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Title: A Culture Method to Maintain Quiescent Human Hematopoietic Stem 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? **No.**

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**.

   ☒ Interview Statements are read by JoVE's voiceover talent.

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

Current Protocol Length

Number of Steps: 10
Number of Shots: 21

Introduction

1. Introductory Interview Statements

NOTE to VO talent: Please record the introduction and conclusion statements

1.1. Cell cycle quiescence is a key feature of hematopoietic stem cells. This protocol helps to understand the behavior of quiescent human HSCs in vitro under near-physiological conditions.

1.1.1. *4.2.1.*

1.2. Using this protocol, researchers can test the effect of various compounds, nutrients, or proteins that regulate the cell cycle status as well as differentiation of HSCs in a scalable manner without using animal models.

1.2.1. *3.4.3.*

1.3. Anti-cancer drugs have varying effects on the survival of quiescent HSCs and cycling progenitors. This protocol makes it possible to find agents that spare quiescent HSCs or agents that selectively damage quiescent leukemic stem cells.

1.3.1. *4.1.3.*

Introduction of Demonstrator on Camera

NOTE to VO talent: Please record the introduction and conclusion statements

1.4. Demonstrating the procedure will be Hiroshi Kobayashi, a Senior Research Fellow from the Takubo laboratory.

1.4.1. INTERVIEW: Author saying the above.

1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Ethics Title Card

1.5. Procedures involving animal subjects have been approved by the National Center for Global Health and Medicine animal experiment committee.

Protocol

2. Lipid Preparation

2.1. To begin, dissolve 16 milligrams per milliliter sodium palmitate, 30 milligrams per milliliter sodium oleate, and 4 milligrams per milliliter cholesterol in methanol in glass tubes **[1]**. Store the lipid solutions at -30 degrees Celsius and thaw them before use **[2]**.

2.1.1. WIDE: Establishing shot of talent adding lipids to methanol.

2.1.2. Talent placing the lipid solutions in the refrigerator.

2.2. In a fresh glass tube, mix the lipid solutions to obtain the final concentration of 100 micrograms per milliliter palmitate, 100 micrograms per milliliter oleate, and 20 micrograms per milliliter cholesterol **[1]**. Evaporate the methanol by passing nitrogen gas through the lipid solution **[2]**.

2.2.1. Talent mixing the lipid solutions.

2.2.2. Talent passing nitrogen through the lipid solutions.

2.3. Completely evaporate the remaining methanol by heating the glass tube in a water bath at 37 degrees Celsius **[1]**. *Videographer: This step is important!*

2.3.1. Talent placing the glass tube in a water bath.

3. Media Preparation

3.1. Prepare DMEM-F12 medium with HEPES and glutamine **[1]**. Add penicillin and streptomycin sulfate for a final concentration of 50 units and 50 micrograms per milliliter, respectively **[2-TXT]**. The medium can be stored at 4 degrees Celsius for at least 2 months **[3]**. NOTE: Step 3.1.1. and Step 3.1.2. were filmed in the same shot.

3.1.1. Prepared DMEM/F12.

3.1.2. Talent adding antibiotics to the medium. **TEXT: Store at 4 °C for at least 2 months**

3.1.3. Talent putting the medium in the refrigerator.

3.2. Add 4% of BSA to DMEM-F12 medium with HEPES and glutamine **[1]**, then adjust the pH of the medium to 7.6 using sodium hydroxide solution **[2]**. Add the medium to the glass tube with the lipids **[3]**. *Videographer: This step is important!*

3.2.1. Talent adding BSA to the medium.

3.2.2. Talent adding NaOH to the medium.

3.2.3. Talent adding the medium to the lipid mix.

3.3. Completely dissolve the lipids by sonication. If the medium is opaque after sonication, extend the sonication time **[1]**. When the BSA and lipids are dissolved, the sample should be stored at -80 degrees Celsius and used within 2 months **[2-TXT]**. *Videographer: This step is difficult and important!*

3.3.1. Talent sonicating the lipids.

3.3.2. Talent putting the medium into the freezer. **TEXT: Thaw immediately before use**

3.4. Add 0.001X of insulin, transferrin, sodium selenite, and ethanol amine mixture to the DMEM-F12 **[1]**, then filter the mixed medium using a 0.22-micrometer filter **[2]**. Before use, add human stem cell factor, or SCF, and human thrombopoietin, or TPO, to the culture media at a final concentration of 3 nanograms per milliliter each **[3]**.

3.4.1. Talent adding the ITS-X mixture to the medium.

3.4.2. Talent filtering the medium.

3.4.3. Talent adding the SCF and TPO to the medium.

4. Cell culture

4.1. Transfer 200 microliters of the previously prepared culture media with cytokines to flat-bottom 96-well plates **[1]**. To avoid evaporation of the medium, fill all unused wells with 100 to 200 microliters of PBS **[2]**. Resuspend the sorted hematopoietic stem cells, or HSCs, in culture media without cytokines at 60 cells per microliter **[3]**.

4.1.1. Talent adding media to wells, with the media container in the shot.

4.1.2. Talent adding PBS to wells, with the PBS container in the shot.

4.1.3. Talent resuspending the cells.

4.2. Aliquot 600 cells into each well. Fewer than 300 cells will lead to larger technical variation and culturing more than 1000 cells in a single well should be avoided because of nutrient deprivation or accumulation of unfavorable cytokines or chemokines **[1]**. *Videographer: This step is important!*

4.2.1. Talent adding cells to a few wells.

4.3. Culture the cells in a humidified multi-gas incubator at 37 degrees Celsius in a 5% carbon dioxide and 1% oxygen atmosphere **[1]**.

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

Results

5. Results: Cell numbers after 7 days of culturing

5.1. After 7 days of culturing the purified HSCs up to 80% of cells displayed marker CD34-positive and CD38-negative phenotypes **[1]**. The total cell number depended on the cytokine concentration **[2]**. Higher concentrations of SCF and TPO induced entry into the cell cycle, proliferation, and differentiation **[3]**.

5.1.1. LAB MEDIA: Figure 4 A.

5.1.2. LAB MEDIA: Figure 4 B. *Video Editor: Emphasize the Total Cell plot.*

5.1.3. LAB MEDIA: Figure 4 B.

5.2. The number of phenotypic HSCs characterized by the marker CD34-positive, CD38-negative, CD90 positive, and CD45RA-negatice phenotypes increased in proportion to the SCF or TPO concentrations **[1]**, whereas the frequency among the total cells decreased **[2]**.

5.2.1. LAB MEDIA: Figure 4 B. *Video Editor: Emphasize the $CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$ plot.*

5.2.2. LAB MEDIA: Figure 4 C.

5.3. Following 3 months of transplantation of cultured adult bone marrow HSCs, reconstitution can be evaluated as a function of their frequency in the peripheral blood of human CD45-positive murine CD45-negative Ter119-negative cells **[1]**.

5.3.1. LAB MEDIA: Figure 5.

5.4. Three lineages, including CD19-positive B cells, CD13-negative-CD33-positive myeloid cells, and CD3-positive T cells were reconstituted in NOG *(spell out 'N-O-G')* mice transplanted with either freshly thawed or cultured HSCs **[1]**.

5.4.1. LAB MEDIA: Figure 5.

Conclusion

6. Conclusion Interview Statements

NOTE to VO talent: Please record the introduction and conclusion statements

6.1. Following culture, HSCs can be subjected to gene expression profiling such as real-time PCR and RNA-sequencing. Functional validation using transplantation into immune-deficient mice can also be performed.

6.1.1. *4.3.1.*

6.2. Researchers can directly compare cycling and quiescent HSCs under defined conditions by adjusting cytokine concentrations. This will help to understand the quiescent-HSC-specific self-renewal programs, stress-resistance mechanisms, and metabolic properties, which are hard to test in vivo settings.

6.2.1. *4.1.1.*