Chapter 10

Hair Induction by Cultured Mesenchymal Cells Using Sphere Formation

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Abstract

Isolated dermal cells possess the capacity to induce hair growth. The cells cannot be expanded while they retain the capacity for hair induction, and lose their potential immediately after cultivation. Sphere-forming multipotent cells derived from the dermis (skin-derived precursors [SKPs]) possess hair-inducing activity. These observations provide two possibilities for the determination of the capacity for hair induction: capacity is dependent on either identity as a dermal cell or on the process of sphere formation. We developed a method that demonstrates cultivated mesenchymal cells derived from dermis and lung tissue possess in vivo hair-inducing capacity via sphere formation.

Key words Mesenchymal cells, Sphere formation, Skin-derived precursors (SKPs)

1 Introduction

Interactions between epithelial and dermal cells are essential for hair follicle morphogenesis and maintenance [1]. The hair-inducing capacity of dermal components is critical for realization of regenerative medicine of hair follicles. Indeed, dermal papilla cells and fetal dermal mesenchymal cells have been shown to possess the capacity to regenerate hair follicles [2–5].

Miller and colleagues first demonstrated that a distinct population of multipotent dermal cells, skin-derived precursors (SKPs), is found in juvenile and adult rodent skin and the adult human scalp [6]. When skin tissues are dissociated and cultured in uncoated flasks, a small population of floating cells proliferates to generate larger spheres. These sphere-forming cells can differentiate into neurons, adipocytes, and smooth muscle cells. Moreover, clonally expanded SKPs induce hair morphogenesis, and form a hair follicle niche upon transplantation [7]. Hoffman and colleagues isolated multipotent cells, utilizing nestin-EGFP mice, from the hair

Robert M. Hoffman (ed.), *Multipotent Stem Cells of the Hair Follicle: Methods and Protocols*, Methods in Molecular Biology, vol. 1453, DOI 10.1007/978-1-4939-3786-8_10,

follicle bulge area [8–10]. These findings raise the following question: On what does hair induction capacity depend, a history of sphere formation or identity as a dermal cell?

We have shown that sphere-forming dermal cells, but not two-dimensionally cultured dermal cells, possess the capacity to induce hair morphogenesis in vivo [11]. Moreover, sphere-forming mesenchymal cells derived from adult lungs of mice also possess the capacity for in vivo hair induction, suggesting that sphere formation alone is sufficient to impart capacity to mesenchymal cells other than dermal cells. Here, we present the technique of regenerating hair from dermal and lung mesenchymal cells by making spheres on agarose gels.

2 Materials

Animal care and all experimental procedures should be performed in accordance with organizational guidelines and policies.

2.1 Mice

1. Pregnant, newborn and 8-week-old male C57bl/6 J mice from a breeder.
2. Seven-week-old SCID mice from a breeder.
3. CAG-EGFP transgenic mice from the Jackson Laboratory (Bar Harbor, ME, USA).

2.2 Two-Dimensional (2D) Expansion of Mesenchymal Cells

1. Microscope, scissors, and forceps.
2. Enzymatic dissociation solution: 0.2% collagenase Type I (Wako, Osaka, Japan) diluted in phosphate-buffered saline (PBS).
3. Cell strainer: 40 μM (BD Falcon, Franklin Lakes, NJ, USA).
4. Culture medium for expansion: Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco-BRL, Carlsbad, CA, USA).
5. Passage solution: 0.25% trypsin/EDTA (Sigma Chemical Co., St. Louis, MO, USA).

2.3 Sphere-Forming Induction of Mesenchymal Cells

1. Agarose gel: 1% (Takara, Osaka, Japan) in PBS.
2. Plastic dishes: 10 cm in diameter.
3. Culture medium for sphere formation: three parts DMEM, one part F-12 containing B-27 (Gibco-BRL), 20 ng/ml EGF and 40 ng/ml bFGF (both from Sigma).

2.4 Cell Transplantation into the Skin of SCID Mice

1. Microscope, scissors, micro forceps, and needle with a 4-0 nylon suture.
2. Dispase: 1000 units (Godo Shusei, Tokyo, Japan).

3. Trypsin/EDTA, 0.25% (Sigma).
4. DMEM containing 10% FBS.
5. Cell strainer: 40 μm nylon filter (Cell Strainer R, BD Falcon).
6. Type I collagen solution: Cellmatrix type I-A (Nitta Gelatin, Osaka, Japan).
7. Cap separated from a 1.5-ml polypropylene microfuge tube. Heat an 18 G needle and make four tiny holes, one of which must be in the center of the cap (for transplantation) while the other three are for suturing.
8. Vinyl tape.
9. Pipette and tips: 200 μl.

3 Methods

3.1 Two-Dimensional Expansion of Mesenchymal Cells

1. Anesthetize mice and perform cervical spine dislocation for euthanasia. Dissect E17 fetal skin and adult lungs (*see* **Note 1**). Mince the tissues with a scalpel, add 0.2% collagenase Type I and incubate at 37 °C for 20–30 min, depending on the type of tissue.
2. Pass the tissues through a 40-μm cell strainer and centrifuge at 1500 rpm (380 × *g*) for 5 min. Place the suspended cells in a plastic flask with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in 5% CO_2.
3. Passage the cultured cells with 0.25% trypsin. Use cells from passages 8–10 for sphere formation.

3.2 Sphere-Forming Induction of Mesenchymal Cells

1. Make a 1% agarose solution by dissolving 5 g of agarose in 495 ml of PBS. Heat the mixture in a microwave oven until completely dissolved.
2. Pour the agarose solution into 10-cm plastic dishes and keep them covered or in a hood until they cool and can be stacked (*see* **Note 2**) (Fig. 1).
3. Prepare sphere-forming culture medium.
4. Suspend 2D-cultured mesenchymal cells with 0.25% trypsin, neutralize with culture medium, centrifuge, resuspend in sphere-forming culture medium, and spread on solidified agarose. Incubate the dishes at 37 °C in 5% CO_2 (*see* **Note 3**) (Fig. 2). Change medium twice a week (*see* **Note 4**).

3.3 Cell Transplantation into the Skin of SCID Mice (see **Note 5***)*

1. Prepare type I collagen gel solution using the Cellmatrix type I-A: mix eight parts collagen, one part concentrated medium, and one part reconstitution buffer. Mix well by pipetting. Keep gel mixture on ice in a cooler box to prevent gelling (*see* **Note 6**).

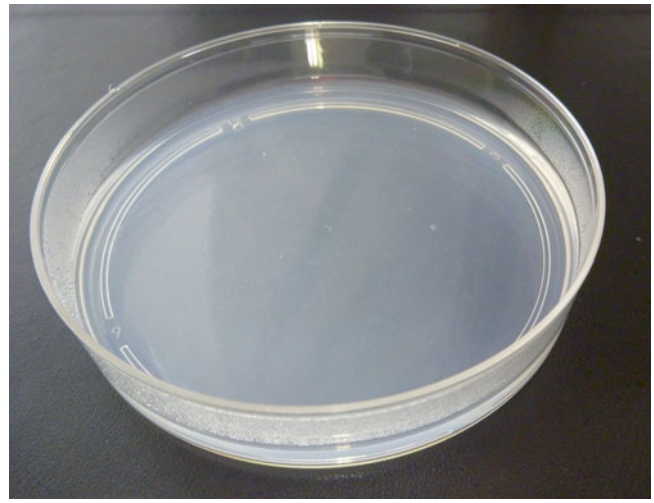

Fig. 1 Agarose gel spread over the bottom of a 10-cm plastic dish. It is important to cover the entire surface of the bottom of the dish

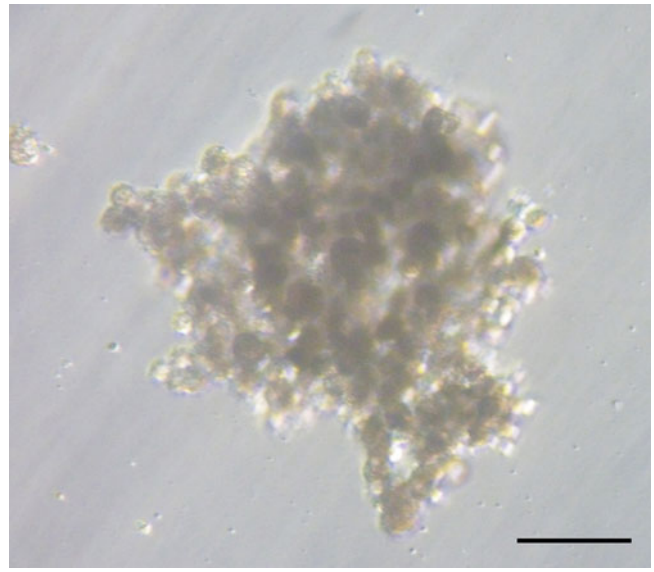

Fig. 2 Spheroid on an agarose gel 1 day after cultivation. Bar = 50 μm

2. Dissect approximately 1 cm^2 of full thickness skin from newborn C57bl/6J mice under a microscope.
3. Immerse and incubate the skin in 1000 units of dispase solution for 30 min at 37 °C.
4. Detach the epidermal sheets from the dermis using two microforceps (*see* **Note** 7).

5. Incubate the epidermal sheets with 0.25% trypsin-EDTA for 15 min at 37 °C. Stop the digestion reaction with a two-fold volume of DMEM containing 10% FBS. Suspend the mixture by pipetting up and down through an 18 G needle. Pass the suspended epidermal cells through a cell strainer and centrifuge at 1500–2000 rpm (380–670 × *g*) for 5 min. Discard the supernatant (*see* **Notes 8** and **9**).
6. Use a pipet to collect the cultured spheroids and centrifuge them at 1500 rpm (380 × *g*) for 5 min. Discard the supernatant. Each transplant contains approximately 1×10^7 epidermal cells and 1×10^7 mesenchymal cells.
7. Mix the newborn epidermal cells with either centrifuged mesenchymal-cell spheroids or 2D cultured mesenchymal cells. Mix the same volume of type I collagen-gel solution with the pellet. Keep on ice until transplantation.
8. Inject pentobarbital into the abdominal cavity. Make full skin thickness wounds, 7 mm in diameter, on both sides of the back skin of 7-week-old male SCID mice.
9. Separate a polypropylene cap from a 1.5-ml microfuge tube and push it into the skin wounds. Suture the surrounding skin with 4-0 nylon (Fig. 3).
10. Inject the mixture through the tiny hole in the center of the cap. Cover with vinyl tape (Fig. 4).
11. One week later, remove the polypropylene caps under anesthesia.
12. Four weeks later, sacrifice the transplanted animals with an inhalational overdose of ether followed by cervical-spine dislocation. Collect the transplants (Fig. 5).

Fig. 3 Polypropylene cap from a microfuge tube containing three holes for a suture, and one central hole for transplantation

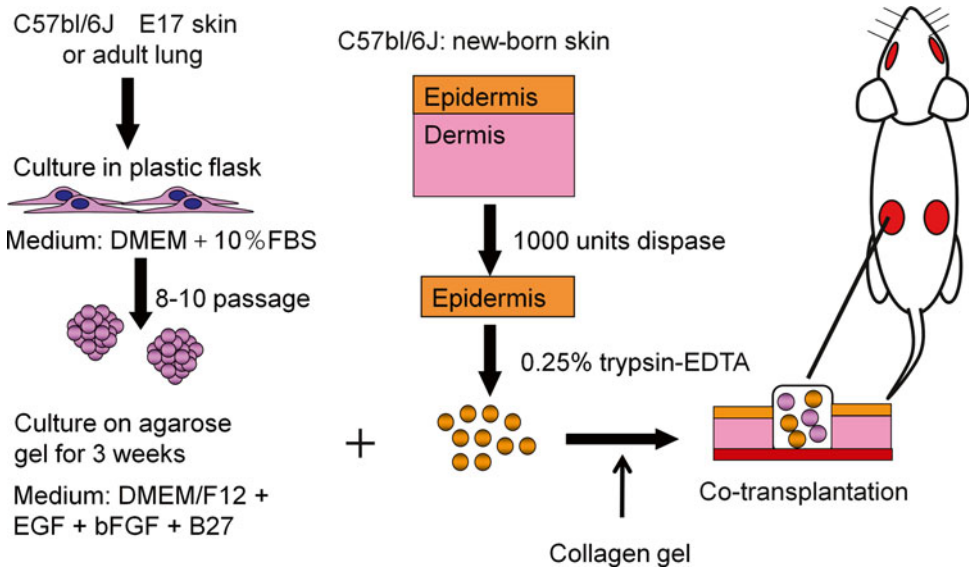

Fig. 4 Schematic of the transplantation process

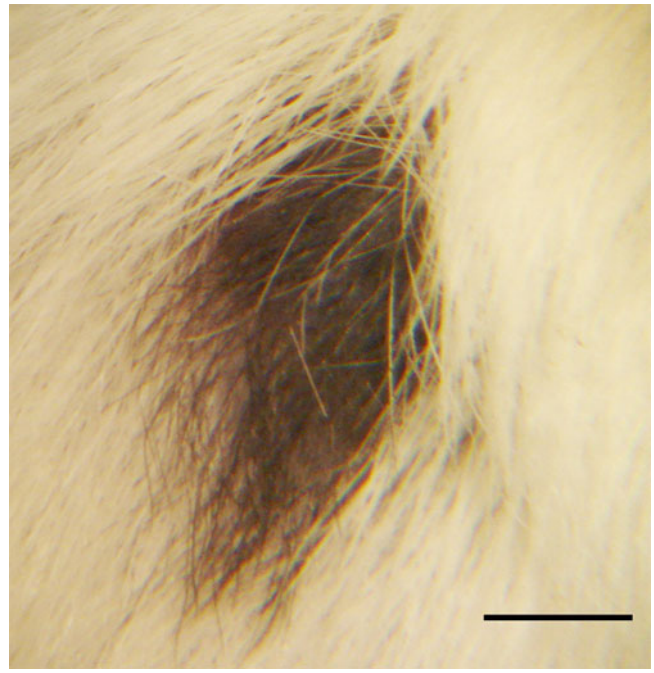

Fig. 5 Regenerated hairs induced by dermal fibroblasts. Bar = 5 mm

4 Notes

1. We tested hair induction using cells from E17 skin and adult lungs, and fibroblasts from passages 8–10. Other fibroblast sources or conditions have not been tested.
2. It is important to cover the entire area of the base of the dish with agarose. Thus, the amount of agarose solution may be increased.
3. Most of the cells start making aggregates within a day after culturing on agarose in dishes.
4. Spheroids can be observed with the naked eye. The spheroids sink to the bottom of the culture medium (approximately 20 ml/dish). One-half to two-thirds of the supernatant is removed when the medium is changed.
5. Cell and animal preparation for the transplantation procedure should be performed simultaneously. Thus, it is recommended that two teams perform the procedures.
6. The collagen solution does not gel when the procedure is performed on ice. However, it easily gels when it becomes warm. To prevent gel formation before transplantation, all equipment and reagents that might attach to the collagen gel solution, including the cell mixture, pipet tips, cylinder, and 18 G needle, should be cooled.
7. If the epidermis does not detach from the dermis, the incubation time may be increased. In order to separate the epidermis effectively, the skin should be spread thoroughly in the dispase solution.
8. To dissolve the epidermal cells in trypsin solution, pipet up and down using a 1-ml syringe with an 18 G needle every 5 min during incubation.
9. Since the cells of fetal or newborn mice are sticky, apply pressure to the filter with a syringe.

References

1. Yang CC, Cotsarelis G (2010) Review of hair follicle dermal cells. J Dermatol Sci 57:2–11
2. Jahoda CA, Horne KA, Oliver RF (1984) Induction of hair growth by implantation of cultured dermal papilla cells. Nature 311:560–562
3. Jahoda CA, Reynolds AJ, Oliver RF (1993) Induction of hair growth in ear wounds by cultured dermal papilla cells. J Invest Dermatol 101:584–590
4. Kishi K, Katsube K, Satoh H et al (2005) The fetal dermal but not loose fascial mesenchymal cells possess regenerative activity of dermal structure. Cell Transplant 14:709–714
5. Young TH, Lee CY, Chiu HC et al (2008) Self-assembly of dermal papilla cells into inductive spheroidal microtissues on poly(ethylene-co-vinyl alcohol) membranes for hair follicle regeneration. Biomaterials 29:3521–3530

6. Toma JG, Akhavan M, Fernandes KJ et al (2001) Isolation of multipotent adult stem cells from the dermis of mammalian skin. Nat Cell Biol 3:778–784
7. Biernaskie J, Paris M, Morozova O et al (2009) SKPs derive from hair follicle precursors and exhibit properties of adult dermal stem cells. Cell Stem Cell 5:610–623
8. Li L, Mignone J, Yang M et al (2003) Nestin expression in hair follicle sheath progenitor cells. Proc Natl Acad Sci U S A 100: 9958–9961
9. Amoh Y, Li L, Yang M et al (2004) Nascent blood vessels in the skin arise from nestin-expressing hair-follicle cells. Proc Natl Acad Sci U S A 101:13291–13295
10. Amoh Y, Li L, Katsuoka K et al (2005) Multipotent nestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons. Proc Natl Acad Sci U S A 102:5530–5534
11. Shimizu R, Okabe K, Kubota Y et al (2011) Sphere formation restores and confers hair-inducing capacity in cultured mesenchymal cells. Exp Dermatol 20(8):679–681