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Title: Cancer-Associated Fibroblasts from Mouse Mammary Tumors as Tools for Molecular and Computational Studies

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar?
Yes

SCOPE: 2.14.1-2.14.2
Videographer: Please capture shots labelled SCOPE as backup

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. Filming location: Will the filming need to take place in multiple locations? **No**

Current Protocol Length
Number of Steps: 26
Number of Shots: 56

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

1.1. **Avalle Lidia:** Our research investigates how cancer-associated fibroblasts shape breast cancer progression, using molecular, *in vitro, in vivo*, and computational approaches to dissect molecular mechanisms and identify potential therapeutic targets.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll: 5.1*

What are the most recent developments in your field of research?

1.2. **Avalle Lidia:** Recent tools such as tumor organoids, single-cell and spatial transcriptomics are changing our ability to model the tumor microenvironment, allowing a precise and dynamic understanding of tumor–stroma crosstalk.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What are the current experimental challenges?

1.3. **Mirzaaghaei Somayeh:** Cancer research faces microenvironment heterogeneity and stromal–epithelial complexity, the dissection of which both in vitro and in mouse models can reveal key molecular mechanisms amenable to therapeutic intervention.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What significant findings have you established in your field?

1.4. **Poli Valeria:** We have established a key role in mediating the pro-oncogenic functions of breast cancer CAFs for the transcription factor STAT3 and several STAT3 target genes, amenable to therapeutic intervention.

1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

What research questions will your laboratory focus on in the future?

1.5. **Daniele Viavattene:** We will take advantage of patient-derived CAFs in order to validate the biological and clinical relevance of our findings in a human setting, including the induction of drug resistance.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the institutional OPBA (Animal Welfare Committee) at the University of Torino

2. Quantifying 4T1 Cell Migration and Invasion via Transwell and Crystal Violet Staining

Demonstrator: Daniele Viavattene, Lidia Avalle

2.1. To begin, obtain the reagents and labware required for the experiment **[1].** Pipette 700 microliters of supplemented DMEM into each well of a 24-well plate **[2-TXT]**.

2.1.1. WIDE: Talent arranging reagents and labware for the experiment, on the workspace.

2.1.2. Talent pipetting medium into a 24-well plate using a pipette. **TXT: DMEM supplemented with 1% heat-inactivated FBS, 1% Pen/Strep**

2.2. Place an 8-micrometer transwell insert into each well, coated with a biologically active matrix for invasion assays or uncoated for migration assays **[1]**. Then transfer the plate to a 37 degrees Celsius incubator to equilibrate **[2].**

2.2.1. Talent placing coated or uncoated transwell inserts into the wells.

2.2.2. Talent placing the plate inside an incubator.

2.3. Now, obtain a 100-millimeter dish containing 4T1 *(Four-T-One)* cells pre-treated for 48 hours with immortalized cancer-associated fibroblast conditioned medium **[1].** Pipette 1 milliliter of 5 millimolar EDTA into the dish to detach the cells **[2]**.

2.3.1. Shot of dish containing 4T1 cells.

2.3.2. Talent using a pipette to add EDTA to the dish.

2.4. Then add 5 milliliters of complete DMEM to the dish **[1].** Transfer the cell suspension into a 15-milliliter conical tube **[2]**.

2.4.1. Talent adding medium to the dish.

2.4.2. Talent transferring the cell suspension into a 15 mL conical tube.

2.5. Centrifuge the tube to pellet the cells **[1]**. Resuspend the pellet in supplemented DMEM **[2]**. Count the cells using a Neubauer chamber **[3-TXT].**

2.5.1. Shot of the tube being removed from the centrifuge with visible pellet.

2.5.2. Talent resuspending the pellet in sDMEM.

2.5.3. Talent transferring cell suspension to Neubauer chamber. **TXT: Dilute cell suspension to 1 x 10^5 cells/100 µL**

2.6. Pipette 100 microliters of the prepared cell suspension into the upper chamber of each transwell insert **[1]**. As a plating control, seed 100 microliters of the same cell suspension into a 24-well plate containing 500 microliters of complete medium **[2-TXT]**.

2.6.1. Talent pipetting cell suspension into transwell inserts.

2.6.2. Talent plating control cells in a separate well. **TXT: Incubation: Migration assay : 16 h; Invasion assay : 24 h**

2.7. Label the upper side of each insert using a thin marker **[1]**. Using tweezers, submerge the inserts in a 50-milliliter conical tube filled with PBS for washing **[2]**. Then use a cotton swab to carefully clean the internal part of the transwell **[3]**.

2.7.1. Talent labeling transwell inserts.

2.7.2. Talent submerging inserts in 50 mL tubes with PBS using tweezers.

2.7.3. Talent cleaning the internal surface of the transwells with a cotton swab.

2.8. Under a fume hood, place each insert into 700 microliters of 4 percent paraformaldehyde and incubate **[1-TXT]**.

2.8.1. Talent placing inserts into paraformaldehyde under a fume hood and setting a timer. **TXT: 10 min, RT**

2.9. Then wash the inserts by placing them three times into 700 microliters of PBS **[1].** After letting them dry for 10 minutes, incubate the inserts in 700 microliters of 0.1 percent crystal violet and incubate for 10 minutes at room temperature **[2]**.

2.9.1. Talent dipping the inserts in wells with PBS.

2.9.2. Talent dipping the inserts in wells containing crystal violet.

2.10. Using tweezers, submerge the inserts three times in a 50-milliliter conical tube containing double-distilled water **[1]**. Dry the internal surface using a cotton swab **[2].** Store samples at room temperature for up to three days before imaging **[3]**.

2.10.1. Talent dipping inserts in a 50 mL tube with double-distilled water.

2.10.2. Talent drying internal surfaces with a cotton swab.

2.10.3. Talent placing them in storage.

2.11. Using a phase-contrast microscope, capture one image with a 10X *(Ten-Ex)* objective to visualize most of the well surface **[1]**. Acquire images from five independent areas of the membrane using a 20X objective **[2]**.

2.11.1. SCOPE: 68364_2.11.1.mp4 00:05-00:15,00:27-00:34

2.11.2. SCOPE: 68364_2.11.2.mp4 00:03-00:20.

2.12. Add acetic acid to dissolve the crystal violet. For wells, add 100 microliters of 10 percent acetic acid **[1].** Mix on an orbital shaker for 10 minutes at room temperature **[2]**.

2.12.1. Talent adding acetic acid to wells.

2.12.2. Shot of the well plate being placed on an orbital shaker.

2.13. For transwells, lay them with the lower surface facing up **[1].** Deposit 50 microliters of acetic acid on the surface **[2]** and mix by pipetting until the dye dissolves **[3]**. Transfer 50 microliters of the dissolved crystal violet from each sample to a 96-well plate for spectrometric analysis **[4]**.

2.13.1. Shot of the transwells being placed with lower surface facing up.

2.13.2. Talent pipetting 50 µL acetic acid over the lower surface of transwells.

2.13.3. Shot of the acetic acid-crystal violet mix being pipetted repeatedly to dissolve the dye.

2.13.4. Talent transferring solution to a 96-well plate.

3. Histological Analysis of Lung Metastases in Tumor-Bearing BALB/c Mice

Demonstrator: Lidia Avalle

3.1. At day 21, post-injection, in a fume hood, open the chest cavity of a euthanized tumor-injected BALCB/c *(Bal-C-B-C)* mouse, using surgical scissors **[1]**. Remove the skin from the submandibular area and the salivary glands to expose the trachea clearly **[2]**.

3.1.1. Talent cutting the chest open using surgical scissors.

ADDED SHOT: Removal of the sternum and rib section.

3.1.2. Talent peeling back the skin and removing salivary glands to reveal the trachea.

ADDED SHOT: Removal of muscles surrounding the trachea.

3.2. Prepare a 22-gauge needle syringe filled with 4 percent paraformaldehyde **[1]**. Insert the needle into the trachea **[2]** and direct it toward the lungs while clamping the trachea toward the head **[3]**. Inject the solution slowly until the lungs swell, typically using around 2 milliliters of solution **[4]**.

3.2.1. Talent filling a syringe with paraformaldehyde.

3.2.2. Talent inserting the needle into the trachea.

3.2.3. Shot of the trachea being clamped toward the head.

3.2.4. Shot of the PFA being injected and the lungs swelling.

3.3. Dissect the lungs **[1]** and transfer them into a 15-milliliter tube containing 4 percent paraformaldehyde **[2]**.

3.3.1. Talent excising the lungs.

3.3.2. Talent placing excised lungs into a labeled 15 mL conical tube with 4% PFA.

3.4. Then gently invert the tube a few times **[1].** Incubate the lungs at 4 degrees Celsius for 24 hours to allow fixation before sectioning and staining **[2]**.

3.4.1. Talent inverting the tube a few times.

3.4.2. Talent placing the tube into a refrigerator set at 4 degrees Celsius.

4. Transcriptomic Analysis of Breast Tumor Stroma Using the MetaLCM Web Application

Demonstrator: Aurora Savino

4.1. Access the MetaLCM *(Meta-L-C-M)* application for the analysis of transcriptomic datasets of laser-capture microdissected breast tumors **[1-TXT]**. To begin the analysis, select the features representing the conditions to be compared **[2]**.

4.1.1. SCREEN: 68364_4.mp4 00:03-00:06 **TXT: Web address: https://aurorasavino.shinyapps.io/metalcm/**

4.1.2. SCREEN: 68364_4.mp4 00:07-00:15.

4.2. Choose the comparison direction by selecting either up-regulated or down-regulated genes **[1]**. When up-regulated genes are selected, click **Run** to see genes with higher expression in condition 1 will be shown **[2]**.

4.2.1. SCREEN: 68364_4.mp4 00:16-00:18 .

4.2.2. SCREEN: 68364_4.mp4 00:19-00:28.

4.3. Now select the p-value threshold for differential expressions **[1]**. Press the **Run** button to execute the analysis each time a parameter is adjusted **[2]**.

4.3.1. SCREEN: 68364_4.mp4 00:29-00:37 .

4.3.2. SCREEN: 68364_4.mp4 00:38-00:41 .

4.4. To limit the analysis to a specific gene list, type gene symbols separated by commas or upload a CSV file with gene symbols in a single column **[1]**.

4.4.1. SCREEN: 68364_4.mp4 00:43-00:50 .

4.5. Press **Submit gene list** to load the gene symbols into the analysis **[1]**. Then press **Reset** to analyze all genes **[2]**. Save the resulting table of differentially expressed genes, which includes average log fold-change, collapsed p-value, and detailed values for each dataset **[3]**.

4.5.1. SCREEN: 68364_4.mp4 00:51-00:54.

4.5.2. SCREEN: 68364_4.mp4 00:55-00:58 .

4.5.3. SCREEN:68364_4.mp4 00:59-01:04 .

Results

5. Results

5.1. Treatment with conditioned medium from super-activated immortalized cancer-associated fibroblasts or iCAFs *(I-Caffs)* significantly enhanced 4T1 cell proliferation compared to control medium **[1]**. iCAFs-conditioned medium doubled the migration capacity of 4T1 cells relative to control **[2]**. A moderate but significant increase in invasion was observed in 4T1 cells treated with iCAFs-conditioned medium compared to control **[3]**.

5.1.1. LAB MEDIA: Figure 1B *Video editor: Show only "Proliferation" graph and highlight the red curve for "iCAFs"*

5.1.2. LAB MEDIA: Figure 1B *Video editor: Show only "Migration" graph and highlight the taller white bar for "iCAFs" with red dots.*

5.1.3. LAB MEDIA: Figure 1B *Video editor: Show only "Invasiona" graph and highlight the taller bar labeled "iCAFs."*

5.2. Co-injection of iCAFs with 4T1 cells in mice significantly increased tumor volume by day 10 compared to injection of 4T1 cells alone **[1]**. Tumor weights were significantly higher in mice co-injected with iCAFs and 4T1 cells compared to those injected with 4T1 cells alone **[2]**. The presence of iCAFs led to a substantial increase in lung metastases in mice, as shown by a higher percentage of nodule area per lung section **[3]**.

5.2.1. LAB MEDIA: Figure 2B *Video editor: Show only "Tumor volume" graph and highlight the red curve for iCAFs from day 6 to day 10.*

5.2.2. LAB MEDIA: Figure 2B *Video editor: Show only "Tumor weight" graph and highlight the bar with red dots*

5.2.3. LAB MEDIA: Figure 2B *Video editor: Show only "Lung Metastases" graph and highlight the bar with red dots*

5.3. Four STAT3 *(Stat-Three)*-regulated genes, MMP13 *(M-M-P-Thirteen)*, MMP3 *(M-M-P-Three)*, LGI2 *(L-G-Eye-Two)*, and TIMP1 *(Tim-P-One)*, were consistently upregulated in the tumor stroma of human breast cancer samples compared to normal stroma **[1]**.

5.3.1. LAB MEDIA: Figure 3B, heatmap . *Video editor: Show right side heatmap only. Highlight the panels labeled MMP13, MMP3, LGI2, and TIMP1 sequentially*

Pronunciation Guide:

1. MMP13

- **Pronunciation link**: No confirmed link found.
- **IPA**: /ˌɛmˌɛmˌpiː ˈθɜːˈtiːn/
- **Phonetic Spelling**: em-em-pee thirteen

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2. MMP3

- **Pronunciation link**: No confirmed link found.
- **IPA**: /ˌɛmˌɛmˌpiː ˈθriː/
- **Phonetic Spelling**: em-em-pee three

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3. LGI2

- **Pronunciation link**: No confirmed link found.
- **IPA**: /ˌɛlˌdʒiːˌaɪ ˈtuː/
- **Phonetic Spelling**: el-jee-eye two

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4. TIMP1

- **Pronunciation link**: No confirmed link found.
- **IPA**: /ˈtɪmp wʌn/
- **Phonetic Spelling**: timp one