Isolation of Normal and Cancer-associated Fibroblasts from Fresh Tissues by Fluorescence Activated Cell Sorting (FACS)

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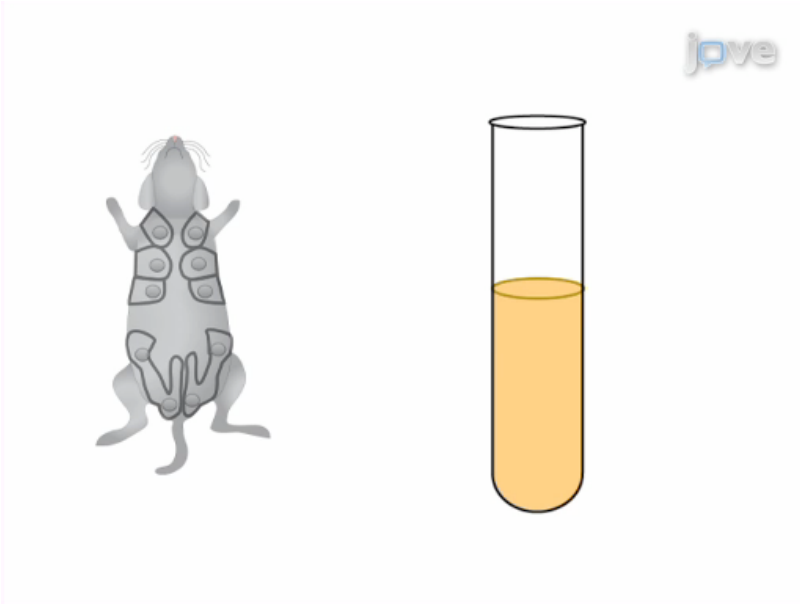

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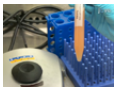

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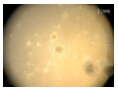

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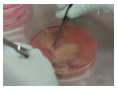

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Abstract

Cancer-associated fibroblasts (CAFs) are the most prominent cell type within the tumor stroma of many cancers, in particular breast carcinoma, and their prominent presence is often associated with poor prognosis[1,2]. CAFs are an activated subpopulation of stromal fibroblasts, many of which express the myofibroblast marker α-SMA[3]. CAFs originate from local tissue fibroblasts as well as from bone marrow-derived cells recruited into the developing tumor and adopt a CAF phenotype under the influence of the tumor microenvironment[4]. CAFs were shown to facilitate tumor initiation, growth and progression through signaling that promotes tumor cell proliferation, angiogenesis, and invasion[5-8]. We demonstrated that CAFs enhance tumor growth by mediating tumor-promoting inflammation, starting at the earliest pre-neoplastic stages[9].

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Despite increasing evidence of the key role CAFs play in facilitating tumor growth, studying CAFs has been an on-going challenge due to the lack of CAF-specific markers and the vast heterogeneity of these cells, with many subtypes co-existing in the tumor microenvironment[10]. Moreover, studying fibroblasts *in vitro* is hindered by the fact that their gene expression profile is often altered in tissue culture[11,12] . To address this problem and to allow unbiased gene expression profiling of fibroblasts from fresh mouse and human tissues, we developed a method based on previous protocols for Fluorescence-Activated Cell Sorting (FACS)[13,14]. Our approach relies on utilizing PDGFRα as a surface marker to isolate fibroblasts from fresh mouse and human tissue. PDGFRα is abundantly expressed by both normal fibroblasts and CAFs[9,15] . This method allows isolation of pure populations of normal fibroblasts and CAFs, including, but not restricted to α-SMA+ activated myofibroblasts. Isolated fibroblasts can then be used for characterization and comparison of the evolution of gene expression that occurs in CAFs during tumorigenesis. Indeed, we and others reported expression profiling of fibroblasts isolated by cell sorting[16]. This protocol was successfully performed to isolate and profile highly enriched populations of fibroblasts from skin, mammary, pancreas and lung tissues. Moreover, our method also allows culturing of sorted cells, in order to perform functional experiments and to avoid contamination by tumor cells, which is often a big obstacle when trying to culture CAFs.

Protocol

1. Dissecting Mammary or Skin Tissue from Mice

1. Before dissecting the desired tissue from mice, prepare the following supplies and reagents:

   Supplies:

   - Surgery tools (washed with detergent and then dipped in 70% ethanol).
   - Styrofoam surface to rest the mouse on during dissection.
   - Pins or needles to hold mice during dissection.
   - Glass jar with lid for digestion, containing magnetic stir bar (autoclaved).
   - Water bath at 37 °C, containing a submersible magnetic stirrer and enough water to submerge the digestion jar, while resting on the stir plate.

   Reagents:

   - FACS buffer I: (4 °C): Dulbecco's Phosphate Buffered Saline CMF (Calcium Magnesium Free) + 0.5% BSA
   - Collagenase solution (made immediately before use):
     0.05 g Collagenase II
     0.05 g Collagenase IV
     0.01 g Deoxyribonuclease
     20 ml FACS buffer I. Keep collagenase solution on ice until use.
   - PharmLyse (Ammonium Chloride Lysing Reagent).
   - FACS buffer II: Dulbecco's Phosphate Buffered Saline CMF + 1% FCS.

2. **Dissection of mammary glands** (see also[17]).

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- Euthanize female mice using carbon dioxide (CO_2) inhalation.
- On a Styrofoam surface, place the mouse on its back and pin firmly all four limbs using 25 gauge needles (**Figure 1A**). Spray the mouse generously with 70% ethanol.
- Using forceps, pull up the abdominal skin at the midline and make a small incision with sharp scissors.
- Starting from the incision, cut the skin up to the neck of the animal while avoiding puncturing the abdominal or thoracic cavities.
- Cut the skin on the rear legs from the midline incision towards the leg, resulting in a "Y shape".
- Pull skin away from mouse body and pin down, exposing the mammary glands attached to the underside of the skin (**Figure 1B**).
- Use Q-tips to gently separate mammary glands from skin while cutting conjunctive tissue with small sharp scissors to separate the mammary gland towards the spine of the mouse.
- The abdominal glands (#4, 5) are best for this protocol. You can also use the 2nd mammary gland, but bear in mind that these glands are anatomically located in close proximity to the pectoralis muscle which may be hard to separate, resulting in cells of mixed tissue origin.
- **Note:** When dissecting tumor tissue, it is recommended to remove necrotic regions.

3. **Dissection of skin tissue:** The easiest way to obtain skin tissue, without having to deal with hair removal is to use mouse ears. If a larger amount of tissue is required, you can shave off hair from mouse torso and dissect skin.

    - Euthanize mice using CO_2 inhalation.
    - Using sharp scissors, cut off both ears and place immediately in digestion jar containing a small volume (~0.5 ml) of PBS on ice.

2. Preparation of Mammary Gland/skin Single Cell Suspension

- Place mammary glands/skin in digestion jar containing a small volume of PBS on ice. If tissue is bloody wash x2 with PBS, and then discard excess PBS.
- Mince thoroughly with curved scissors.
- Add 20 ml collagenase solution (**Note:** this amount of collagenase solution will efficiently dissociate approximately 5 g of mammary or tumor tissue and ears from up to 15 mice).
- Place immediately on stir plate in 37 °C water bath, and incubate for 15 min while stirring at medium rate.

Note: optimal incubation times may vary if digesting other tissues.

- To stop the reaction, add 30 ml cold DMEM + 10% FCS.
- Strain the tissue/media mixture through a 70 µm cell strainer placed on top of a 50 ml conical tube.
- Centrifuge the conical tube for 5 min at 450 x g at 4 °C.

3. Red Blood Cells Lysis

Note: this step is not necessary if mice were heart-perfused with PBS before they were sacrificed.

- Aspirate the supernatant using a glass pipette attached to vacuum, without disturbing the cell pellet.
- To lyse RBC, resuspend cell pellet in 10 ml PharmLyse solution and incubate for 5 min at room temperature.
- Neutralize the PharmLyse solution by adding approximately 40 ml of FACS buffer I.
- Spin the conical tube with cells for 5 min at 450 x g at 4 °C.

- Aspirate the supernatant.

4. Blocking of Endogenous Fc

- Optional: count cells to determine the appropriate volume for the following steps.
- Resuspend cells in 1-3 ml FACS buffer I (depending on cell number, and amount of antibody you want to use).
- Add FcBlock (anti-mouse CD16/CD32) to reach 1:50 dilution (10 µg/ml), and incubate on ice for 10-20'. No need to wash off FcBlock.

5. Staining

- Take out 100 µl aliquots to eppendorf tubes to be used as an un-stained control and single color controls (according to the number of fluorescent antibodies used).
- To label fibroblasts: add anti-PDGFRα (directly conjugated to fluorophore) to a dilution of 1:50 (10 µg/ml).
- To label other cell populations (*e.g.* immune cells, macrophages, endothelial cells etc.): add appropriate fluorescently conjugated antibodies to surface markers, to a 1:50 dilution.

Note: although PDGFRα is a robust marker for fibroblasts, it is recommended to include antibodies for immune cells and for epithelial cells in order to exclude any double positive populations and thus enhance the purity of isolated fibroblasts.

- Add 2 µl of each antibody to each of the single color control eppendorf tubes.
- Incubate cells with antibodies for 1 hr on ice in the dark.
- To wash: fill tubes with FACS buffer I and spin for 5 min at 450 x g at 4 °C.
- Aspirate supernatant, resuspend cells in FACS buffer I to a concentration of approximately 2 x 10^6 cells/ml, or whichever concentration most appropriate for sorting with your available FACS sorter.

Optional: Cells may be resuspended in FACS buffer II for the duration of sorting. This may improve cell viability. However, exposure to factors in serum may have an effect gene expression, which should be tested, and taken into account.

- Transfer labeled cells into FACS tubes with filter top: filter cells into the tubes to prevent cell clumps.
- To exclude dead cells, add 7AAD (0.5 µg/ml) or Propidium Iodide (PI) to samples (except for the un-stained control).

Note: To avoid wasting cells, you can add 7AAD/PI to the un-stained sample after recording in the FACS, and use again as 7AAD-only control.

- Keep samples on ice while analyzing.

6. Isolation of Cells by FACS

(**Note:** this part of the protocol requires previous knowledge in operating a FACS sorter, *e.g.* BD FACS AriaII, or the assistance of a skilled technician).

- Using the FACS sorter software (BD FACSDiva), prepare appropriate analysis plots to analyze forward scatter (FSC), side scatter (SSC), 7AAD, FITC, PE and any other fluorophore used to label cells (**Figure 2**).
- Before loading each sample to the cell sorter, vortex briefly to resuspend cells.

- Start by analyzing the unstained sample in order to set the gating for the total cell population, to gate out cell debris, and to determine autofluorescence or background staining.
- Analyze unstained sample + 7AAD to determine and gate the population of live cells from the total cell population.
- Analyze each single color control to determine and calibrate parameters such as compensation.
- Analyze the stained sample to define the position of gates for sorting.
- Once parameters and gates for the desired cell populations have been set, begin sorting the labeled cell populations into eppendorf tubes or 15 ml conical tubes containing culture medium or RNA lysis buffer (such as RLT buffer or Trizol).

Note: If sorting a large number of cells, it is not recommended to sort directly into RNA lysis buffer, as the large volume of sheath fluid accompanying the cells will dilute the lysis buffer and reduce the yield.

- Spin cells down immediately after sorting is finished, and transfer into fresh medium or RNA lysis buffer, depending on the purpose of your experiment.
- If sorting in order to culture isolated cells, perform sterile sort. For better recovery of cells, use phenol red free DMEM + 5% FCS instead of FACS buffer II and collect cells into culture medium with 20% FCS.
- If culturing sorted fibroblasts, alwaysplate on Collagen coated plates and never directly on plastic as this results in activation of fibroblasts leading to changes in their gene expression.

Representative Results

Using PDGFRα as a marker for fibroblasts results in isolation of highly enriched populations of tissue fibroblasts. The level of purity after sorting was 99%, as quantified by post-sort analysis (**Figure 2A**). Estimating the percentage of contaminating non-fibroblast cells by the relative expression of cell-specific control genes (**Figure 2B**) typically shows 0.1-0.6% contamination. This level of purity allows high quality transcriptome profiling of isolated fibroblasts[9].

In mammary glands, the percentage of fibroblasts in the tissue varies between 5-20% in normal mammary tissue, and 1-5% in MMTV-PyMT tumors. The relative percentage of fibroblasts in tumor tissue is lower than in pre-neoplastic tissue, despite increase in the total number of fibroblasts, due to the massive expansion of the epithelial compartment. The reduced % of fibroblasts isolated from tumor tissue is also a result of the added technical difficulty and decreased efficiency of cell sorting from a highly necrotic tissue.

Isolated fibroblasts can be cultured directly after sorting, but may require a few days to recover (**Figure 2D**). It is essential to perform all further experiments with low passage cells, as primary fibroblasts undergo senescence during propagation in culture.

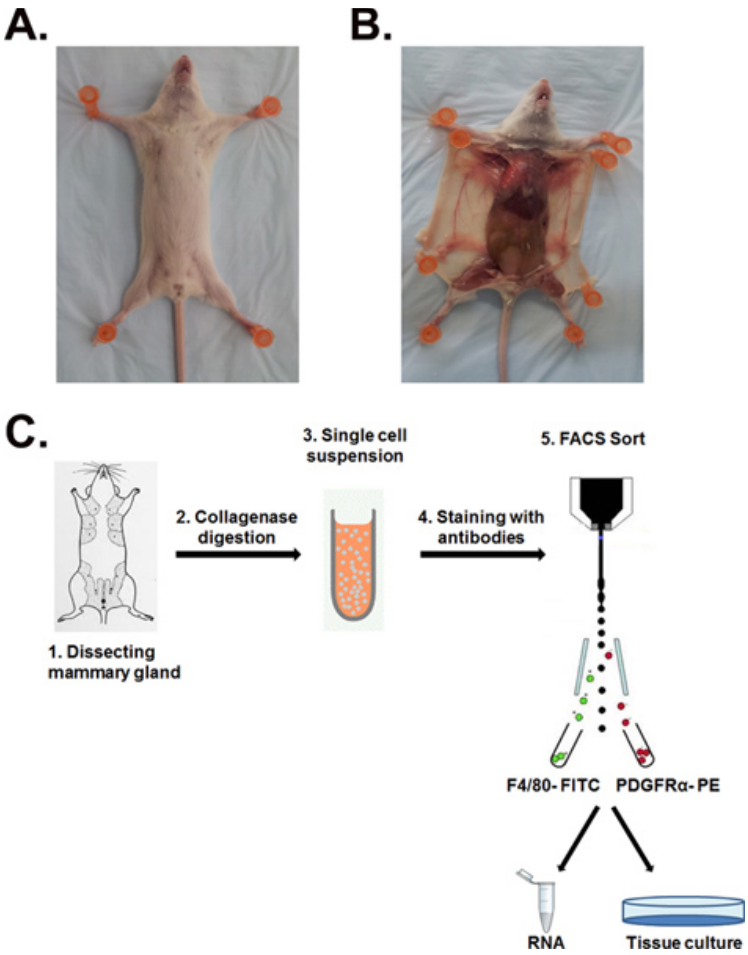

Figure 1. Purification of fibroblasts from fresh mammary glands. A- FVB/n /PyMT mouse. **B-** Exposed mammary glands. **C-** Fresh mammary glands were dissected from FVB/n /PyMT mice and digested with collagenase to a single cell suspension. Cells were immune-labeled with antibodies for fibroblast surface marker (PDGFRα) and macrophages surface marker (F4/80) followed by separation by FACS. Sorted fibroblasts were either cultured or lysed for RNA purification. Click here to view larger figure (http://www.jove.com/files/ftp_upload/4425/4425fig1large.jpg).

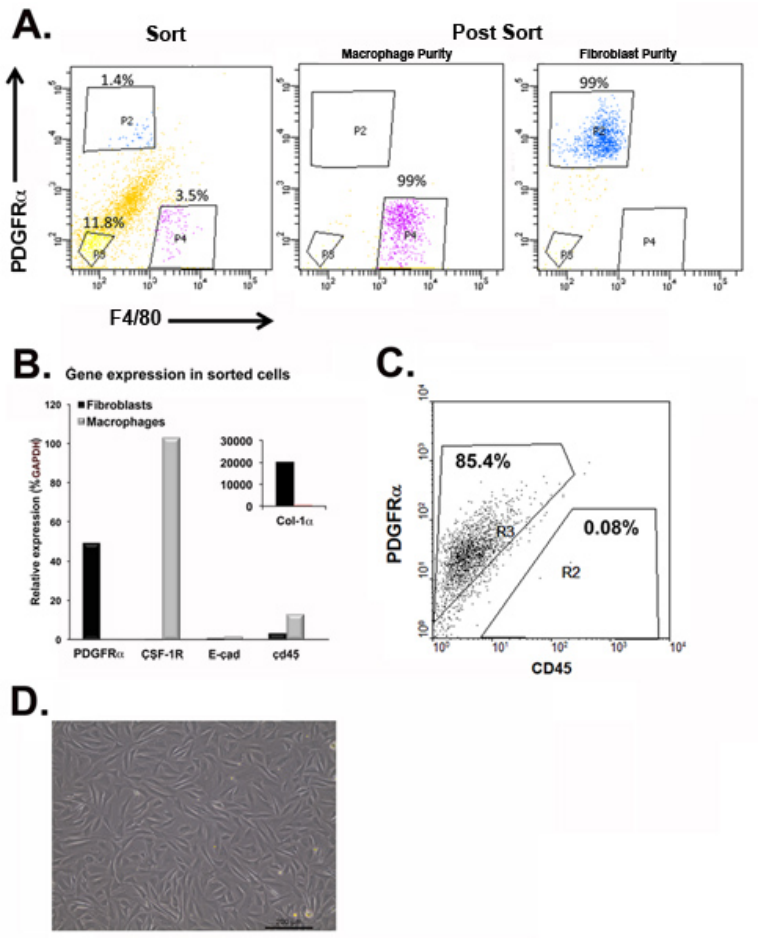

Figure 2. Profiling of sorted fibroblasts. A- Single cell suspensions of mouse mammary glands were stained with PDGFRαand F4/80 antibodies. FACS sorting plot is shown (left panel). P2 is the fibroblasts gate (PDGFRα+ cells) and P4 is the macrophages gate (F4/80+ cells). A post sort analysis was performed to determine the purity of sorted fibroblasts and macrophages (middle and right panels). **B-** Analysis of sorting purity by qRT-PCR of cell-specific control genes for fibroblast (PDGFRα, Col-1α), immune cells (CD45, CSF-1R) and epithelial cells (E-cadherin). Results were normalized to two house keeping genes (GAPDH and mGUS). Relative expression to GAPDH is shown. **C-** Quantification of PDGFRα expression in total unsorted population of mammary fibroblasts. **D-** Tissue culture of sorted fibroblasts. Click here to view larger figure (http://www.jove.com/files/ftp_upload/4425/4425fig2large.jpg).

Discussion

While experiments performed in tissue culture can be informative and suggest functional principles that can be verified *in vivo*, it is known that large changes occur in gene expression of cells in culture[11,12] . In order to avoid a tissue culture step when profiling gene expression in fibroblasts, we developed a protocol that allows isolation of normal, as well as cancer-associated fibroblasts from fresh mouse or human tissue. This protocol was successfully applied with skin, pancreas and breast tissues from mouse and from human[9]. Isolation of fibroblasts by FACS sorting requires a surface marker that labels fibroblasts. We established that PDGFRα is a robust surface marker that enables the isolation of highly enriched populations of fibroblasts[9]. Moreover, PDGFRα is expressed on both normal tissue fibroblasts and on CAFs, thus allowing unbiased isolation and profiling of tissue fibroblasts rather than focusing on a specific subpopulation.

Fibroblasts are a heterogeneous cell population in regard to the expression of marker molecules as well as in

their origin and signaling properties[8,10,18]. While PDGFRα is a robust surface marker for fibroblasts, it does not label 100% of fibroblasts in all tissues resulting in an unlabeled subpopulation of cells, depending on tissue type. In skin, approximately 90% of fibroblasts are PDGFRα+ (not shown) while in mammary glands approximately 85% of total tissue fibroblasts are PDGFRα+ (**Figure 2C**). The percentage of PDGFRα expressing fibroblasts may vary in other tissue types, and needs to be defined for each tissue. Nevertheless, in skin and in mammary glands, utilizing PDGFRα as a cell surface marker will result in highly enriched populations of the majority of tissue fibroblasts, allowing expression profiling or culturing of sorted cells.

Disclosures

No conflicts of interest declared.

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Materials

Name	Company	Catalog Number	Comments
DMEM	Gibco	41965	
PBS	Biological Industries	02-023-1A	
Collagenase II	Worthington	LS4176	
Collagenase IV	Worthington	LS4188	
Deoxyribonuclease	Worthington	LS2007	
PharmLyse	BD	555899	
Cell strainer 70 µm	SPL	93070	
Purified anti-mouse CD16/CD32	BD Pharmingen	553142	
Via probe (7AAD)	e-Bioscience	00-6993-50	
Anti-mouse CD140a-PE (PDGFRa)	e-Bioscience	12-1401-81	
Anti-mouse F4/80- FITC	Cederlane	CL8940F	
DMEM w/o Phenol Red	Gibco	31053	
Collagen Type I	BD Biosciences	354236	

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