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Title: Hepatic Progenitor Specification from Pluripotent Stem Cells Using a Defined Differentiation System

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **yes**

    If **Yes**, can you record movies/images using your own microscope camera?

    Images already taken.

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, only different lab spaces within the building/location.**

    If **Yes**, how far apart are the locations? **Two different floors. Elevator available.**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Linda Ding:** This protocol provides a stem cell derived hepatic progenitor differentiation system that offers a reproducible tool to study liver biology for basic and clinical research. By combining a standardized and easy-to-follow protocol with off-the-shelf cell culture medium, this system can be used to produce hepatic progenitors at a large scale.

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

NOTE: Step 1.2. was not filmed separately. Instead, 1.1 and 1.2 and their corresponding VO text was combined into a single shot and was delivered by a **Linda Ding** instead of **Jose Meseguer-Ripolles.**

Protocol

2. Human Pluripotent Stem Cell Seeding

2.1. Maintain human pluripotent cells at 37 degrees Celsius and 5% carbon dioxide in a 10-cm dish on laminin-521 **[1]**, feeding them daily with 10 milliliters of stem cell maintenance medium per well **[2]**.

2.1.1. WIDE: Establishing shot of talent taking a stack of culture dishes out of the incubator.

2.1.2. Talent adding medium to the culture dishes.

2.2. To prepare laminin-521 plates, dilute thawed laminin-521 in ice-cold DPBS with calcium and magnesium to a final concentration of 8 micrograms per milliliter **[1]**. Add 250 microliters of the laminin solution to each well of a 24-well plate or 50 microliters to each well of a 96-well plate **[2]**. *Videographer: This step is important!*

2.2.1. Talent diluting laminin-521 in DPBS.

2.2.2. Talent adding laminin solution to a few wells of a 24-well plate.

2.3. Gently rock the plate from side to side to evenly coat the wells **[1]**, then seal the plates with a semitransparent, flexible film **[2]** and store them at 4 degrees Celsius overnight **[3]**. *Videographer: This step is important!*

2.3.1. Talent rocking the plate from side to side.

2.3.2. Talent sealing a plate.

2.3.3. Talent putting the plate in a refrigerator.

2.4. On the day of cell seeding, warm the precoated plates in a cell culture incubator for 30 to 60 minutes **[1-TXT]**. Aspirate the laminin-521 solution **[2]** and add stem cell maintenance medium supplemented with 10 micromolar ROCK inhibitor to each well **[3-TXT]**, then return the plate to the incubator **[4]**.

2.4.1. Talent putting plates in the incubator and closing the door. **TEXT: 37 °C** *Videographer: Obtain multiple usable takes of this shot because it will be reused in 2.4.4.*

2.4.2. Talent aspirating laminin from a few wells.

2.4.3. Talent adding medium to a few wells of a plate. **TEXT: 24 well plate: 0.5 mL per well ; 96 well plate 0.05 mL per well**

2.4.4. *Use 2.4.1.*

2.5. When cell confluency reaches 70 to 80%, aspirate the spent medium from the culture dish **[1]** and wash each culture dish with 5 milliliter of DPBS without calcium or magnesium at room temperature **[2]**.

2.5.1. Talent aspirating medium from a 10-cm dish.

2.5.2. Talent adding DPBS to the culture dish, with the DPBS container in the shot.

2.6. Aspirate the DPBS wash from the culture dish then add 5 milliliter of enzyme free dissociation reagent to the dish **[1]** and incubate the plate at 37 degrees Celsius for 8 to 10 minutes until cells visibly detach from the dish **[2]**.

2.6.1. Talent aspirating DPBS from a 10-cm dish and adding dissociation reagent.

2.6.2. Talent putting the plate in the incubator and closing the door.

2.7. Gently detach the cells from the culture dish with a cell scraper **[1]**, then pipette the contents of each well up and down with a 5-milliliter serological pipette to yield a single-cell suspension **[2]**. For each cell line, pool cells from all maintenance wells into a sterile 50 milliliter tube **[3]**. *Videographer: This step is important!*

2.7.1. Talent detaching cells with a cell scraper.

2.7.2. Talent pipetting the contents of a 10-cm dish up and down.

2.7.3. Talent transferring the cells into a 50-milliliter tube.

2.8. Wash each emptied culture dish with 5 milliliter of the stem cell maintenance medium **[1]** and add the washes to the corresponding tube with the pooled cells **[2]**. Perform 3 viable cell counts on each pooled sample **[3]**. *Videographer: This step is important!*

2.8.1. Talent adding medium to a 10-cm dish.

2.8.2. Talent adding the wash medium to the 50mL tube.

2.8.3. Talent counting cells using a nucleocounter.

2.9. Centrifuge the pooled samples at 250 *x g* for 5 minutes **[1]**, then aspirate the supernatant **[2]** and resuspend the cells in 1 to 3 milliliters of room temperature stem cell maintenance medium supplemented with 10 micromolar ROCK inhibitor Y27632 **[3]**. *Videographer: This step is important!*

2.9.1. Talent putting a 50mL tube with cells in the centrifuge and closing the lid.

2.9.2. Talent aspirating the supernatant.

2.9.3. Talent resuspending the cells.

2.10. After calculating the required number of cells needed to achieve the desired seeding density, resuspend the cells to the appropriate concentration **[1]** and dispense them into the pre-coated plates. The total volume per well should be 1 milliliter for a 24-well plate and 0.1 milliliter for a 96-well plate **[2]**. *Videographer: This step is difficult and important!*

2.10.1. Talent resuspending the cells in medium, with the medium container in the shot.

2.10.2. Talent adding cells to wells in the 24-well precoated plate.

2.11. Gently rock the plates from side to side and back and forth to ensure even cell dispersion **[1]** and place the seeded plates into the incubator, rocking them back and forth and from side to side **[2-TXT]**. *Videographer: This step is difficult and important!*

2.11.1. Talent rocking the plate.

2.11.2. Talent placing the plate in the incubator, while rocking them side to side and back and forth. **TEXT: 37 °C and 5% CO_2**

3. Differentiating hPSCs to Hepatic Progenitors on Laminin-521

3.1. Prepare media for definitive endoderm induction and for the subsequent hepatic progenitor cell specification differentiation as described in the text manuscript **[1]**. On day 1 of the differentiation, remove the spent medium from the wells **[2]** and replace it with Stage 1 Medium 1 **[3-TXT]**.

3.1.1. Containers with prepared media, all clearly labeled.

3.1.2. Talent removing media from a few wells.

3.1.3. Talent adding Stage 1 Medium 1 to wells, with the medium container in the shot and labeled. **TEXT: 24 well plate: 0.5 mL per well ; 96 well plate: 0.5 mL per well**

3.2. On days 2, 3, and 4, remove the spent medium **[1]** and feed each well with Stage 1 Medium 2 **[2-TXT]**. On day 5, fix the wells intended for definitive endoderm differentiation analysis **[3]**. For the remaining wells, remove the spent medium and feed each well with STEMdiff™ Hepatic Progenitor Medium **[4-TXT]**.

3.2.1. Talent removing the spent medium.

3.2.2. Talent adding Stage 1 Medium 2 to the wells, with the medium container in the shot and labeled. **TEXT: 24 well plate: 0.5 mL per well ; 96 well plate: 0.5 mL per well**

3.2.3. Talent removing the spent medium and adding fixative to the wells.

NOTE: The first part of shot 3.2.3. was added later.

3.2.4. Talent removing the spent medium and adding STEMdiff™ Hepatic Progenitor Medium to the wells, with the medium container in the shot and labeled. **TEXT: Refresh the medium again on days 6, 7, and 9**

NOTE: The first part of shot 3.2.4. was added later.

3.3. On day 10, harvest cells for hepatic progenitor differentiation analysis **[1]** or proceed with further hepatocyte-like cell differentiation **[2-TXT]**.

3.3.1. *Use 3.2.3.*

3.3.2. Added shot: Talent removing the spent medium and adding STEMdiff™ Hepatocyte Medium to the wells, with the medium container in the shot and labeled. **TEXT: Refresh the medium again on days 12, 14, 16, 18 and 20. Hepatocyte-like cells are ready for analysis on day 21**

3.4. To characterize the hepatic progenitor differentiation cultures, use immunostaining to detect expression of definitive endoderm-specific markers on day 5 and hepatic progenitor-specific markers on day 10 **[1]**. Measure alpha fetoprotein and albumin secretion via ELISA and quantify the percentage of HNF4-alpha positive cells **[2]**.

3.4.1. Talent at the microscope imaging cells.

3.4.2. Talent using the plate reader for ELISA.

Results

4. Results: Hepatic Progenitor Differentiation from hPSCs

4.1. This protocol was used to differentiate hepatic progenitor cells from both human embryonic stem cells **[1]** and human induced pluripotent stem cells **[2]**.

4.1.1. LAB MEDIA: Figure 2. *Video Editor: Emphasize the H9 images when VO says "human embryonic stem cells 'D0'".*

4.1.2. LAB MEDIA: Figure 2. *Video Editor: Emphasize the P106 images 'D0'.*

4.2. At day 5 of the differentiation protocol, definitive endoderm specification was assessed via Sox17 *(pronounce 'socks-17')* expression **[1]**. In both cell lines, Sox17 was highly expressed, with 80 and 87.8% of Sox17-positive cells for H9 and P106, respectively **[2]**.

4.2.1. LAB MEDIA: Figure 2 just the D10 images. *Video Editor: Get rid of everything else, but please keep the H9 and P106 labels*

4.2.2. LAB MEDIA: Figure 3. *Video Editor: Emphasize the H9 and P106 images.*

4.3. At day 10, hepatic progenitors displayed a cobblestone-like morphology **[1]**. In addition, hepatic progenitor specification was assessed for HNF4-alpha, AFP, ALB, and cytokeratin-19 expression **[2]**. Both hepatic progenitor cultures expressed fetal hepatic markers such as HNF4-alpha *(pronounce 'H-N-F-4-alpha')*, AFP, and CK19 **[3]**.

4.3.1. LAB MEDIA: Figure 2, just the D10 images. *Video Editor: Get rid of everything else, but please keep the H9 and P106 labels.*

4.3.2. LAB MEDIA: Figure 4.

4.3.3. LAB MEDIA: Figure 4. *Video Editor: Emphasize A, B, and D.*

4.4. Alpha fetoprotein secretion was detected at day 10 in both cell lines **[1]**, while albumin synthesis was observed at lower levels and was not detected with ELISA **[2]**.

4.4.1. LAB MEDIA: Figure 5. *Video Editor: Emphasize the alpha fetoprotein graph.*

4.4.2. LAB MEDIA: Figure 5. *Video Editor: Emphasize the albumin graph.*

4.5. Cell number variability and hepatic progenitor differentiation efficiency was assessed by quantifying HNF4-alpha expression **[1]**. At day 10, hepatic progenitors showed no significant variability across rows with over 94 and 97% of HNF4-alpha-positive cells per well for H9 and P106, respectively **[2]**.

4.5.1. Figure 6 A and C.

4.5.2. Figure 6 B and D.

Conclusion

5. Conclusion Interview Statements

5.1. **Linda Ding:** An even cell distribution prior to the start of the differentiation is key to ensure a homogenous population of hepatic progenitor cells. For this, gently rock the plates from side to side and back and forth to ensure even cell dispersion.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.11.1 - 2.11.2.*

5.2. **Linda Ding:** Hepatic progenitor cells produced by this protocol can be further differentiated to hepatocyte like cells for other assays, offering a reproducible and standardized tool for disease modelling or drug screening.

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

NOTE: Both statements 5.1 and 5.2 were delivered by **Linda Ding** and not **Jose Meseguer-Ripolles** and **Yu Wang.**