 spheroids of each culturing condition (T47D cells alone, T47D and HFF cocultures (2:1 and 1:2)) were pooled, dissociated to single cell suspension using 1:10 diluted TrypLE, then labeled with mouse anti-Pan-keratin (1:200) and rabbit anti-PCNA antibody (1:250) then visualized using anti-mouse secondary antibody that was conjugated with APC (1:600) and anti-mouse secondary antibody that was conjugated with FITC (1:50). The samples were acquired using the BD ISRFortessa Flow Cytometer and analyzed using FlowJo. Scatter plots.

Figure 5. Trichrome staining of matched Normal Breast tissue and adenocarcinoma tissue

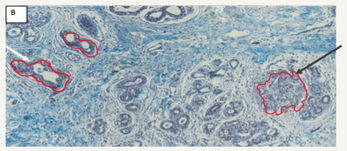
Slides were purchased from Novus biologicals and trichrome staining was performed to highlight the contrast between the stromal compartment (blue) and the rest of the tissue (dark red).

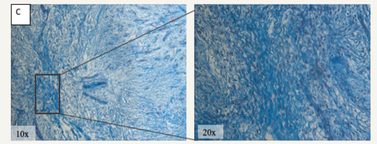


A representative image depicting the normal architecture of the breast. The inset illustrates the structure of the normal ducts (4x magnification, 20x magnification).

3 Data Analysis Continued

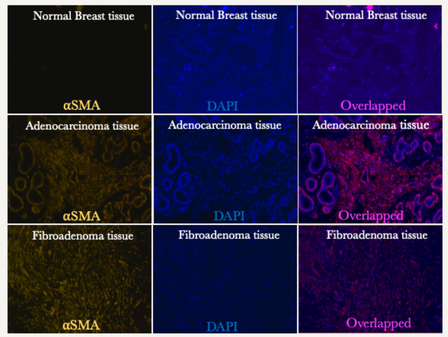
Representative images of a ductal adenocarcinoma, with white arrows pointing to the architecture, which is still maintained, while the black arrow points to the unorganized architecture where cells are hyperplastic (10x magnification).





Representative images of trichrome staining in a fibroadenoma tissue at 10 x and 20x magnification.

Figure 6. Immunofluorescence staining of matched Normal Breast tissue and adenocarcinoma tissue.



Slides were purchased from Novus Biologicals and were stained for αSMA expression to identify CAFs. DAPI was used to label the nuclei and delineate the cellular architecture of the tissue. Pictures were acquired at 20x magnification and overlapped using imageJ.

Results:
In the normal breast tissue, no αSMA staining was observed in the stroma. As expected, in the matched adenocarcinoma tissue, the αSMA was present within the stroma and also surrounding the milk ducts. Fibroadenoma tissue was also stained as a positive control where the staining was all the fibrotic tissue of the tumor.

Key Findings

- Stroma is rich in Cancer associated fibroblast.
- Fibroblasts are attenuating the tumor cells.
- Fibroblasts are exerting effects on the tumor cell population thus we cannot conclude that they are not reprogrammed.
- The decrease in the percentage of cells proliferating compared to T47D cells alone, shows that there was also a down-regulation to the expression of the Pan-keratin in some of those tumor cell.
- Fibroblasts may release pro-inflammatory cytokines when in direct contact with the breast tumor cells and tumor cells are becoming more invasive while attenuating their proliferation.
- We still do not know how the tumor cells are affecting the fibroblasts, nor how tumor cells are actually reprograming the fibroblast.

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[1] Karagiannis, G. S., Poutahidis, T., Erdman, S. E., Kirsch, R., Riddell, R. H., & Diamandis, E. P. (2012). Cancer-associated fibroblasts drive the progression of metastasis through both paracrine and mechanical pressure on cancer tissue. Molecular cancer research : MCR, 10(11), 1403–1418. https://doi.org/10.1158/1541-7786.MCR-12-0307
[2] Liu, T., Han, C., Wang, S., Fang, P., Ma, Z., Xu, L., & Yin, R. (2019). Cancer-associated fibroblasts: an emerging target of anti-cancer immunotherapy. Journal of hematology & oncology, 12(1), 1-15.
[3] Alkasalias, T., Moyano-Galceran, L., Arsenian-Henriksson, M., & Lehti, K. (2018). Fibroblasts in the Tumor Microenvironment: Shield or Spear?. International journal of molecular sciences, 19(5), 1532. https://doi.org/10.3390/ijms19051532

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