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Title: Neuronal Differentiation from Mouse Embryonic Stem Cells In Vitro

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**
3. **Filming location:** Will the filming need to take place in multiple locations? **No**

   If **Yes**, how far apart are the locations?

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Shasha Zhao:** Neural differentiation from mESCs is an excellent model for elucidating the key mechanisms involved in neurogenesis. Here, we demonstrate an optimized method for embryonic neurogenesis using a "combinatorial screening" strategy.

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

1.2. **Xiang Mao:** This technique has the advantages of high efficiency, low-cost, and easy-operation, and is suitable for popularization among laboratories. It is a powerful tool for neuroscience research.

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

Protocol

2. Differentiation from mESCs to NPCs (Phase I)

2.1. To perform differentiation using Protocol 1, seed 20,000 mouse embryonic stem cells **[1]** in 2 milliliters of basal differentiation medium into each well of a 0.1% gelatin-coated 6-well plate **[2]**.

2.1.1. WIDE: Establishing shot of talent adding basal differentiation medium to a plate.

2.1.2. Talent seeding cells into the wells of a 6-well plate. *Videographer: Obtain multiple usable takes of this shot because it will be reused in 2.8.1 and 2.13.1.*

2.2. After cell attachment, wash the cells with 2 milliliters of PBS **[1]** and add 2 milliliters of basal differentiation medium to each well **[2]**, then put the cells back in the incubator **[3-TXT]**. Allow the cells to differentiate for 8 days, replacing the medium every 2 days **[4]**.

2.2.1. Talent adding PBS to a few wells, with the PBS container in the shot.

2.2.2. Talent adding medium to a few wells, with the medium container in the shot.

2.2.3. Talent putting the plate in the incubator and closing the door. **TEXT: 37 °C, 5% CO_2** *Videographer: Obtain multiple usable takes of this shot because it will be reused in 2.6.1, 2.8.3, 2.12.3, and 2.13.3.*

2.2.4. Talent replacing the medium in the plate.

2.3. To perform protocol 2, add 1.5 million mouse embryonic stem cells into a non-adhesive bacterial dish in 10 milliliters of basal differentiation medium **[1]** and incubate the plate at 37 degrees Celsius and 5% carbon dioxide to allow for embryoid body formation **[2]**. *Videographer: This step is important!*

2.3.1. Talent adding cells to a dish. *Videographer: Obtain multiple usable takes of this shot because it will be reused in 2.10.1.*

2.3.2. Talent putting the dish in the incubator and closing the door.

2.4. After 2 days, transfer the cell aggregates into 15-milliliter centrifuge tubes and let them settle by gravity **[1]**. Remove the supernatant **[2]** and add 10 milliliters of fresh basal differentiation medium to resuspend the embryoid bodies **[3]**, then replant them into a new non-adhesive dish and incubate them for another 2 days **[4]**. *Videographer: This step is important!*

2.4.1. Talent transferring cells into a tube and leaving it to sit.

2.4.2. Talent removing the supernatant from the tube.

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

2.4.4. Talent plating the cells into a dish.

2.5. Collect and seed about 50 embryoid bodies in 2 milliliters of basal differentiation medium per well onto gelatin-coated 6-well plates **[1]**. For retinoic acid induction, add 2 microliters of RA stock into each well to a final concentration of 1 micromolar **[2-TXT]**. *Videographer: This step is important!*

2.5.1. Talent seeding the embryonic bodies into a few wells.

2.5.2. Talent adding RA to a few wells. *Videographer: Obtain multiple usable takes of this shot because it will be reused in 2.8.2 and 2.9.2.* **TEXT: RA = retinoic acid**

2.6. Return the plate to the incubator and differentiate the cells for another 4 days. To perform differentiation using protocol 3 **[1]**, repeat protocol 2 but allow only 2 days for embryonic body formation and 6 days for RA induction **[2]**. *Videographer: This step is difficult and important!*

2.6.1. *Use 2.2.3.*

2.6.2. Plate with cells in the incubator.

2.7. **Shasha Zhao**: Quality control of embryoid bodies formation should be carried out using microscopy. Only those with bright cores can differentiate successfully in the subsequent process.

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

2.8. For protocol 4, seed 20,000 mouse embryonic stem cells per well into the gelatin-coated plates and follow protocol 1 **[1]**. After allowing the cells to differentiate for 4 days, add 2 microliters of all-trans RA stock into each well **[2]** and incubate the plate for another 4 days **[3]**.

2.8.1. *Use 2.1.2.*

2.8.2. *Use 2.5.2.*

2.8.3. *Use 2.2.3.*

2.9. To perform protocol 5, repeat protocol 4 **[1]** but begin RA induction after only 2 days of differentiation **[2]**.

2.9.1. Talent taking the plate of cells out of the incubator.

2.9.2. *Use 2.5.2.*

2.10. For protocol 6, plant 1.5 million stem cells into a non-adhesive bacterial dish in 10 milliliters of N2B27 *(pronounce 'N-2-B-27')* medium-2 **[1]** and incubate the plate at 37 degrees Celsius and 5% carbon dioxide to allow for embryoid body formation **[2]**.

2.10.1. *Use 2.3.1.*

2.10.2. Talent placing the dish in the incubator and closing the door.

2.11. After 2 days, collect cell aggregates as previously described **[1]** and resuspend the embryoid bodies with 10 milliliters of fresh N2B27 medium-2 **[2]**. Replant them into a new non-adhesive bacterial dish and allow differentiation for another 2 days **[3]**.

2.11.1. Tube with cells aggregating at the bottom.

2.11.2. Talent resuspending the cells, with the N2B27 medium II container in the shot.

2.11.3. Talent transferring the cells into a dish.

2.12. On the fourth day, collect and seed about 50 embryoid bodies per well onto gelatin-coated 6-well plates with 2 milliliters of N2B27 medium-2 **[1]**. Add 2 microliters of all-trans RA stock into each well **[2]** and induce differentiation for another 4 days **[3]**.

2.12.1. Talent seeding embryoid bodies into wells.

2.12.2. Talent adding RA stock to a few wells.

2.12.3. *Use 2.2.3.*

2.13. To perform protocol 7, seed about 20,000 cells in 2 milliliters of basal differentiation medium per well onto the gelatin-coated plates **[1]**. After cell attachment and 2 washes with PBS, add 2 milliliters of N2B27 medium-2 to each well **[2]** and allow the cells to differentiate for 8 days at 37 degrees Celsius and 5% carbon dioxide **[3]**.

2.13.1. *Use 2.1.2.*

2.13.2. Talent adding N2B27 medium II to a few wells, with the medium container in the shot.

2.13.3. *Use 2.2.3.*

3. Differentiation from NPCs to neurons (Phase II)

3.1. After performing Phase 1 differentiation using Protocol 3, seed about 500,000 mouse embryonic stem cell derivatives in 2 milliliters of basal differentiation medium per well onto the 0.1% gelatin-coated plates **[1]**. Randomly divide the derivatives into 3 groups in order to test three Phase 2 differentiation protocols **[2]**. *Videographer: This step is important!*

3.1.1. Talent seeding cells into wells.

3.1.2. Three plates with cells, each labeled with Phase II-Protocol 1, 2, or 3.

3.2. Incubate the plate for 6 hours **[1-TXT]**, then wash the cells twice with 2 milliliters of PBS **[2]** and add 2 milliliters of basal differentiation medium, N2B27 medium-1, or N2B27 medium-2, to each well, depending on the protocol **[3]**. *Videographer: This step is important!*

3.2.1. Talent putting the plates in the incubator and closing the door. **TEXT: 37 °C, 5% CO_2**

3.2.2. Talent washing wells with PBS.

3.2.3. Talent adding medium to wells on a plate, with all 3 medium containers in the shot.

3.3. Place the plates into the incubator and allow the cells to differentiate for another 10 days, changing the corresponding medium every 2 days **[1]**.

3.3.1. Plates in the incubator.

Results

4. Results: Optimal Protocols for Phase I and Phase II Differentiation

4.1. Seven protocols were tested to determine the optimal protocol for the differentiation of mouse embryonic stem cells into neural precursor cells, or NPCs **[1]**. The protocols were tested on both A2lox *(pronounce 'A-2-lox')* and 129 cells and the differentiation status of each group was monitored using a light microscope **[2]**.

4.1.1. LAB MEDIA: Table 1.

4.1.2. LAB MEDIA: Figure 3 A.

4.2. Most A2lox and 129 derivatives showed well-stacked and neurite-like morphologies under protocol 3, indicating the formation of NPCs **[1]**. However, cells differentiated using protocol 2 appeared apoptotic, which may be due to the lack of nutrients within the embryoid bodies **[2]**.

4.2.1. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize the protocol 3 images.*

4.2.2. LAB MEDIA: Figure 3 A. *Video Editor: Emphasize the protocol 2 images.*

4.3. To further confirm the formation of NPCs, Nestin-positive cells were detected using an immunofluorescence assay **[1]**. Protocol 3 resulted in the highest percentage of Nestin-positive cells **[2]**, reaching up to 78 and 69% in A2lox and 129 derivatives, respectively **[3]**.

4.3.1. LAB MEDIA: Figure 3 B.

4.3.2. LAB MEDIA: Figure 3 B. *Video Editor: Emphasize the protocol 3 images.*

4.3.3. LAB MEDIA: Figure 3 B, just the bar graphs. *Video Editor: Zoom in on the bar graphs and emphasize the protocol 3 bars in both graphs.*

4.4. Three protocols were tested for the differentiation of NPCs into neurons **[1]**. It was determined that protocol 3 most effectively induces the differentiation. Most A2lox and 129 derivatives in phase 2-protocol 3 had prolonged neuron-like structures with clear neurites and cell body extensions **[2]**.

4.4.1. LAB MEDIA: Figure 4 A.

4.4.2. LAB MEDIA: Figure 4 A. *Video Editor: Emphasize the protocol 3 images.*

4.5. Immunofluorescence assays further confirmed the generation of neurons **[1]**, with the percentage of Beta-Tubulin-3-positive cells up to 68 and 59% in A2lox and 129 derivatives, respectively, using protocol 3 **[2]**.

4.5.1. LAB MEDIA: Figure 4 B.

4.5.2. LAB MEDIA: Figure 4 B, just the bar graphs. *Video Editor: Zoom in on the bar graphs and emphasize the protocol 3 bars in both graphs.*

Conclusion

5. Conclusion Interview Statements

5.1. **Xiang Mao:** When attempting this procedure, it is important to ensure that mESCs are healthy and undifferentiated before differentiation. In addition, strict quality control should be carried out after the formation of embryoid bodies and before phase II differentiation.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.6.2, 3.1.1.*

5.2. **Shasha Zhao:** This technique provides an excellent model for researchers of neurobiology and developmental biology and is expected to be a powerful tool for elucidating the key mechanisms involved in neurogenesis.

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