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Generation and Culture of Mouse Embryonic Fibroblasts

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i. Summary

The innate immune system is evolutionarily conserved and shared by a wide spectrum of cells, including epithelial cells, endothelial cells, fibroblasts, macrophages, dendritic cells and lymphocytes. The extensive utilization of genetically manipulated animals in innate immunity studies has become the standard approach to confirm functional data acquired in cell lines. The easy generation and physiological relevance of mouse embryonic fibroblasts (MEFs) have made them a powerful tool in discovering novel signaling pathways, investigating regulatory networks, and exploring biochemical profiling of protein complexes involved in innate immune responses. Due to their extensive use, this chapter aims to provide a protocol for generating, maintaining and storing primary MEFs for those who have minimal experience in animal models. Precautions and notes are integrated into the description of each step of the protocol for the benefits of minimizing unnecessary cross-referencing.

Keywords

Innate immunity; mouse embryonic fibroblasts; MEF

1. Introduction

Recent advances in immunology have heavily relied on the generation and exploration of gene deletion or transgenic mouse models. The extensive utilization of these genetically manipulated animals has become the standard approach to confirm the functional data acquired in cell lines. Innate immunity studies witnessed exponential development in recent years thanks to the discoveries of novel families of pathogen-associated molecular patterns receptors, immune adaptor molecules, intricate regulatory networks of innate immune responses, and novel co-stimulatory/co-inhibitory pathways [1–3]. In contrast to the involvement of highly specialized antigen presenting cells and lymphocytes in the adaptive immune system, innate immunity is an evolutionarily conserved system shared by a wide spectrum of cells, including but not limited to epithelial cells, endothelial cells, fibroblasts,

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macrophages, dendritic cells and lymphocytes. Importantly, due to the anatomic features of mammalian tissues, epithelial cells and fibroblasts often constitute the primary physical barrier of the host against a variety of microbial or environmental insults. Hence, these cells are equipped with cytoplasmic sensors and the necessary innate immune signaling pathways to be readily engaged with host-pathogen interactions. Given the functional relevance and easy generation of mouse embryonic fibroblasts (MEFs), these cells are extensively used in biochemical identification and functional characterization of novel players in the innate immune system. In fact, some emerging families of these sensors are extensively studied in MEFs [4–9]. This chapter will provide a standard protocol for the generation and culture of MEFs. Of special note, the protocol involving the generation and the use of MEFs must be reviewed and approved by the appropriate regulatory bodies at each institution (i.e. the Institutional Animal Care and Use Committee (IACUC)). Likewise, the breeding and care for mice must also follow all official guidelines.

2. Materials

2.1. Mice.

1. At least 2 breeding cages of mice for each genotype need to be tested. Each breeding cage should include 1 male and 2 female mice, 6 to 12 weeks of age (*see* Note 1).

2.2. Reagents.

1. 70% (v/v) ethanol
2. Phosphate-buffered saline (1X PBS), pH 7.4
2. Dulbecco's Modified Eagle Medium (DMEM)
3. Fetal bovine serum (FBS)
4. 100 × Penicillin-streptomycin (10,000 units of penicillin and 10,000µg of streptomycin/ml)
5. 0.25% Trypsin-EDTA solution
6. Freezing medium: 10% Dimethyl sulfoxide (DMSO), 20% FBS, 70% DMEM high glucose, and 1% Penicillin-Streptomycin (*see* Note 2).

2.3. Supplies and Equipment.

1. 60-mm and 100-mm tissue culture dishes
2. 15ml and 50ml conical tubes
3. 2ml screw cap cryovials.
4. Laminar flow hood (*see* Note 3)
5. Sterile 40µm cell strainers
6. Surgical instruments: Dissecting forceps, scissors and single-edge razor/scalpel blades (*see* Note 4)

7. Examination gloves
8. Freezing containers
9. Inverted microscope
10. 37°C tissue culture incubator with 5% CO₂

3. Methods

3.1. Establish timed breeding cages(see Note 5)

1. Place 1 male mouse with 2 female mice.
2. Examine the female mice for the presence of copulation plugs each morning until the plug is identified.
3. Once the plug is identified, transfer the female mice to a new cage and note the time of embryo development as 0.5 days.
4. Allow the embryos to develop for 13.5 – 15.5 days. The most widely accepted ages for the embryos used in MEF generation are between 13.5 and 15.5 days.

3.2. Pre-surgical preparations.

1. Pregnant mice should be euthanized as specified in an approved animal protocol. The most widely used methods include the use of carbon dioxide, isoflurane overdose or avertin overdose. The primary method should be followed by a secondary physical method, such as cervical dislocation.
2. Spray the entire mouse with 70% ethanol.
3. The dissection should be performed in a laminar flow hood (*see* Note 6).
4. In a clean area under the hood, label PBS-filled 100-mm tissue culture plates with the genotype of each mouse and a unique number to identify each embryo (*see* Note 7).

3.3. Dissect the embryos.

1. Lift the abdominal wall with blunt forceps and make a shallow 3-mm transverse incision. Next, make a longitudinal incision to expose the abdominal wall. At this point, the uterus with embryos should be easily visible. Take all precautions to keep the surgical site uncontaminated. If the surgical instruments are contaminated by hair, fully rinse the instrument in 70% ethanol or use a new set of instruments.
2. Expose the embryos by opening the abdominal wall. Take special precautions to avoid damaging the internal organs in the peritoneal cavity, especially the small intestine and colon. Damage to these organs increases the risk of contaminating the embryos with bacteria.
3. Carefully transfer each embryo, with intact yolk sac, to the tissue culture plates prepared in Protocol 3.2.4.

3.4. Prepare the fetuses.

1. Tease apart the yolk sac with a pair of fine forceps and discard the yolk sac.
2. Carefully remove the fetus head and keep it in a separate PBS-filled conical tube labeled with its genotype and identifying number. This identification should match the respective 100-mm tissue culture plate. The fetal remains that are not used for MEF generation, including the head, are used for genotyping.
3. Carefully remove the heart and liver, which are both dark red tissues, and discard.
4. Wash each prepared fetus in PBS and transfer it to a new 60-mm tissue culture dish that has been identically labeled as the original plate.

3.5. Prepare the MEF suspension.

1. Add 3ml of pre-chilled 0.25% trypsin-EDTA to each dish.
2. Mince the fetus tissue thoroughly with two razor or scalpel blades. This step is critical for enhancing the final yield of MEFs. It is important that this step be thoroughly conducted and not rushed.
3. Gently pipet all of the tissue fragment suspensions up and down 10 times.
4. Incubate each tissue culture dish at 37°C for 10 minutes. Do not expose cells to extended trypsin treatment. Extensive exposure to trypsin will markedly reduce cell viability.
5. Transfer the suspension to a 40µm cell strainer placed on a 50ml conical tube and allow cells suspension to flow through the cell strainer with gravity.
6. Spin the cells down at 500×g and 4°C for 5 minutes. Wash the cell pellets once with pre-chilled complete DMEM medium containing 10% FBS to deactivate and remove any residual trypsin.

3.6. Passage 0 MEFs culture.

1. Cell pellets from the previous step are resuspended in complete culture media.
2. Add 11ml of culture medium to each of the 100-mm culture dishes or 75-mm² culture flasks.
3. Culture the cells in a standard humidified 37 °C, 5% CO₂ incubator for 24 hours. If the all of the previous steps are followed properly, the cells should be close to confluence. These cells are considered to be at passage 0 (*see* Note 8).

3.7. Harvest MEFs (see Note 9).

1. Following the initial passage, split cells when they reach approximately 70% confluence.
2. Remove culture medium and gently wash cells two times with PBS.

3. Add 4ml of 0.05% trypsin-EDTA solution to the culture dish, and incubate at 37°C for 3 minutes. Shake 1–2 times during the incubation.
4. Deactivate the trypsin and resuspend the cells with 15ml complete culture medium. Spin down the cell pellets at 500xg and 4°C for 5 minutes.
5. Wash the pellets one time with PBS and resuspend in 15ml complete culture medium for passage.

3.8. Freeze and thaw MEFs.

1. Resuspend 1.0×10^6 MEFs in 1ml of freezing medium, and transfer to a pre-labeled cryovial.
2. Place cryovials in the controlled rate freezer for 24 hours and then transfer them to liquid nitrogen for long-term cryopreservation (*see* Note 10).
3. When thawing cells from frozen stocks, remove cells from liquid nitrogen and immediately place the cryovials in a 37°C water bath.
4. Transfer the cell suspension to a 50ml conical tube, and add pre-warmed complete culture media dropwise into the suspension. Then spin down the cells and proceed with Protocols 3.6 and 3.7.

4. Notes

1. Although the use of wild type mice from the same genetic background is commonly accepted as controls for MEF generation, we highly recommend generating littermate control cells for better accuracy. If gene manipulation does not result in breeding failure or death at early embryonic stages, then the use of heterozygous breeding pairs are recommended. The male to female mouse ratio should be 1:2 in the breeding cages.
2. Be very careful when handling DMSO, which is a known carcinogenic chemical and could penetrate intact skin.
3. We recommend using different hoods for generating MEFs and regular cell culture to minimize the chances of contamination.
4. All instruments should be sterilized by autoclaving prior to experiments.
5. Timed breeding is critical for the successful preparation of MEFs. The most widely used approach to determine the starting date of embryo development is the identification of a copulation plug (semen plug) in the vagina. Although it is usually identifiable before 8:00 am, it should be noted that the copulation plug can be hard to recognize 12 hours after mating. In addition, it is more difficult to identify the plug in very young mice. Hence, copulation plugs need to be examined early in the morning every day until it is identified. The time when a copulation plug is identified will be marked as day 0.5 for embryo development. All female mice should then be transferred to a new cage for the next 13–15 days.

6. Due to the thick hair, it can be challenging to keep the dissection process sterile. Thus, it is of critical importance to take all available precautions to reduce the potential for contamination. It is advisable to separate relatively “clean areas” and relatively “dirty areas” in the hood after transferring mice into the working space. Embryo dissection should be confined to the “dirty area”, and all subsequent procedures should be performed in the “clean area”.
7. Clear numbering of the plates will greatly benefit subsequent genotyping and matching the results to each of the dissected embryos.
8. Primary MEFs maintain active proliferation for about 4 passages. The proliferation rates will considerably drop after 5 passages. Hence, only early passage cells are appropriate for downstream applications. Active MEFs typically double in cell number every 48 hours. Hence, it is recommended to freeze passages 0 and 1 MEFs for the convenience of further experimentation.
9. The culture and maintenance of MEFs are similar to other fibroblast cell lines.
10. There are a number of affordable controlled rate freezers available from different vendors. The principle is to decrease the ambient temperature gradually. Do not directly place cryovials into liquid nitrogen. This will significantly reduce cell viability.

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