Video Article

Skin Punch Biopsy Explant Culture for Derivation of Primary Human Fibroblasts

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Abstract

Tissues and cell lines derived from an individual with disease are ideal sources to study disease-related cellular phenotypes. Patient-derived fibroblasts in this protocol have been successfully used in the derivation of induced pluripotent stem cells to model disease[1]. Early passages of these fibroblasts can also be used for cell-based functional assays to study specific disease pathways, mechanisms[2] and subsequent drug screening approaches. The advantage of the presented protocol over enzymatic procedures are 1) the reproducibility of the technique from small amounts of tissue derived from older patients, *e.g.* patients affected with Parkinson's disease, 2) the technically simple approach over more challenging methodologies using enzymatic treatments, and 3) the time consideration: this protocol takes 15-20 min and can be performed immediately after biopsy arrival. Enzymatic treatments can take up to 4 hr and have the problems of overdigestion, reduction of cell viability and subsequent attachment of cells when not handled properly. This protocol describes the dissection and preparation of a 4-mm human skin biopsy for derivation of a fibroblast culture and has a very high success rate which is important when dealing with patient-derived tissue samples. In this culture, keratinocytes migrate out of the biopsy tissue within the first week after preparation. Fibroblasts appear 7-10 days after the first outgrowth of keratinocytes. DMEM high glucose media supplemented with 20% FBS favors the growth of fibroblasts over keratinocytes and fibroblasts will overgrow the keratinocytes. After 2 passages keratinocytes have been diluted out resulting in relatively homogenous fibroblast cultures which expresses the fibroblast marker SERPINH1 (HSP-47). Using this approach, 15-20 million fibroblasts can be derived in 4-8 weeks for cell banking. The skin dissection takes about 15-20 min, cells are then monitored once a day under the microscope, and media is changed every 2-3 days after attachment and outgrowth of cells.

Video Link

The video component of this article can be found at http://www.jove.com/video/3779/

Protocol

The skin punch biopsy obtained using standard procedure[3] (*e.g.* obtained with 4mm round Visipunch instrument) should be kept in complete DMEM 20% FBS media on ice. Once the sample has arrived in the laboratory, process the biopsy as soon as possible.

1. Preparation of the Skin Punch Biopsy

STEPS 1.1- 1.3 ARE TO BE PERFORMED INSIDE A BIOSAFETY CABINET

1. Prepare in advance: Add 1 ml of 0.1% Gelatin to each well of a 6-well plate. Set the plate aside for 30-60 min. Aspirate the gelatin solution and add 800 µl of complete DMEM/20% FBS media to each well. Ensure that the entire surface of the well is covered with media.
2. Invert the lid of a sterile 10 cm tissue culture dish and add 1.5 ml of DMEM/ 20% FBS media to the middle of the lid and spread out the media drop with the tip of the serological pipette.
3. Using a sterile forceps, place the skin biopsy piece in the media on the dish.

2. Dissection of the Skin Punch Biopsy

STEPS 9 2.1-2.2 ARE TO BE PERFORMED INSIDE A HORIZONTAL LAMINAR FLOW HOOD

1. Place the bottom portion of 10 cm dish on inverted lid, and transfer the skin biopsy to the dissecting microscope in the laminar flow hood.
2. Dissect a 4-mm round skin biopsy into 12-15 evenly sized pieces with sharp edges by cutting pieces in equal halves using one scalpel to hold the biopsy in place and the second scalpel to cut with a rolling motion in one direction. Pieces with ragged edges contribute to poor attachment/cell outgrowth.

3. Transfer of Dissected Skin Biopsy Pieces into Tissue Culture Plates

STEPS 3.1-3.6 ARE TO BE PERFORMED INSIDE A BIOSAFETY CABINET

1. Place the bottom portion of the 10 cm tissue culture dish on top of the inverted lid, and transfer dish back into to the biosafety cabinet.
2. Using a pointed forceps, place 2-3 biopsy pieces into each well of the prepared 6-well plate containing 800 µl and not on dry wells. Use tapping or sliding motion to get the pieces to attach to the bottom of the well. A scalpel is useful to remove any biopsy pieces from the forceps.
3. Place the 6-well plate in the 37 °C incubator. Monitor daily to ensure there is a film of media coating the bottom of the well for the first week; add ~200 µl every 2 days to replace any evaporated media.
4. After one week, increase amount of media to 2 ml of complete DMEM/20%FBS and change media every 2-3 days.
5. Once fibroblasts are confluent in each well to the point where the fibroblasts are reaching the edges of the well, trypsinize and passage 6-well plate into 2X T75 flasks (passage 1). The tissue pieces can be transferred as well. They will not attach and be washed out during the next media change.
6. Once fibroblasts are confluent, transfer them to 3X T175 flasks (passage 2), freeze in complete DMEM media plus 10% DMSO at $1x10^6$ cells/ml per vial.

4. Characterization the Fibroblasts through Immunostaining

1. Culture fibroblasts in 8 well chamber slide coated with gelatin.
2. When cells are at 80% confluency, aspirate the medium from each well.
3. Fix cells in 4% Paraformaldehyde for ten minutes at room temperature.
4. Wash the wells 3 times with 1X PBS.
5. Permeabilize the cells with 150 µl of 0.3% Triton X-100 (in PBS) for 5 min at room temperature.
6. Wash the wells 3 times with 1X PBS.
7. Add 200 µl blocking solution (PBS + 5% Normal Goat Serum (Vector, S-1000)) for 1 hr at room temperature.
8. Prepare a 1:250 dilution of Anti-SERPHIN-1 (Anti-rabbit, mAB, Sigma, S5950-200 µl) in blocking solution.
9. Aspirate the blocking solution, and add 150 µl of the 1:250 dilution of AntiSERPINH-1. Incubate at 1-2 hr at room temperature, or at 4 °C overnight.
10. Wash the wells 3 times with 1X PBS.
11. Prepare a 1:200 goat-anti-rabbit monoclonal antibody (Alexa Fluor Goat anti-rabbit IgG(H+L), Invitrogen, A11008) in blocking solution.
12. Add 150 µl of 1:200 goat-anti-rabbit monoclonal antibody to each well. Incubate for 1 hr, in dark, at room temperature.
13. Wash wells once with 1xPBS.
14. Aspirate the PBS, and carefully lift the chambers off to reveal just the slide.
15. Mount the slide with a 2-3 drops of mounting medium containing DAPI (Vector, H-1200).
16. Analyze the slide under a fluorescent microscope.

Representative Results

Keratinocytes growing out of the biopsy pieces as soon as 48 hr after dissection. First fibroblasts outgrowth can be observed about one week after processing. Once the wells have reached confluency, fibroblasts are passaged for two more passages to reach three 150 cm flasks (T150) and cells are cryopreserved. We generate with this method 15-20 million cells. The fibroblasts are positive for anti-SERPINH1 (also known as HSP-47) (**Figure 1**), which is a collagen-specific molecular chaperone localized in the endoplasmic reticulum. HSP47 plays an essential role in collagen biosynthesis in skin fibroblasts (Kuroda, *et al.*, 2004)[5].

TIME LINE

Day	Expectation
Day 3- Day 7	Attachment and outgrowth of keratinocytes
Day 7- Day 14	Outgrowth of fibroblasts
Day 25-Day 35	Fibroblasts should cover the 6-well plate and should be for passaged into 75cm flasks, passage a second time into 150cm flasks
Day 30-Day 50	Freeze down as 1Mio cells/vial about 15-20 Mio cells in complete DMEM media plus 10% DMSO

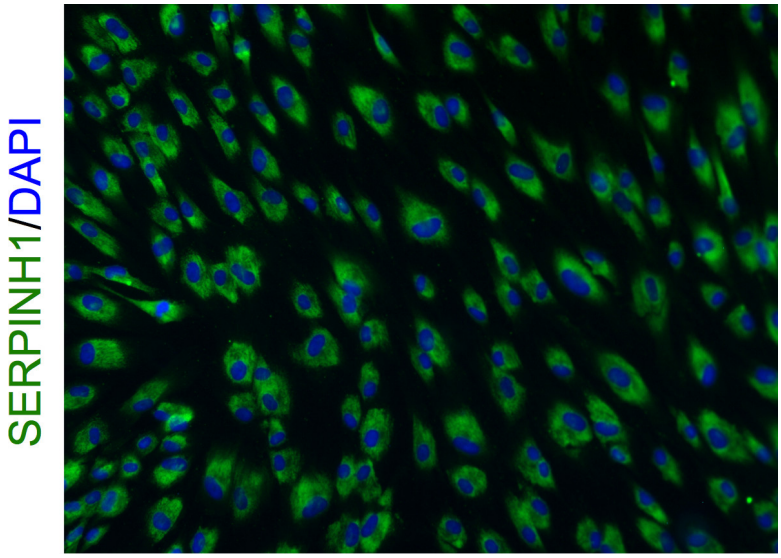

Figure 1. Anti-SERPHIN1 immunohistochemistry of patient-derived fibroblast culture with DAPI counterstaining.

Discussion

With this protocol, relatively pure cultures of skin fibroblasts can be obtained. The fibroblasts have characteristic morphological features of elongated, spindle-like cell bodies, round to oval cell nuclei, and the fibroblasts grow aligned and in bundles when confluent. The media supports the growth of fibroblasts whereas other cell populations *e.g.* keratinocytes need additional supplements and growth factors, or are mitotically less active, this allows for relatively pure fibroblast cultures.

The fibroblasts are subsequently passaged with trypsin at a 1:3 to 1:5 ratio. The culture is expanded to the desired quantity of fibroblasts (15-20 Million) within 4-8 weeks.

Using this technique, our laboratory has derived over 70 fibroblast lines successfully. We have tested every line for mycoplasma contamination and add antibiotics to the growth media to avoid other bacterial contamination. For the initial explant culture we found that 20% FBS in the media supports the growth, however, in later passages 10% FBS is sufficient. Occasionally, we observe a direct outgrowth of fibroblasts from the skin pieces presumably due to the cutting angle of the skin and the removal of the epidermis which contains keratinocytes.

A similar technique using a coverslip to hold down the skin pieces was less effective in our hands[4]. Due to the movement of the coverslip within culture dish -even when handled with great care- the skin pieces did not attach properly.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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