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Title: Studying TGF-β Signaling and TGF-β-induced Epithelial-to-mesenchymal Transition in Breast Cancer Cells

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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, need the scope kit for SP8 confocal microscope**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No.**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees self-record interview statements. JoVE can provide support for this option.

4. **Filming location:** Will the filming need to take place in multiple locations? **No, same building different floors**

Current Protocol Length

Number of Steps: 9
Number of Shots: 29

Introduction

1. Introductory Interview Statements

NOTE: Videographer took new headshots, please use the ones that the videographer took. There are also some shots of the authors together that can be used for social media, if needed.

REQUIRED:

1.1. **Peter ten Dijke:** TGF-β is a main driver of epithelial to mesenchymal transition of normal and cancer cells. The methods in our protocol allow researchers to examine the impact of modulators on TGF-β/SMAD signaling and TGF-β-induced EMT. The untransformed NMuMG cells established from a mouse mammary gland are frequently used as a model system to investigate TGF-β-induced EMT. TGF- β provokes a particular strong EMT response in these cells. A similar EMT response also occurs in breast cancer cells and contributes to cancer progression.

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

1.2. **Jing Zhang:** The combined staining of the epithelial marker E-cadherin and organization of filamentous actin helps to validate and better visualize the morphological changes that occur during epithelial to mesenchymal transition.

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

OPTIONAL:

1.3. **Peter ten Dijke:** The acquisition of a mesenchymal phenotype of cancer cells has been linked to increased ability to migrate, invade, and metastasize and also chemotherapy resistance.

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

1.4. **Peter ten Dijke:** This method may provide important insights into cancer progression, but also into other processes such as tissue fibrosis.

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

1.5. **Jing Zhang:** When attempting this protocol, keep in mind that the cell density should be well controlled. High density of the cells makes the epithelial to mesenchymal transition difficult to observe.

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

1.6. **Jing Zhang:** With this visual demonstration, researchers will get a good grasp on how to perform TGF-β signaling and TGF-β-induced EMT experiments.

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

Protocol

2. Analysis of TGF-β-induced EMT

2.1. To perform indirect immunofluorescence staining of E-cadherin, begin by placing sterile 18-millimeter square glass coverslips in 6-well plates **[1]**, one coverslip per well **[2]**.

2.1.1. WIDE: Establishing shot of talent placing coverslips in a plate. NOTE: 2.1.1 tale 3 is also 2.1.2.

2.1.2. Talent placing a coverslip in a well.

2.2. Seed 100,000 NMuMG *(spell out 'n-m-u-m-g')* cells with 2 milliliters of complete DMEM into each well and allow the cells to adhere overnight. Non-transformed NMuMG cells are frequently used as a model system to investigate TGF-β *('beta')*-induced EMT **[1]**.

2.2.1. Talent seeding cells into a well.

2.3. On the next day, gently move the coverslips with adherent cells to a new 6-well plate **[1]** and add 2 milliliters of culture medium to the wells **[2]**. Treat the cells with ligand buffer as a control **[3.0]** or 5 nanograms per milliliter TGF-β *(pronounce 'T-G-F-beta')* for 2 days. The added ligand buffer should be the same volume as added TGF-β**[3]**.

2.3.1. Talent transferring a coverslip to a new plate.

2.3.2. Talent adding medium to a few wells.

2.3.3.0 Added shot: adding ligand buffer to a few wells.

2.3.3. Talent adding TGF-β to a few wells.

2.4. After the treatment, remove the culture medium **[1]** and gently wash the cells twice with 1 milliliter of prewarmed PBS **[2]**. Fix the cells by adding 1 milliliter of 4% paraformaldehyde and incubating for 30 minutes at room temperature **[3]**. Then, gently wash the cells twice with 1 milliliter of PBS **[4]**.

2.4.1. Talent removing the medium.

2.4.2. Talent washing the cells in PBS.

2.4.3. Talent adding PFA to the cells, with the PFA container in the shot.

2.4.4. Talent washing the cells with PBS.

2.5. Permeabilize the fixed cells with 0.1% Triton X-100 for 10 minutes at room temperature **[1]** and wash the cells twice with PBS **[2]**. Block the nonspecific protein binding to cells with 5% BSA in PBS for 1 hour at room temperature, then repeat the washes with PBS **[3]**. *Videographer: This step is difficult and important!*

2.5.1. Talent adding with 0.1% Triton X-100 to the cells, with the Triton X-100 container in the shot.

2.5.2. Talent washing the cells with PBS.

2.5.3. Talent adding BSA to the cells.

2.6. Add the primary antibody against E-cadherin to the top of each coverslip **[1-TXT]** and incubate for 1 hour at room temperature **[2]**. Remove the primary antibody **[3]** and wash each coverslip with PBS three times **[4]**. *Videographer: This step is important!*

2.6.1. Talent adding the E-cadherin antibody to a coverslip. **TEXT: diluted 1:1000 in PBS**

2.6.2. Coverslips incubating.

2.6.3. Talent removing the antibody.

2.6.4. Talent washing the coverslips in PBS.

2.7. Add the Alexa Fluor 555 secondary antibody and the Alexa Fluor 488 Phalloidin to the top of each coverslip **[1-TXT]**, then cover with aluminum foil and incubate for 1 hour at room temperature **[2]**. *Videographer: This step is important!*

2.7.1. Talent adding the Alexa Fluor 555 secondary antibody and the Alexa Fluor 488 Phalloidin to the coverslip. **TEXT: diluted 1:500 in PBS**

2.7.2. Talent covering the coverslips with aluminum foil.

2.8. Remove the secondary antibody and wash the coverslip three times with PBS **[1]**. Mount the coverslip, with the cells facing downward, onto glass slides using mounting medium with DAPI **[2]** and store the mounted slides in a box at 4 degrees Celsius, protected from light **[3]**. Observe staining with SP8 confocal microscopy **[4]**. *Videographer: This step is important!*

2.8.1. Talent removing the secondary antibody.

2.8.2. Talent mounting the coverslip.

2.8.3. Talent placing the coverslips in a box.

2.8.4. SCREEN: 61830_2.8.4.mkv.

Results

3. Results: TGF-β Signaling Responses

3.1. In the MCF10A-Ras cell line, the phosphorylation of SMAD2 significantly increased in response to TGF-β *(pronounce 'T-G-F-beta')* stimulation **[1]** while the expression of total SMAD2/3 *(pronounce 's-mad-two-and-three')* was not affected **[2]**. TGF-β also markedly induced the luciferase reporter in the MCF10A-Ras cell line compared to non-treated cells **[3]**.

3.1.1. LAB MEDIA: Figure 4 A. *Video Editor: Emphasize the p-SMAD2 image.*

3.1.2. LAB MEDIA: Figure 4 A. *Video Editor: Emphasize the SMAD2/3 image.*

3.1.3. LAB MEDIA: Figure 4 B.

3.2. Well-characterized direct transcriptional gene targets of TGF-β, including *SMAD7* and *SERPINE1*, were highly expressed in TGF-β-treated MCF10A-Ras breast cells **[1]**.

3.2.1. LAB MEDIA: Figure 4 C.

3.3. NMuMG *(spell out 'n-m-u-m-g')* epithelial cells treated with TGF-β changed from a classic epithelial morphology to a spindle-shaped mesenchymal-like morphology **[1]**. Consistent with the morphological changes, TGF-β treatment led to an increase in the protein expression of mesenchymal markers **[2]**.

3.3.1. LAB MEDIA: Figure 5 A.

3.3.2. LAB MEDIA: Figure 5 B. *Video Editor: Emphasize the N-cadherin, Snail, and Slug images.*

3.4. In contrast, E-cadherin, an epithelial marker, was downregulated after 2 days of TGF-β treatment **[1]**.

3.4.1. LAB MEDIA: Figure 5 B. *Video Editor: Emphasize the E-cadherin images.*

3.5. Quantitative real-time-polymerase PCR was used to investigate the gene expression of EMT markers after TGF-β stimulation **[1]**. *CDH1* was significantly decreased **[2]** while mesenchymal markers were increased **[3]**

3.5.1. LAB MEDIA: Figure 5 C.

3.5.2. LAB MEDIA: Figure 5 C. *Video Editor: Emphasize the CDH1 graph.*

3.5.3. LAB MEDIA: Figure 5 C. *Video Editor: Emphasize the SNAIL and ZEB2 graphs.*

3.6. Upon TGF-β stimulation, NMuMG cells expressed less E-cadherin than cells in the uninduced control group **[1]**. Moreover, the cells formed more actin stress fibers in the presence of TGF-β **[2]**.

3.6.1. LAB MEDIA: Figure 5 D.

3.6.2. LAB MEDIA: Figure 5 E.

3.7. Treatment with GW788388 *(pronounce 'G-W-seven-eight-eight-three-eighty-eight')* inhibited TGF-β-induced SMAD2 phosphorylation in a dose-dependent manner **[1]**. Additionally, the TGF-β-mediated phosphorylation of SMAD2 was blocked by SB431542 *(pronounce 'S-B-four-three-one-five-forty-two')* treatment **[2]**.

3.7.1. LAB MEDIA: Figure 6 A. *Video Editor: Emphasize the P-Smad2 bands in columns 4 – 6 and 10 – 12.*

3.7.2. LAB MEDIA: Figure 6 A. *Video Editor: Emphasize the P-Smad2 bands in column 9.*

3.8. Both SB431542 and GW788388 significantly inhibited the TGF-β-induced nuclear translocation and accumulation of SMAD2/3 in NMuMG cells **[1]**.

3.8.1. LAB MEDIA: Figure 6 B.

Conclusion

4. Conclusion Interview Statements

4.1. **Jing Zhang:** Following this procedure, immunofluorescence staining of other epithelial and mesenchymal marker proteins can be performed by changing the specific primary and secondary antibodies to investigate the expression level and localization of these proteins within the cell.

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