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Title: Isolation of Exosome-Enriched Extracellular Vesicles Carrying Granulocyte-Macrophage Colony-Stimulating Factor from Embryonic Stem Cells

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Author Questionnaire:

1. Microscopy: Does your protocol involve video microscopy? N

2. Does your protocol demonstrate software usage? N

3. Which steps from the protocol section below are the most important for viewers to see?

3.1.-3.3., 4.2., 4.3.

4. What is the single most difficult aspect of this procedure and what do you do to ensure success?

3.3., You have to carefully follow the protocol to ensure success.

5. Will the filming need to take place in multiple locations (greater than walking distance)? N

Section - Introduction

Videographer: Interviewee Headshots are required. Take a headshot for each interviewee.

1. **REQUIRED Interview Statements (Said by you on camera): All interview statements may be edited for length and clarity.**

    1.1. **Shuhan Meng**: Our protocol can be used to produce high-quality, exosome-enriched extracellular vesicles from embryonic stem cells that express the immune stimulatory factor GM-CSF **[1]**.

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

    1.2. **Shuhan Meng**: Exosome-enriched extracellular vesicles carrying GM-CSF have the potential to serve as cell-free immune regulatory vesicles that can modulate the immune response **[1]**.

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

OPTIONAL Interview Statements: (Said by you on camera) - All interview statements may be edited for length and clarity.

    1.3. **Aaron Whitt**: These extracellular vesicles may then have the potential to modulate the immune response under different disease conditions **[1]**.

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

    1.4. **Aaron Whitt**: Since GM-CSF activates and regulates the immune response, these vesicles could also provide insight into the role of immune regulation in various diseases **[1]**.

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

    1.5. **Chi Li**: Researchers with basic molecular and cellular biology training should be able to easily execute this protocol, but anyone performing this protocol for the first time should follow the directions closely **[1]**.

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

    1.6. **Chi Li**: Since our protocol is complicated, visualizing the intricate details of each step will help other researchers to quickly master the technique **[1]**.

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

Section - Protocol

2. ES-D3 Cell Culture

2.1. To generate exosome-free FBS (F-B-S), ultracentrifuge the desired volume of FBS **[1-TXT]** and collect the exosome-free supernatant **[2-TXT]**.

2.1.1. WIDE: Talent adding tube(s) to centrifuge **TEXT: 16 h, 100,000 x g, 4 °C**
2.1.2. Shot of pellet if visible, then supernatant being collected **TEXT: FBS: fetal bovine serum**

2.2. Before plating the ES-D3 (E-S-D-three) cells, coat 15-centimeter tissue culture dishes with 0.1% gelatin at room temperature for 30 minutes **[1]**.

2.2.1. Talent adding gelatin to dish(es), with gelatin container visible in frame

2.3. Remove gelatin by aspiration, and culture the ES-D3 cells without feeder layer cells in ES-D3 cell culture medium **[1-TXT]** at 37 degrees Celsius in a 5% carbon dioxide humidified incubator until the cells reach 90% confluency **[2]**.

2.3.1. Talent adding cells to plate, with medium container visible in frame **TEXT: See text for all medium/solution preparation details**
2.3.2. Talent placing plate(s) into incubator

2.4. Wash the almost-confluent cultures with 5 milliliters of 0.05% trypsin per dish **[1]** followed by a 5-minute incubation at 37 degrees Celsius in fresh trypsin **[2]**.

2.4.1. Dish being washed, with trypsin container visible in frame
2.4.2. Talent placing plate(s) into incubator

2.5. At the end of the incubation, pool the detached cells in a centrifuge tube **[1]** and inactivate the trypsin with 5 milliliters of fresh culture medium **[2]**.

2.5.1. Talent adding cells to tube, with plate(s) visible in frame
2.5.2. Talent adding medium to tube, with medium container visible in frame

2.6. Sediment the cells by centrifugation **[1-TXT]** and resuspend the pellets in fresh medium for counting **[2]**.

2.6.1. Talent placing tube(s) into centrifuge **TEXT: 5 min, 390 x g, RT**
2.6.2. Shot of pellet if visible, then medium being added to tube, with medium container and hemocytometer visible in frame

2.7. For passaging, plate 5 x 10^6 of the ES-D3 cells in 15 milliliters of cell culture medium onto new gelatin-coated plates for 3 days of culture **[1]** before subculturing the cells **[2]**.

2.7.1. Talent adding cells to plate(s), with medium container visible in frame

2.7.2. Talent adding plate(s) to incubator

2.8. To collect the cell culture supernatant for the isolation of exosome-enriched extracellular vesicles, plate 1 x 10^7 ES-D3 cells in 15 milliliters of cell culture medium **[1]** per new gelatin-coated plate for 3 days prior to collecting the cell culture supernatants **[2-TXT]**.

2.8.1. Cells being added to plate, with medium container visible in frame
2.8.2. Talent collecting supernatant **TEXT: Store supernatant at 4 °C ≤1 wk**

3. Exosome-Enriched Extracellular Vesicle (EV) Isolation

3.1. For exosome-enriched extracellular vesicle isolation, first sediment the large cell fragments within the supernatants collected from 72-hour-cultured ES-D3 cells by centrifugation **[1-TXT]**.

3.1.1. WIDE: Talent adding tube(s) to centrifuge *Videographer: Important step* **TEXT: 60 min, 5000 x g, 4 °C**

3.2. After collecting the supernatant, ultracentrifuge the samples **[1-TXT]** and discard the supernatants **[2]**.

3.2.1. Talent adding supernatant to ultracentrifuge tube(s) *Videographer: Important step* **TEXT: 90 min, 100,000 x g, 4 °C**
3.2.2. Shot of pellet(s) if visible, then supernatant being aspirated/decanted *Videographer: Important step*

3.3. Gently rinse each pellet two times with 1 milliliter of PBS per wash to remove any residual culture supernatant **[1]** and quantify the exosome-enriched extracellular vesicle protein content with a bicinchoninic acid assay according to the manufacturer's instruction **[2-TXT]**.

3.3.1. Talent adding PBS to tube, with PBS container visible in frame *Videographer: Important step*
3.3.2. Talent opening assay kit, with exosome sample visible in frame *Videographer: Important/difficult step* **TEXT: *e.g.*, 4 micrograms protein/mL cell culture supernatant**

3.4. Then resuspend the exosome-enriched extracellular vesicles in PBS at a 6 micrograms/microliter concentration for storage at minus 80 degrees Celsius **[1]**.

3.4.1. PBS being added to sample, with PBS container visible in frame

4. Exosome-Enriched EV Transmission Electron Microscopy (TEM) Characterization

4.1. To visualize the exosome-enriched extracellular vesicles by transmission electron microscopy, fix 3-5 micrograms/milliliter of the extracellular vesicles **[1]** with a final concentration of 2% electron microscope-grade paraformaldehyde at room temperature for 2 hours **[2]**.

4.1.1. WIDE: Talent adding PFA to tube, with PFA container visible in frame

4.1.2. Talent setting timer, with tube and PFA container visible in frame

4.2. At the end of the incubation, load 10 microliters of the fixed samples onto copper grids with carbon support film for 1 minute **[1]** before draining the grids with filter paper **[2]**.

4.2.1. Sample(s) being loaded onto grid(s) *Videographer: Important step*
4.2.2. Grid(s) being drained *Videographer: Important step*

4.3. Stain the grids with an appropriate staining solution according to the manufacturer's protocol **[1]** and use tweezers to transfer the grids to a piece of filter paper **[2]**.

4.3.1. Stain being added to grid, with stain container visible in frame *Videographer: Important step*
4.3.2. Grid(s) being placed onto paper *Videographer: Important step*

4.4. Then use a transmission electron microscope with a 50,000x magnification to acquire electron microscopy images according to the standard protocols **[1]**.

4.4.1. LAB MEDIA: Figure 8-4-1_EM_Figure3.tif

5. Whole Cell Extract (WCE) and EV Lysate Preparation

5.1. To prepare whole cell extracts, collect ES-D3 cells from culture as demonstrated **[1]** and resuspend the harvested cells in PBS for counting **[2]**.

5.1.1. WIDE: Talent rinsing plate with trypsin, with trypsin container visible in frame
5.1.2. Talent adding PBS to tube, with PBS container and hemocytometer visible in frame

5.2. After a second centrifugation **[1-TXT]**, resuspend the cells at a 5 x 10^3 cells/microliter of SDS-PAGE (S-D-S-page) loading buffer containing 0.5% SDS concentration **[2-TXT]** and sonicate the samples for 10 seconds on a sonicator with a 10% amplitude **[3]**.

5.2.1. Talent adding tube(s) to centrifuge **TEXT: 5 min, 390 x g, RT**
5.2.2. Shot of pellet if visible, then pellet being resuspended, with SDS-PAGE buffer container visible in frame **TEXT: SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis**
5.2.3. Sample being sonicated

5.3. Then heat the samples at 100 degrees Celsius for 5 minutes **[1]**.

5.3.1. Talent placing sample(s) at 100 °C

5.4. To prepare lysates from the exosome-enriched extracellular vesicles, resuspend the exosome-enriched extracellular vesicles in SDS-PAGE loading buffer containing 0.5% SDS at a 1.2 micrograms/microliter concentration **[1]** and sonicate the samples for 10 seconds as demonstrated **[2]**.

5.4.1. Talent adding buffer to tube, with buffer container visible in frame

5.4.2. Talent sonicating sample(s)

5.5. Then heat the samples at 100 degrees Celsius for 5 minutes **[1]**.

5.5.1. Talent placing sample(s) at 100 °C

6. Western Blot Analysis

6.1. For western blot analysis, load 10-microliter whole cell extract **[1]** and exosome-enriched extracellular vesicle lysate samples into individual wells of a Bis-Tris (biss-triss) PAGE gel **[2]**.

6.1.1. WIDE: Talent loading sample into well(s), with WCE sample container visible in frame
6.1.2. EV lysate being added to well(s), with EV lysate container visible in frame

6.2. At the end of the run, transfer the proteins onto PVDF (P-V-D-F) membranes **[1-TXT]** and incubate the membranes with the appropriate primary and secondary antibodies of interest **[2-TXT]**.

6.2.1. Talent placing membrane onto gel **TEXT: PVDF: polyvinylidene fluoride**
6.2.2. Antibody being added to membrane, with antibody containers visible in frame **TEXT: See text for Ab suggestion/dilution details**

6.3. Then detect the protein expression using an enhanced chemiluminescence detection kit according to the manufacturer's instructions **[1]**.

6.3.1. Talent adding reagent to membrane, with kit visible in frame

7. Enzyme-Linked Immunosorbent Assay (ELISA)

7.1. To evaluate the amount of GM-CSF within exosome-enriched extracellular vesicles, use a kit for murine GM-CSF **[1-TXT]** to coat an ELISA (eliza) plate with anti-GM-CSF capture antibody **[2]**.

7.1.1. WIDE: Talent opening kit **TEXT: GM-CSF: granulocyte-macrophage-colony stimulating factor**
7.1.2. Talent adding antibody to well(s), with antibody container visible in frame

7.2. Next, treat 0.6 micrograms of exosome-enriched extracellular vesicle samples with 100 microliters of PBS alone **[1]** or PBS plus 0.05% Tween-20 at room temperature for 30 minutes **[2]**.

7.2.1. Talent adding PBS to sample, with PBS container visible in frame
7.2.2. Talent adding PBS + Tween-20 to sample, with Tween-20 container visible in frame

7.3. At the end of the incubation, add the treated samples to individual wells of the prepared ELISA plate for a 1-hour incubation at room temperature **[1]** followed by washing of the appropriate corresponding wells with PBS alone or PBS plus 0.05% Tween-20 **[2]**.

7.3.1. Sample(s) being added to well(s), with sample containers visible in frame
7.3.2. Well(s) being washes, with PBS and PBS + Tween-20 containers visible in frame

7.4. After the wash, add the detection antibody to the samples for a 1-hour incubation at room temperature **[1]** followed by a wash with PBS alone or PBS + 0.05% Tween-20 as demonstrated **[2]**.

7.4.1. Talent adding detection antibody to plate, with detection antibody container visible in frame
7.4.2. Talent adding PBS or PBS + Tween-20 to well(s), with both containers visible in frame

7.5. Then add avidin-horse radish peroxidase to the samples for a 30-minute incubation at room temperature **[1]** followed by a wash and measuring the absorbance in each well on a microplate reader at 450 nanometers **[2]**.

7.5.1. Avidin-HRP being added to plate, with container visible in frame
7.5.2. Talent placing plate onto plate reader

Section – Results

8. Results: Representative GM-CSF Expression Analyses

8.1. The GFP fluorescence intensity of a GM-CSF-expressing ES-D3 cell line **[1]** or an ES-D3 cell line expressing an empty vector **[2]** is much higher than that of their parental counterparts **[2]**.

8.1.1. LAB MEDIA: Figure 1B: *Video Editor please emphasize GM-CSF data line*
8.1.2. LAB MEDIA: Figure 1B: *Video Editor please emphasize vector data line*
8.1.3. LAB MEDIA: Figure 1B: *Video Editor please emphasize parental data line*

8.2. ELISA reveals that ES-D3 cells expressing GM-CSF produce markedly higher levels of GM-CSF in their cell culture supernatant **[1]** than do empty vector control cells **[2]**.

8.2.1. LAB MEDIA: Figure 2: *Video Editor please emphasize ES-D3-GM-CSF data bar*
8.2.2. LAB MEDIA: Figure 2: *Video Editor please emphasize ES-D3-vector data bar*

8.3. Furthermore, the amount of GM-CSF generated by GM-CSF-expressing ES-D3 cells is similar to that produced by STO (S-T-O) fibroblasts expressing GM-CSF **[1-TXT]**.

8.3.1. LAB MEDIA: Figure 2: *Video Editor please emphasize STO-GM-CSF data bar* **TEXT: STO: (SIM)-derived 6-thioguanine- and ouabain-resistant**

8.4. Transmission electron microscopy of extracellular vesicles isolated from vector-transduced **[1]** and GM-CSF-transduced cell cultures reveal vesicles of different sizes that fall within the expected 30-100-nanometer-diameter range **[2]**.

8.4.1. LAB MEDIA: Figure 8-4-1_EM_Figure3.tif: *Video Editor please emphasize vesicles indicated by arrows in original Figure 3 vector image*
8.4.2. LAB MEDIA: Figure 3: *Video Editor please emphasize vesicles indicated by arrows in original Figure 3 GM-CSF image*

8.5. The expression of exosomal markers, including CD81 (C-D-eighty-one), annexin five, and Flotillin-1, is markedly enhanced in extracellular vesicles isolated from ES-D3 cells **[1]** compared to corresponding whole cell extracts by western blot analysis **[2]**.

8.5.1. LAB MEDIA: Figure 4 top blot: *Video Editor please emphasize Flotillin-1, CD81, and Annexin V bands in exosome GM-CSF lane*
8.5.2. LAB MEDIA: Figure 4 top blot: *Video Editor please emphasize (lack of bands in) WCE GM-CSF lane*

8.6. Importantly, the presence of other subcellular compartment markers in ES-D3-derived extracellular vesicles was not detected **[1]**, including the endoplasmic reticulum marker protein disulfide isomerase **[2]**, the mitochondrial markers cytochrome c and Oxphos Complex four-subunit four **[3]**, and the cytosolic marker GAPDH (gap-D-H) **[4]**.

8.6.1. LAB MEDIA: Figure 4 bottom blot

8.6.2. LAB MEDIA: Figure 4 bottom blot: *Video Editor please emphasize lack of band in exosome GM-CSF PDI row*

8.6.3. LAB MEDIA: Figure 4 bottom blot: *Video Editor please emphasize lack of band in exosome GM-CSF cytochrome c and Oxphos Complex IV-subunit IV rows*

8.6.4. LAB MEDIA: Figure 4 bottom blot: *Video Editor please emphasize lack of band in exosome GM-CSF GAPDH row*

8.7. After washing with 0.05% Tween-20, the background GM-CSF levels detected in the control extracellular vesicles were significantly reduced **[1]**.

8.7.1. LAB MEDIA: Figure 5: *Video Editor please emphasize lack of white vector data bar*

8.8. In contrast, GM-CSF levels in the extracellular vesicles of GM-CSF-expressing cells were significantly increased by Tween-20 **[1]**.

8.8.1. LAB MEDIA: Figure 5: *Video Editor please emphasize white vector GM-CSF bar*

Section - Conclusion

9. Conclusion Interview Statements: (Said by you on camera) - All interview statements may be edited for length and clarity.

9.1. **Shuhan Meng**: (Step: 3.1.-3.3.) Acquiring high-quality, exosome-enriched extracellular vesicles carrying GM-CSF is the most important step for the success of this protocol **[1]**.

9.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.1.-3.3.*

9.2. **Shuhan Meng**: Researchers can perform additional experiments, such as studies in GM-CSF-dependent cell lines and animals, to determine whether exosome-enriched extracellular vesicles carrying GM-CSF can function as cell-free immune regulatory vesicles **[1]**.

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

9.3. **Aaron Whitt**: These exosome-enriched extracellular vesicles can then be used to explore how modulation of the immune response effects different disease conditions **[1]**.

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