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# Isolation, Characterization, and Differentiation of Human Multipotent Dermal Stem Cells

## Materials

## Medium andSolution Preparation

1. **Foreskin transporting medium:** Dulbecco’s modification of Eagle’s medium (DMEM; Cellgro #10-017-CM) supplemented with gentamycin (100 mg/mL; Cellgro #30-005-CR). After sterilization through a 0.2 mm filter, the medium is transferred into sterile containers in 20 mL aliquots and stored at 4°C for up to 1 month.
2. **Dispase solution ( 0.48% ):** Dispase (grade II, 0.5 U/mg; Boehringer Mannheim #165859) 0.48 g is dissolved in 100 mL phosphate-buffered saline (PBS) without Ca 2+ and Mg 2+ (Cellgro #MT21-031-CM). Sterilize the enzyme solution through a 0.2 mm filter, aliquot into 5 mL/tubes, and store at −20°C for up to 3 months.
3. **Collagenase solution ( 1 mg/mL ):** Collagenase type IV (Invitrogen #17104-019) 100 mg is dissolved in 100 mL DMEM to yield a final concentration of 1 mg/mL. Sterilize the enzyme solution through a 0.2 mm filter, aliquot into 5 mL/tubes, and store at −20°C for up to 3 months.
4. **Mouse embryonic fi broblast ( MEF ) derivation medium :** The medium contains 87% DMEM (Invitrogen #11965-092), 10% defined FBS (Invitrogen #16000-044; heat inactivate for 30 min at 57°C), 1% 200 mM L -glutamine (Invitrogen #21051024), 1% nonessential amino acids 100× (Invitrogen #11140) and 1% penicillin-streptomycin 100×.
5. MEF growth medium: MEF derivation medium withoutpenicillin–streptomycin.
6. **Human embryonic stem cell medium (HES):** 78% DMEM/F-12 (Invitrogen #11330-032), 20% Knockout-Serum Replacer (Invitrogen #10828-028), 1% 100 mM L -glutamine + b -mercaptoethanol (Invitrogen #21051-024—add 7 mL b -mercaptoethanol to 10 mL L -glutamine), 1% nonessential amino acids 100× (Invitrogen #11140), 4 ng/mL basic fi broblast growth factor (bFGF; Fitzgerald Industries #30R-AF015).
7. **Human embryonic stem cell medium 4 (HESCM4):** 70% MEF conditioned HES medium and 30% HES medium, sterilize through a 0.2 mm filter.
8. **L-Wnt3a cell growth medium and conditioning medium:** 90% DMEM (Cellgro #10-017-CM), add 10% FBS and 0.4 mg/ mL G418 (Sigma #G-8168). Conditioning medium including 99% DMEM and 1% FBS.
9. **Differentiation medium:**

* **For melanocyte differentiation (100 mL):** 50 mL Wnt3a conditioned medium, 30 mL DMEM-Low Glucose (Invitrogen #11885), 20 mL MCDB201 (Sigma #M6770), 1× ITS Liquid Medium Supplement (Sigma #I-3146), 1 mg/mL linoleic acid-BSA (LA-BSA; Sigma #L-9530), 10 −4 M L -ascorbic acid (Sigma #A-4403), 100 ng/mL stem cell factor (SCF; Fitzgerald Industries, #RDI-307- 255X), 0.05 m M dexamethasone (Sigma #D-2915), 20 pM Cholera toxin (Sigma #C-3012), 50 nM TPA (Sigma #P-1583), 4 ng/mL bFGF (Fitzgerald Industries #30RAF015), 100 nM endothelin-3 (ET-3; American Peptide Co. #88-5-10).
* **For neuronal cell differentiation (100 mL):** (1) 60 mL DMEM, 30 mL F12 (GIBCO #11765), 10 mL FBS, 40 ng/mL bFGF. (2) 60 mL DMEM, 30 mL F12, 10 mL FBS, 10 ng/mL nerve growth factor (Millipore #GF028), 10 ng/mL brain-derived neurotrophic factor (Peprotech #450-02-10), 10ng/mL NT-3 (Stem Cell Technologies #02508).
* **For smooth muscle cell differentiation (100 mL):** 90 mL DMEM-F12 (GIBCO m#11330), 10 mL FBS, 0.1 M nonessential amino acids solution and 60 pM transforming growth factor- b 1 (TGF- b 1; R&D Systems #240B).
* **For adipocyte differentiation (100 mL):** 90 mL lowglucose DMEM (Sigma #D6046), 10 mL horse serum (Invitrogen #26050-070), 1× ITS, 1 mg/mL LA-BSA, 1 m M hydrocortisone (Sigma #H4001), 60 m M indomethacin (Sigma #I7378), 0.5 mM isobutylmethylxanthine (Sigma #I5879).
* **For chondrocyte differentiation (100 mL):** 90 mL highglucose DMEM, 10 mL FBS, 1× ITS, 1 mg/mL LA-BSA, 50 nM dexamethasone, 60 pM TGF- b 1.
* **For Schwann cell differentiation (100 mL):** (1) 60 mL DMEM, 30 mL F12 (GIBCO #11765), 10 mL FBS. (2) Medium I plus 4 m M foskolin (Sigma #F3917).
* **Skin reconstruct medium: Basic medium:** 400 mL Keratinocyte-SFM (Invitrogen #10724), 2% dialyzed FCS (Gibco LTI #16440-034), 60 ng/mL bovine pituitary extract (BPE; Invitrogen #13028-014), 4.5 ng/mL bFGF, 100 nM ET3 (American Peptide Co. #88-5-10), 10 m g/mL SCF (Fitzgerald Industries #RDI-307-255X). (1) Add 1 ng/mL EGF (Invitrogen #10450-013) to 100 mL basic medium; (2) Add 0.2 ng/mL EGF (Invitrogen #10450-013) to 100 mL basic medium; (3) Add 2.4 mM CaCl 2 (Sigma; #C-7902) to 200 mL basic medium.

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## Dermal StemCell Culture

## Day 1

* Take out a foreskin from the transfer tube; rinse it with 70% ethanol for 1 min
* Transfer the foreskin to a sterile 100 mm culture dish, add 20 mLHBSS without Ca 2+ and Mg 2+, wait for 2 min
* Open the foreskin ring with scissors and cut the foreskin into several pieces of approximately 5 × 5 mm 2 using a surgical scalpel blade
* Transfer the skin pieces into a 50 mL Falcon tube with 5 mL0.48% dispase II, and incubate at 4°C overnight

## Day 2

* Remove the Falcon tube containing the skin sample from 4°Cand incubate it at 37°C for 5 min
* Pour the skin pieces with the disease into a sterile 10 cm culture-dish, transfer all skin pieces to a new dish using forceps
* Separate the epidermis from the dermis by holding the dermal part of each skin piece with one pair of forceps and gently remove the epidermal part with a surgical scalpel blade. Discard the epidermis. Repeat the procedure for each piece of skin
* Transfer all dermal pieces to a new dish and mince them as small as possible.
* Collect the minced dermal pieces using a pipette and transfer to a 50 mL Falcon tube containing 2 mL 1 mg/mL collagenase IV. Incubate at room temperature for 24

## Day 3

* Add 25 mL HBSS without Ca 2+ and Mg 2+ to the tube containing the dermis with collagenase IV. Mix well by pipetting up and down; serially filter through 100, 70, and 40 cell strainers
* Centrifuge the cell suspension at 200 × g for 5 min
* Resuspend the cell pellet in 10 mL HESCM4 medium and seed the cells in two T25 flasks
* Put the flasks into an incubator with 5% CO 2 at 37°C
* After 48 h, aspirate 2.5 mL medium from the flask, and replace with 2.5 mL fresh HESCM4 medium. Change half the volume of the medium every 3–4 days

## Dermal Stem Cell Differentiation to Melanocytes, Neuronal Cells, Schwann Cells, Smooth Muscle Cells, Adipocytes, and Chondrocytes

* Tissue culture-grade 4-well chamber slides (Becton Dickinson) are precoated with: 0.5 mL/well 10 ng/mL fibronectin (Advanced Biomatrix #5050; for melanocyte, chondrocyte, and adipocyte differentiation), 0.1% Matrigel (BD Biosciences #354234; for neuronal and smooth muscle cell differentiation), and a mixture of 20 mg/mL laminin (BD Biosciences #354232) with 200 mg/mL poly- D -lysine (BD Biosciences #354210) (for Schwann cell differentiation)
* Collect dermal spheres and transfer into a 50 mL tube, spindown, remove the supernatant as much as possible
* Add 0.5 mL 1 mg/mL collagenase IV and 0.5 mL 0.25%trypsin/EDTA. Incubate at 37°C for 5 min
* Pipette up and down for 1 min, then add 9 mL soybean trypsin inhibitor. Spin down. Resuspend the sphere cell pellet in the various differentiation media and seed the cells in coated chamber slides
* For neuronal and Schwann cell differentiation, use the medium-I during the first week and switch to medium-II during the second week
* Incubate for 2–3 weeks, replace 1/2 fresh medium twice a week. Cells are ready to fix for staining using differentiation markers

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## DSCs Differentiation to Epidermal Melanocytes in Three-Dimensional Skin Reconstruct Culture

* Coat the transwell (Organogenesis) with the collagen mixture: 0.59 mL 10× minimal essential medium (EMEM), 50 m L 200 mM L -glutamine, 0.6 mL FBS, 120 m L 7.5% sodium bicarbonate, 4.6 mL bovine collagen I and mix well. Add 1 mL of the mixture into one transwell of tissue culture trays
* Collect dermal spheres by centrifugation and resuspend 6,600dermal spheres in 0.75 mL HESCM4 medium
* Trypsinize fibroblasts and collect cells by centrifugation and resuspend 0.45 × 10 6 cells in 0.75 mL skin reconstruct medium-I
* Mix the following reagents in a 50 mL tube: 1.65 mL 10× MEM, 150 m L 200 mM L -glutamine, 1.85 mL FBS, 350 m L 7.5% sodium bicarbonate, 14 mL bovine collagen I, 0.75 mL dermal spheres from step 2 , 0.75 mL fi broblasts suspension from step 3 and mix well. Add 3 mL to each coated transwell. Incubate for 45 min at 37°C in a 5% CO 2 tissue culture incubator. Add skin reconstruct medium I (2 mL inside and 10 mL outside of the transwell). Incubate for 4 day
* Harvest human keratinocytes, resuspend 3 × 10 6 cells in 600 m Lskin reconstruct medium-I
* Remove the skin reconstruct tray from the incubator, aspiratemedium from both inside and outside of transwells
* Add skin reconstruct medium I (1.5 mL inside and 10 mL outside of insert). Drop 100 m L keratinocyte suspension to each inside transwell. Incubate for 2 days
* Remove skin reconstruct medium I from both inside and outside of transwells. Add skin reconstruct medium II (2 mL inside and 10 mL outside). Incubate for another 2 days
* Aspirate skin reconstruct medium II both inside and outside of transwells, add 7.5 mL skin reconstruct medium III to only the outside of the transwells ( see Note 12 ). Change medium-III every other day until day 18
* Harvest the skin reconstruct at day 18: Remove the transwellfrom the tray with forceps. Cut out the reconstruct (including the polycarbonate fi lter) by tracing a circle close to the edge with a scalpel blade. Place the reconstruct in a histology cassette (Surgipath #02275-BX) between two black TBS biopsy papers (Triangle Biomedical Sciences, #BP-B) and soak the whole cassette in 10% formalin (Fisher Healthcare #245-685) for 4–6 h. Then place the cassette in 70% for paraffin embedding