Isolation of skin-derived precursors (SKPs) and differentiation and enrichment of their Schwann cell progeny

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This protocol describes methods of isolating skin-derived precursors (SKPs) from rodent and human skin, and for generating and enriching Schwann cells from rodent SKPs. SKPs are isolated as a population of non-adherent cells from the dermis that proliferate and self-renew as floating spheres in response to fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF). Their differentiation into Schwann cells and subsequent enrichment of these differentiated progeny involves culturing SKPs as adherent cells in the absence of FGF2 and EGF, but in the presence of neuregulins, and then mechanically isolating the Schwann cell colonies using cloning cylinders. Methods for expanding and characterizing these Schwann cells are provided. Generation of primary SKPs takes approximately 2 weeks, while differentiation of Schwann cells requires an additional 4–6 weeks.

INTRODUCTION

The therapeutic utility of Schwann cells has been widely recognized since Aguayo and colleagues first demonstrated the growth-promoting abilities of peripheral nerves grafted into the CNS[1,2]. Since then, numerous animal studies have confirmed that exogenous transplants of Schwann cells provide a trophic, growth-promoting environment, and that they can significantly enhance remyelination, axonal growth and functional recovery following spinal cord injury[3–12]. Although Schwann cells thus provide a potential cell source for treatment of nervous system disorders such as spinal cord injury, obtaining Schwann cells for autologous transplantation has proven difficult. Isolation of Schwann cells requires a highly invasive surgical biopsy to remove a segment of peripheral nerve. Subsequent techniques then employ enzymatic digestion and/or mechanical dissociation of embryonic or neonatal peripheral nerves to liberate Schwann cells for further expansion[13–23]. Unfortunately, these techniques have proven much less effective with adult nerves because of their fully developed epineurium and perineurium, which consist entirely of fibroblasts that typically overtake cultures and hinder purification[23]. Expansion of adult cultures also requires serum[24,25], which permits growth of contaminating fibroblasts. Finally, adult nerve-derived Schwann cells have displayed a relatively limited proliferative capacity.

We have isolated a self-renewing, multipotent precursor population from the dermis of both rodent and human skin[26]. These cells, termed skin-derived precursors (SKPs), exhibit a similar gene expression profile to embryonic neural crest stem cells and appear to generate only neural crest derivatives[26,27], including Schwann cells and neurons of the peripheral nervous system. In this regard, Fernandes *et al.*[28] have described procedures for generating neurons from rodent SKPs and have characterized them with regard to their neurotransmitter phenotype, demonstrating that they are primarily catecholaminergic. Our laboratory[29] as well as others[30] have shown that precursors isolated from human dermis can also generate neurons.

We have recently devised cell culture conditions to enhance the generation and survival of Schwann cells from SKPs and, importantly, have demonstrated that these SKP-derived Schwann cells express a myelinating phenotype when cocultured with peripheral axons, and generate compact myelin when they encounter axons within the injured peripheral and central nervous systems *in vivo*[31]. These SKP-derived Schwann cells are more than 95% pure, and can be kept in culture for long periods of time (more than 12 passages or 8 months), with little change in survival, proliferative ability or myelinating capacity. Here we provide a detailed protocol for generating SKPs from rodent and human skin, and protocols for the subsequent differentiation and selection of rodent Schwann cells.

There are several limitations to this procedure that must be considered prior to implementation. First, the entire procedure takes 6–10 weeks, and the generation of Schwann cells is dependent on first successfully generating SKPs. Second, there are limitations related to the age and species of the initial skin sample. As one example, we have not yet optimized procedures for generating SKPs from adult human skin, although another group has reported a protocol for doing so[30]. As another example, it cannot be assumed that SKPs obtained from various skin types (i.e., hairy versus glabrous) will have equivalent potential to generate Schwann cells (J.A.B. and F.D.M., unpublished observations). Third, although infant human SKPs are capable of generating myelinating Schwann cells[31], we have not yet optimized the protocol for obtaining large numbers of human SKP-derived Schwann cells. In this regard, prospective isolation of multipotent SKPs that display both neural and mesodermal potential may ultimately be required to further enhance the efficiency of generating Schwann cells from human skin.

MATERIALS

REAGENTS

- Fresh embryonic, neonatal or adult skin from mouse, rat or human
! CAUTION All animal and human experiments must be performed in accordance with relevant authorities' guidelines and regulations.
- Culture medium (see REAGENT SETUP)
- Neurobasal medium (Invitrogen, cat. no. 21103)
- Hank's Balanced Salt Solution (HBSS) (Invitrogen, cat. no. 14175-095)
- B27 supplement (Invitrogen, cat. no. 17504-044)
- N_2 supplement (Invitrogen, cat. no. 17502)
- Fungizone (Invitrogen, cat. no. 15290-018)
- Trypsin (Calbiochem, cat. no. 6502) diluted to 1% in HBSS and frozen at −20 °C until use
- Trypsin versene or trypsin EDTA (Cambrex, cat. no. 17-161E)
- Collagenase type XI (Sigma, cat. no. C9407) diluted in sterile H_2O to a concentration of 1 mg ml^{-1}
- Heat-inactivated FBS (Cambrex, cat. no. 14-502F)
- Basic fibroblast growth factor (bFGF) (Collaborative, cat. no. 356061A)
- Epidermal growth factor (EGF) (Collaborative, cat. no. 354052)
- Heparin sulfate (Sigma, cat. no. H-3149)
- Forskolin (Sigma, cat. no. F6886) dissolved to a concentration of 25 mM in DMSO, aliquoted and frozen at −80 °C **▲ CRITICAL** If DMSO is used as a diluent, prepare stocks so that the final concentration of DMSO in each culture does not exceed 1%.
- DMSO, anhydrous above 99% (Sigma, cat. no. 276855)
- Heregulin-1β (R&D Systems, cat. no. 377-HB) (see REAGENT SETUP)
- Laminin (Collaborative, cat. no. 354232) dissolved in 10 ml of sterile H_2O
- Fibronectin (Biomedical Technologies, cat. no. BT-225S) dissolved in sterile H_2O
- Poly-D-lysine (Collaborative, cat. no. 354210) dissolved in 10 ml of sterile H_2O
- Trypan blue (Cambrex, cat. no. 17-942E)
- Liberase Blendzyme 1 (10×) (Roche, cat. no. 1988417) (see REAGENT SETUP)
- DNaseI (Sigma, cat. no. D5025) dissolved in sterile HBSS to a concentration of 2 mg ml^{-1} and stored as 400 µl aliquots at −20 °C (needed only for human tissue culture)

EQUIPMENT

- Fine-tipped stainless steel forceps (sizes 5 and 55; Fine Science Instruments)
- 10 ml sterile pipettes (Falcon, cat. no. 13-675)
- 75 cm^2 flask (Falcon, cat. no. CABD353136)
- 25 cm^2 flask (Falcon, cat. no. CA29185-300)
- Table-top centrifuge (Central CL2, thermo IEC, product no. 426). This centrifuge is used in all centrifugation steps unless otherwise indicated
- 15 ml conical tubes (Falcon, cat. no. 352097)
- 50 ml conical tubes (Falcon, cat. no. 352070)
- 10 cm tissue-culture-treated culture dishes (Falcon, cat. no. 353003)
- Chamber slides (four or eight wells) (Nunc, cat. no. 177437 or 177445)
- Cell strainer 40 µm diameter (Falcon, cat. no. CA21008-949)
- Cloning cylinders (Corning, cat. no. 3166-8 or 3166-10)
- Vacuum grease (Dow Corning, cat. no. 2021854-0499)
- #10 or #15 sterile scalpel blades (Feather, cat. no. 089275A)
- Hemacytometer (Brightline, Hausser Scientific)
- Sterile syringe filters 0.2 µm pore size (Nalgene, cat. no. 190-2520)
- Cyrogenic vial (Corning, cat. no. 430659)
- Cryo freeze container (Nalgene, cat. no. 5100 0001)
- Cell strainer 70 µm diameter (Falcon, cat. no. CA21008-949) (needed only for human tissue culture)

REAGENT SETUP

Heregulin-1β Also referred to as neuregulin-1β (R&D Systems, cat. no. 377-HB); dissolve in sterile PBS containing 0.1% BSA, aliquot at a concentration of 10 µg ml^{-1} and store at −80 °C.
Liberase Blendzyme 1 (10×) Make up stock solution by dissolving in sterile H_2O to a concentration of 9 mg ml^{-1}. Distribute into 553 µl aliquots and freeze at −20 °C (needed only for human tissue culture).
Culture medium DMEM + Glutamax (Invitrogen, cat. no. 10567-014) mixed 3:1 with F-12 nutrient supplement (Invitrogen, cat. no. 31765-035) containing 1% penicillin/streptomycin (Cambrex, cat. no. 17-602E).
Wash medium DMEM/F12 (3:1), containing 1% penicillin/streptomycin.
SKPs proliferation medium DMEM/F12 (3:1) containing 0.1% penicillin/streptomycin, 40 µg ml^{-1} fungizone, 40 ng ml^{-1} FGF2, 20 ng ml^{-1} EGF, 2% B27 supplement. For a T75 flask, we use 30–40 ml of medium. **▲ CRITICAL** Prepare medium fresh on the day of the experiment.
SKP-derived Schwann cell differentiation medium I DMEM/F12 (3:1) containing 0.1% penicillin/streptomycin, 40 µg ml^{-1} fungizone, 40 ng ml^{-1} FGF2, 20 ng ml^{-1} EGF, 2% B27 supplement, 5–10% FBS. **▲ CRITICAL** Prepare medium fresh on the day of the experiment.
SKP-derived Schwann cell differentiation medium II DMEM/F12 (3:1) containing 0.1% penicillin/streptomycin, 5 µm forskolin (diluted from 25 mM frozen aliquots), 50 ng ml^{-1} heregulin-1β (diluted from frozen 50 µg ml^{-1} aliquots), 2% N_2 supplement, 1–5% FBS (as determined by the experiment).
▲ CRITICAL Make fresh on the day of the experiment and for every feeding.

PROCEDURE

1 | SKPs can be prepared from rodent (option A) or human (option B) skin. We have generated SKPs from both infant and adult human skin[26,29], although the protocol for adult skin has not yet been optimized. In option B we will highlight the differences involved in isolating SKPs from infant human skin as opposed to the rodent skin protocol described in option A.

(A) Generation of SKPs from rodent skin ● TIMING 1–3 weeks

(i) Prior to dissection, fill six 10 cm plastic plates with cold sterile HBSS and place on ice.
(ii) Thaw aliquots of collagenase type XI. For each neonatal back skin sample we used approximately 1 ml of collagenase (1 mg ml^{-1}).
(iii) Clean and sterilize all instruments using an autoclave and 70% ethanol prior to dissection.
(iv) Euthanize animals using an institute-approved protocol.
(v) Using fine forceps and scissors, dissect dorsal back skin by making incisions from shoulder to shoulder down either side of the back, and remove a rectangle of tissue from the middle of the back.

▲ CRITICAL STEP If culturing skin from adult animals, shave hair on the back skin to reduce the amount of hair fragments in the cultures. Minimize bacterial contamination by thoroughly wiping the back skin with 70% ethanol.
▲ CRITICAL STEP Prior to and throughout the dissection procedure and dissociation, keep skin tissue and/or dissociated cells on ice or at 4 °C.

▲ **CRITICAL STEP** Gently clean each piece of skin. This involves careful removal of all blood vessels, adipose, fascia and muscle underlying the dermis. This is important to reduce contamination by other cell types in the culture.

(vi) **Optional step**: To further reduce contaminating cell types, the epidermis can be removed from the dermis, the latter being the source of SKPs. To do this, float whole skin sections (epidermal surface face-up) on a solution of 0.1% trypsin in HBSS at 37 °C for 1 h. Incubation times may need to be lengthened for older skin samples. Fine forceps (#55) can then be used to peel the epidermis off the underlying dermis. Although this step is useful, under the culture conditions described below epidermal cells readily adhere to the surface of the flask and do not generate spherical colonies, making this step optional.

■ **PAUSE POINT** Dissected skin can be stored in HBSS for 24–48 h at 4 °C and even shipped at this point without significantly affecting cell yield or health.

(vii) Mince dissected skin tissue into 1–2 mm^2 size pieces using a sterile razor blade, and then transfer into a 15 ml conical tube for digestion. Submerge tissue in 0.1% trypsin or collagenase type XI (1 mg ml^{-1}) for 15–60 min at 37 °C. For each neonatal back skin, we used 1 ml of trypsin solution or 1 ml of collagenase type XI. Appropriate incubation times for digestion are dependent on the age of the animal from which the skin is dissected (approximate times: embryonic skin 15–20 min, neonatal skin 45–60 min, adult skin 60 min), and should be determined by the user. Digestion is complete when tissue becomes pale in color and when cells can be easily liberated by mechanical impact or trituration. Tissue can be re-exposed to collagenase (or trypsin) without compromising the integrity of the cells.

▲ **CRITICAL STEP** Experimenters should determine incubation times within their own laboratory because enzymatic activity varies depending upon the source and batch, which will affect digestion times.

(viii) While tissue is digesting, prepare wash medium (see REAGENT SETUP).

(ix) If the tissue was exposed to trypsin, then add 10 ml of wash medium supplemented with 10% FBS. If tissue was digested with collagenase, then add 20 ml of wash medium alone. Wash the tissue extensively by repeated inversion of the tube or place on a rocker at 4 °C for 5 min.

(x) Centrifuge tissue samples using a tabletop centrifuge at 1,200 r.p.m. for 6–8 min to pellet all cells and skin pieces. Discard all supernatant, which contains the medium plus enzyme.

(xi) Add 1 ml of fresh wash medium to the cell/tissue pellet. Using a 10 ml disposable plastic pipette, mechanically dissociate tissue by repeatedly grinding it with the pipette tip. Add another 2–3 ml of medium and triturate the tissue using either a 10 ml pipette or a 1,000 µl pipette tip. Collect the supernatant, add 1 ml more wash medium and repeat the trituration. Repeat this sequential dissociation until tissue pieces become thin and cells are no longer liberated from the tissue.

▲ **CRITICAL STEP** The supernatant from these sequential dissociations is collected and stored on ice. The supernatant should appear 'milky' with suspended cells. We normally repeat the dissociation step 3–4 times, or until cells can no longer be liberated from the tissue sample.

(xii) Pass the dissociated cell suspension through a 40 µm cell strainer into a 15 ml conical tube. Centrifuge the cell suspension in a tabletop centrifuge at 1,200 r.p.m. for 7 min. Remove the supernatant and resuspend the pellet in wash medium plus 2% B27 supplement. Resuspension volumes range from 5–20 ml depending upon the size of the pellet, and can be adjusted to simplify quantification of cell numbers.

(xiii) Determine the total yield of dissociated skin cells using a hemocytometer. We usually dilute 5 µl of the dissociated cell suspension in 45 µl (1:10 dilution) of trypan blue as an indicator of cell viability. Typically, one embryonic mouse back skin sample will provide approximately 10^6 cells, whereas a neonatal back skin sample will provide 10^7 cells.

(xiv) Dilute the dissociated skin cells into proliferation medium to an optimum density of 10,000–25,000 cells per ml of medium; 30 ml total medium in a 75 cm^2 flask and 10 ml in a 25 cm^2 flask. SKPs are then grown in these flasks in a 37 °C, 5% CO_2 tissue culture incubator as floating spherical colonies.

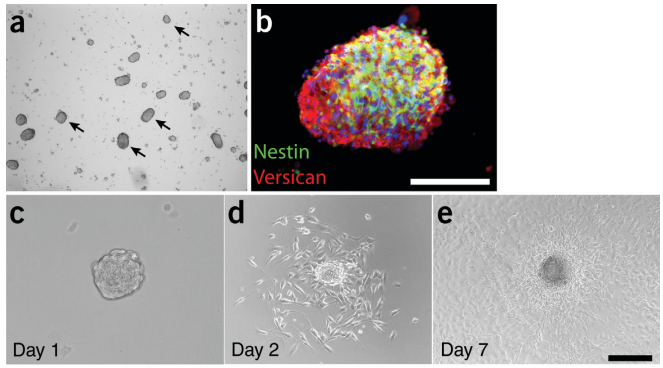

Figure 1 | SKPs grown in FGF2 and EGF. (**a**) Low magnification brightfield image of SKPs generated from adult rat skin at a density of 20,000 cells per ml 10 d after the initial tissue dissection. Arrows indicate SKP spheres. (**b**) High magnification image of a rat SKP sphere immunostained for the intermediate filament nestin (green) and chondroitin sulfate proteoglycan versican (red), which are typical markers of SKP spheres. Nuclei stained with Hoechst dye are shown in blue. Scale bar represents 100 µm. (**c–e**) High magnification brightfield images illustrating sequential stages of SKP differentiation. (**c**) A single SKP sphere, generated at clonal density, minutes after being plated in differentiation media. (**d,e**) Clonal sphere following 2 d (**d**) or 7 d (**e**) of differentiation. Note that cells migrate out from the adherent sphere colony. Scale bar represents 250 µm.

▲ CRITICAL STEP For lower cell densities, it is recommended to add at least 10% conditioned medium to promote survival and proliferation (see Passaging SKPs, Step 2).

(xv) Allow growth of spherical colonies for 7–21 d without passaging. Over this period, cultures must be fed every 3–5 d with an addition of 1–2 ml fresh medium containing all growth factors and supplements (FGF2, EGF, B27) at a concentration that will replenish the entirety of the culture medium. **Figure 1a** shows an example of an adult rodent skin culture following 10 d in SKPs proliferation medium.

▲ CRITICAL STEP Large clusters of cells that appear within the first few days of culturing (1–3 d) are not SKPs but are instead cell aggregates. They are an indication that either the cell density is too high or that the tissue was not adequately dissociated. Moreover cell densities above 25,000 cells ml^{-1} could result in aggregation of cells into structures that resemble spheres but are not proliferating colonies of cells. These aggregates are not SKPs and do not exhibit multipotency or self-renewal, and thus aggregates may reduce efficiency in generating Schwann cells, or other types of cells, from SKPs.

▲ CRITICAL STEP The growth rate of SKPs is cell-density-dependent, so that the lower the cell density, the slower their growth. This can be partially circumvented with the use of conditioned medium.

▲ CRITICAL STEP It is extremely important to use tissue-culture-treated plastic in the flasks to prevent adherence of cells to the plastic. Premature adherence will typically result in a reduced yield of SKPs. Other laboratories have utilized rolling cultures or gentle agitation to promote sphere formation.

▲ CRITICAL STEP Rat SKPs grow significantly faster than do mouse SKPs.

? TROUBLESHOOTING

(B) Generation of SKPs from human skin ● TIMING 1–3 weeks

(i) Wash human foreskin samples extensively in HBSS prior to dissection to remove blood cells. Carefully remove all blood vessels, adipose, fascia and muscle underlying the dermis.

! CAUTION Human tissue should always be treated as a biohazard, and appropriate precautions taken in its handling.

(ii) Prepare liberase blendzyme solution for digestion of human skin by diluting one 553 µl aliquot in 25 ml sterile HBSS. Keep on ice until use. For one infant human foreskin, we typically use 25 ml Blendzyme solution.

(iii) For human SKP isolation, it is essential to remove the dermis from the epidermis to reduce contamination by undesired cell types. To do this, cut the skin into small pieces of 3–5 mm^2. Then float these skin pieces, epidermis side up, in a 10 cm plastic tissue culture dish filled with 25 ml of Blendzyme solution for 24–48 h at 4 °C. This incubation period can be shortened or lengthened without compromising the integrity of dermal cells.

(iv) After 24–48 h, peel the epidermis away from the underlying dermis using fine (#55) forceps. The epidermis can be discarded.

? TROUBLESHOOTING

(v) Using a razor blade, mince the isolated dermal tissue into small, 1–2 mm^2 pieces. This takes approximately 30 min.

(vi) Place these small pieces of human dermis into a 15 ml conical tube containing 5–10 ml of fresh Blendzyme solution. DNaseI (one 400 µl aliquot) can also be added to the suspension to reduce aggregation of cells. For most efficient digestion of the tissue, we gently agitate the sample for 1–2 h at 37 °C.

(vii) Upon completion of the digestion, add 20 ml wash medium plus 10% FBS to inactivate the Blendzyme. Invert the tube 10–15 times. Centrifuge the tissue samples using a tabletop centrifuge at 1,200 r.p.m. for 6–8 min to pellet all cells and skin pieces. Discard the supernatant, which contains the medium plus enzyme.

(viii) Add 1 ml of fresh wash medium. Using a 10 ml disposable plastic pipette, begin mechanically dissociating tissue by repeatedly grinding the tissue against the walls and bottom of the tube with the tip of the pipette. Add 2–3 ml of wash medium and triturate the tissue further using either a 10 ml pipette or a 1,000 µl pipette tip. Briefly centrifuge the undissociated tissues for 20 s to pellet large pieces of skin at 1,200 r.p.m. in a table-top centrifuge. Remove and collect the supernatant into a 50 ml collection tube and keep it on ice. Again, add 1 ml wash medium to the tissue pellet and repeat the trituration step, adding the new supernatant to the collection tube. Repeat this cycle until the tissue pieces become thin and cells can no longer be liberated.

▲ CRITICAL STEP The supernatant should appear 'milky' with suspended cells. We normally repeat these dissociation steps 4–5 times, with each dissociation step lasting 2 min.

(ix) Pass the dissociated cell suspension in the supernatant tube through a 70 µm cell strainer into a 50 ml conical tube. Centrifuge the filtered cell suspension in a tabletop centrifuge at 1,200 r.p.m. for 7 min. Discard the supernatant and resuspend the cell pellet in wash medium plus 2% B27 supplement. Resuspension volumes range from 5 to 20 ml of medium depending on the size of the pellet, and can be adjusted to simplify quantification of cell yield.

(x) Determine the total number of skin cells using a hemocytometer. A dissociated cell suspension of 5 µl is typically diluted into 45 µl (1:10 dilution) of trypan blue as an indicator of cell viability. Typically, one human neonatal foreskin sample will yield approximately 10^7 cells.

(xi) Dilute the dissociated dermal cells into approximately 30 ml of proliferation medium for a 75 cm^2 flask and 10 ml for a 25 cm^2 flask. Conditioned medium is not typically required for primary human SKP cultures. Human cells are generally grown at higher cell densities of 25,000 or more cells ml^{-1} in order to enhance survival and increase yield of sphere formation. Secondary spheres can then be generated at clonal densities, according to experimental demand. Human SKPs, like rodent SKPs, are grown in these flasks in a 37 °C, 5% CO_2 tissue culture incubator as floating spherical colonies.

(xii) Allow growth of spherical colonies for 7–14 d without passaging. Over this period, cultures must be fed every 4–5 d with the addition of 1–2 ml fresh medium containing all growth factors and supplements (FGF2, EGF, B27) at a concentration that will replenish the entirety of the culture medium.

▲ **CRITICAL STEP** See Step 14 of the protocol for isolation of rodent SKPs.

▲ **CRITICAL STEP** Growth rates of human SKPs are significantly faster than those of their rodent counterparts, and spherical colonies are much larger. You can expect to see the first spherical colonies at about 4–5 d following initiation of culture, and these can initially be expanded for 10–14 d.

▲ **CRITICAL STEP** After 7–14 d in proliferation medium, you will observe large, phase bright, spherical colonies. These primary spheres should then be passaged (see below), particularly if the core of the spheres has begun to darken (signifying poor health of the central cells).

Passaging rodent and human SKPs

2 | Remove all medium, which contains the floating SKP spheres, from the tissue culture flask and collect it in a 10 or 50 ml conical tube, whichever is appropriate. Pellet the spheres by centrifugation in a tabletop centrifuge at 1,200 r.p.m. for 5 min (**Box 1**). Remove the conditioned medium supernatant.

▲ **CRITICAL STEP** Save the conditioned medium supernatant because it is essential for successful passaging and expansion of SKPs. This conditioned medium should be filtered through a 0.2 µm sterile filter and frozen at –20 °C for storage.

▲ **CRITICAL STEP** As the SKP spheres grow larger, cells in the central core will begin to darken, suggesting that the cells are being undernourished and require passaging. SKPs may also begin to differentiate, and will begin to adhere to the surface of the flask, suggesting that they require passaging.

? TROUBLESHOOTING

3 | For murine SKPs, resuspend the cell pellet in 1 ml of wash medium and gently triturate the spheres approximately 50 times with a 1,000 µl pipette tip, or until the spheres are dissociated to single cells. For rat or human SKPs, cells in the spheres are

BOX 1 | FREEZING AND SHIPPING SKPS

1. SKPs can be frozen for long-term storage and then recultured with little change in growth characterisics. To initiate freezing, pellet SKP spheres from a culture that is ready to be passaged by centrifuging in a tabletop centrifuge at 1,200 r.p.m. for 5 min. Remove and save the conditioned medium (as described in text), and resuspend the sphere pellet in freezing medium consisting of 90% FBS and 10% DMSO. Put the resuspended cell suspension in a 2 ml screwtop cryovial, and place it in a slow freeze container at –80 °C. Do not dissociate the spheres prior to freezing because this decreases cell viability.

2. Frozen SKPs should be shipped on dry ice. Upon receipt, frozen cells should be resuspended by thawing and immediately resuspending in 20 ml of wash medium to dilute the freezing medium. Mix the contents by repeated inversion of the tube.

3. Pellet the spheres by centrifugation at 1,200 r.p.m. for 5 min.

4. Resuspend spheres in 50% conditioned medium and 50% proliferation medium containing 40 ng ml^{-1} FGF2, 20 ng ml^{-1} EGF, and 2% B27. Put SKP suspension into a vented tissue culture flask and place in a 37 °C incubator. Cells should be not be disturbed for 24–48 h, at which time they should be fed with fresh proliferation medium.

As an alternative method, SKPs can be shipped at 4 °C in conical tubes:

1. Remove all medium and floating spheres from a 7–14 d old SKP culture, and collect it in a 10 or 50 ml conical tube.

2. Add 20% fresh proliferation medium (i.e., 2 ml in 10 ml total). Make sure the conical tube is completely filled with medium to minimize oxidation and changes in pH during shipping. Filtered, conditioned medium should be added if additional volume is required.

3. Upon receipt, SKPs should be resuspended by trituration with a 10 ml disposable, plastic pipette. This will also dissociate any spheres that may have aggregated during transport.

4. Remove entire contents of the tube into a vented tissue culture flask and place in a 37 °C incubator. Cells should be not be disturbed for 24–48 h, at which time they should be fed with fresh proliferation medium.

more adherent, and they require enzyme digestion for dissociation. For these latter two cell types, resuspend the cell pellet in 0.5 ml collagenase type XI (1 mg ml^{-1}) for 10–20 min at 37 °C, triturating intermittently. Trituration with a 100 µl tip may be required to break spheres into single cells. Following successful dissociation, add 20 ml of wash medium plus 10% FBS to the cell suspension to inactivate the collagenase and mix the suspension by repeated inversion. For all types of SKPs, pellet the newly dissociated cells by centrifugation at 1,200 r.p.m. for 6–8 min.

▲ **CRITICAL STEP** SKPs can be passaged either as single cells or as partially dissociated spheres. The partially dissociated spheres show enhanced proliferation and expansion, but cannot be used for experiments that require clonal spheres.

4 | Determine the total number of SKP cells using a hemocytometer. Typically, 5 µl of the dissociated cell suspension is diluted in 45 µl (1:10 dilution) of trypan blue as an indicator of cell viability. Plating density can be determined according to experimental demand. For routine maintenance passaging, we will typically split one flask into two or three new flasks.

5 | Resuspend the pelleted cells in 50% fresh proliferation medium containing twice the concentration of growth factors and supplements (80 ng ml^{-1} FGF2, 40 ng ml^{-1} EGF, and 4% B27) mixed with 50% of filtered, conditioned medium.

6 | Feed passaged SKPs every 4–5 d by adding 1–2 ml of filtered conditioned medium containing all growth factors and supplements (FGF2, EGF, B27) at a concentration sufficient to replenish the medium.

7 | Repeat the passaging every 7–14 d, or as required. Spheres should be passaged before central cells have begun to darken or with increasingly large numbers of spheres in the flask, particularly if spheres are in contact with each other or have begun to aggregate. For human SKPs we typically passage once every 2 weeks, splitting cells 1:2 in order to maintain low density sphere formation.

▲ **CRITICAL STEP** Mass expansion of SKPs spheres after passage is enhanced if spheres are not broken completely into single cells but rather into small clusters. This is especially true at later passages (i.e., passage 5 and later), when proliferation seems to slow down.

Differentiation, expansion and isolation of rodent SKP-derived Schwann cells ● TIMING 2–3 weeks

8 | Prepare culture plates. This step can be done as little as 6 h prior to the experiment or can be performed the night before differentiation is to commence. For successful differentiation and proliferation of SKP-derived Schwann cells, it is essential to coat tissue-culture-plate surfaces with laminin (0.02 mg ml^{-1}) and poly-D-lysine (0.2 mg ml^{-1}) overnight. For coating solution, add 200 µl of laminin and 100 µl poly-D-lysine to 10 ml of sterile H_2O. For each 10 cm dish, 8 ml of coating solution should be added, whereas for four- and eight-chamber plastic slides, 600 ml and 300 ml of coating solution should be added to each chamber, respectively. After incubation in a tissue culture hood for 6 h or overnight at room temperature, 18–24° C, wash slides/dishes with dH_20 and allow to dry.

? TROUBLESHOOTING

9 | Isolate SKP spheres from a culture that is ready to be passaged (see Step 1 on passaging rodent and human SKPs for criteria to make this decision) by transferring the medium containing the SKPs from the flask into conical plastic tubes, and then centrifuging these tubes in a tabletop centrifuge at 1,000 r.p.m. for 3–5 min. Remove the supernatant, and resuspend and dissociate the spheres to single cells as described in Steps 2 and 3. Following dissociation, use a hemacytometer and trypan blue to determine cell number, as described in Step 4.

▲ **CRITICAL STEP** Although this protocol works with mouse SKPs, we generally have much more success differentiating Schwann cells from rat SKPs, consistent with the finding that primary cultures of Schwann cells are easier to generate and expand from rat than from mouse.

? TROUBLESHOOTING

10 | Once you know the number of cells you have and the number of plates you would like to differentiate, determine the amounts of medium and cell suspension that you require to plate your cells at a density of 25,000–50,000 cells per ml with 10 ml cell suspension per 10 cm culture dish. Prepare this volume of SKP-derived Schwann cell differentiation medium I. For example, if you want to make up five 10 cm culture dishes for differentiation and you have a cell suspension of 8×10^6 cells per ml, then you will need to make 50 ml of SKP-derived Schwann cell differentiation medium I and add 313 µl of cell suspension.

▲ **CRITICAL STEP** For the differentiation to occur optimally, first culture cells in SKP-derived Schwann cell differentiation medium I containing 10% FBS for 3 d in order to increase cell numbers and promote differentiation and adherence to the substrate. We have found that, although not absolutely essential, this step greatly enhances the survival of SKPs and

ultimately their Schwann cell progeny. However, increasing levels of FBS for long periods may be detrimental because it also stimulates growth of other differentiating cell types. We suggest that following the proliferation stage, complete removal of FBS, or a reduction to 1% FBS, is optimal. Increasing concentration of FBS should be considered only when differentiating at low cell densities.

▲ **CRITICAL STEP** Although a cell density of 25,000–50,000 cells per ml is optimal at this stage, lower cell densities can also be used.

? TROUBLESHOOTING

11 | Resuspend cells in SKP-derived Schwann cell differentiation medium I using a 1,000 µl pipette tip or 10 ml pipette and add the appropriate volume of the cell suspension to poly-D-lysine/laminin-coated chamber slides or culture dishes. For four- and eight-chamber slides, add 600 or 300 µl to each chamber, respectively, and for a 10 cm culture dish, use 10 ml. Culture these cells in a 37 °C, 5% CO_2 incubator for 3 d.

? TROUBLESHOOTING

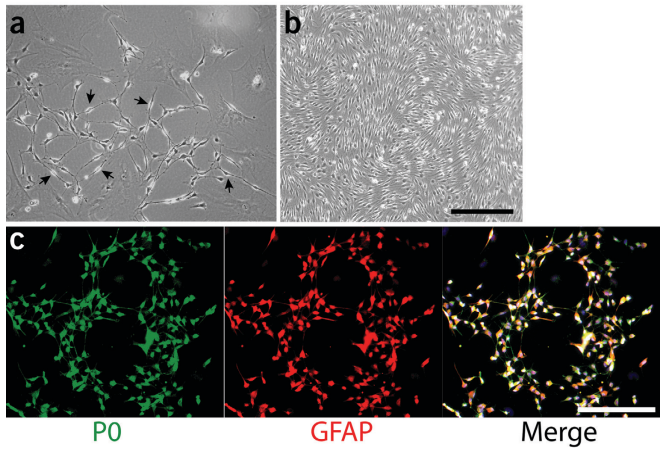

Figure 2 | Morphology of SKP-derived Schwann cells. (**a**) Phase contrast image showing a typical colony of proliferating SKP-derived Schwann cells following 2 weeks of differention in Schwann cell medium. Arrows indicate cells with phase bright, bipolar morphology of Schwann cells. (**b**) High magnification image of an isolated and expanded colony of purified SKP-derived Schwann cells arranged in parallel arrays. Scale bar represents 200 µm. (**c**) SKP-derived Schwann cells immunostained for Schwann cell markers peripheral myelin associated protein P0 (green) and GFAP (red). Nuclei are stained with Hoechst dye and shown in blue. Scale bar represents 200 µm.

12 | After 3 d, change the medium to Schwann cell differentiation medium II. To do this, remove 50% of the medium from the slide or dish and replace with fresh Schwann cell differentiation medium II containing 2× concentration of all the growth factors and supplements. Culture the cells in the incubator, changing the medium every 2–3 d by removing 50% of the medium and replacing it with the same volume of Schwann cell differentiating medium II containing 2× concentration of supplements and growth factors. It should take approximately 2–3 weeks before the appearance of SKP-derived Schwann cell colonies. These colonies can be identified morphologically as colonies of bipolar cells (**Fig. 2a,b**). Alternatively, they can be detected immunocytochemically by their expression of marker proteins such as GFAP, S100β, p75 neurotrophin receptor or peripheral myelin protein 0 (P0) (**Fig. 2c**). If the purpose of the experiment is to isolate and expand the Schwann cells, then it will be necessary to learn how to identify the colonies morphologically.

? TROUBLESHOOTING

13 | To generate large numbers of purified SKP-derived Schwann cells, isolate Schwann cell colonies mechanically using a cloning cylinder, as described in **Box 2**.

? TROUBLESHOOTING

BOX 2 | ISOLATION OF SCHWANN CELL COLONIES

1. Sterilize cloning cylinders by autoclaving.

2. Identify colonies of SKP-derived Schwann cells morphologically under the tissue culture microscope, and then place a pen mark on the underside of the slide or dish to identify the location(s) of these colonies.

3. Remove the medium from the tissue culture dish or slides, and coat one end of the cloning cylinder with sterile vacuum grease. With the aid of a low magnification inverted tissue culture microscope, place the cylinder over an individual Schwann cell colony, and press it down to form a tight seal around the colony. Fill the cylinder with 200–500 µl of trypsin versene or trypsin EDTA (undiluted) and incubate at 37 °C for 5 min. Cells should then be easily detached from the substrate with gentle trituration.

4. Remove all contents from the cylinder (including the putative Schwann cells), into a conical tissue culture tube and dilute the cell suspension in 10 ml of wash medium containing 10% FBS to inactivate the enzyme. Mix the suspension by repeated inversion and then pellet cells by centrifugation in a tabletop centrifuge at 1,200 r.p.m. for 6–8 min. Cells can then be resuspended in fresh Schwann cell differentiation medium.

Note: Plating these trypsinized cells at high density will improve cell viability and the efficiency of expansion.

BOX 3 | SCHWANN CELL FREEZING AND STORAGE

1. Passage cells when they reach confluence (anywhere from 7–14 d, depending upon the initial density of the culture).

2. Remove and discard medium, and remove cells from the substrate enzymatically by adding 2–3 ml of trypsin versene (for a 10 cm dish) and incubating the dishes at 37 °C for 5 min.

3. Rinse the cells from the surface of the plate using wash medium, and collect the cell suspension in a 15 ml conical tube. Add 7 ml of fresh wash medium containing 10% FBS to deactivate the trypsin

4. Following trypsinization and inactivation with FBS, pellet cells by centrifugation at 1,200 r.p.m. for 6 min. Resuspend the cells in freezing medium (90% FBS and 10% sterile DMSO) at a density of 2×10^6 cells ml^{-1}. Aliquot 1 ml of suspension into cryogenic vials and immediately place into a cryofreeze container at –80 °C.

Regarding human SKPs, although the addition of factors such as neuregulin and forskolin enhances the generation of Schwann cells, our protocol for generating human SKP-derived Schwann cells has not yet been optimized.

14 | Plate the resuspended cells in laminin/poly-D-lysine-coated chamber slides or tissue culture dishes, and place them in a 37 °C, 5% CO_2 incubator. These SKP-derived Schwann cells, which grow adherently, should be fed 80% fresh Schwann cell differentiation medium II without serum every 3–4 d. We have found that serum is no longer required for proliferation and maintenance of SKP-derived Schwann cells after the initial differentiation step.
? TROUBLESHOOTING

15 | When they reach confluence (anywhere from 7–14 days, depending upon the initial density of the culture), passage SKP-derived Schwann cells, as follows.
? TROUBLESHOOTING

16 | Remove and discard medium, and remove cells from the substrate enzymatically by adding 2–3 ml of trypsin versene (for a 10 cm dish) and incubating the dishes at 37 °C for 5 min.
? TROUBLESHOOTING

17 | Rinse the cells from the surface of the plate using wash medium, and collect the cell suspension in a 15 ml conical tube. Add 7 ml of fresh wash medium containing 10% FBS to deactivate the trypsin.
? TROUBLESHOOTING

18 | Mix the cell suspension by repeated inversion and then pellet the cells by centrifugation at 1,200 r.p.m. for 6–8 min. The cell pellet can then be gently resuspended in new Schwann cell differentiation medium II using a 1,000 µl pipette tip. For maximum expansion and passaging, we typically split one confluent 10 cm culture dish into four or five 10 cm dishes. This would mean resuspending the cell pellet obtained from one culture dish into 50 ml fresh differentiation medium, and then putting 10 ml of this suspension into each of five different 10 cm culture dishes. SKP-derived Schwann cells can also be frozen and stored indefinitely at –80 °C, as described in **Box 3**.
? TROUBLESHOOTING

● TIMING
Step 1A(i)–(xiv), dissociation of skin and preparation of culture: 4–6 h
Step 1A(xv), growth and expansion of SKPs: 1–3 weeks
Step 1B(i–xi), dissociation of human skin cells and preparation of culture: 48 h
Step 1B(xii), growth and expansion of human SKPs: 2–3 weeks
Steps 2–7, passaging of SKPs: 1–3 weeks
Steps 8–12, SKP-derived Schwann cell differentiation: 1–3 weeks
Steps 13–18, isolation and expansion of SKP Schwann cells: 1–2 weeks

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
Step 1	Too few cells liberated from skin sample	Insufficient digestion	Increase digestion time or concentration of collagenase, or obtain enzyme from a new source
		Insufficient dissociation	Increase trituration and homogenization of sample and ensure all supernatant during dissociation is saved
Step 1	Absence of spheres after 10 d in proliferation media	Poor reagents (FGF2, EGF, B27)	Try new stocks, different lot or change vendor
		Inappropriate number of cells plated	Increase/decrease density of cells plated. Densities that are too high or too low will result in malnourished cultures and will retard proliferation
		Poor dissociation of skin cells	Increase duration of collagenase digestion, or increase trituration of tissue
		Adherence and premature differentiation of SKPs	After 4 d *in vitro*, transfer all media and floating spheres to new flask to avoid cell contact between floating spheres and adherent epidermal cells
Step 1	Epidermis does not peel away from the dermis	Insufficient time of incubation in the Blendzyme	Lengthen the time of incubation in Blendzyme
		Pieces of skin may not be small enough to allow penetration of enzyme	The skin should be cut smaller
Step 2	Passaged cells are not generating spherical colonies	Cells lacking critical survival factors	Grow passaged cells in 100% conditioned medium containing all growth factors and supplements
		Cells compromised during dissociation of SKPs	Reduce concentration or duration in collagenase, or be more gentle during the mechanical trituration
Steps 8–18	Absence of Schwann cells after 3 wk	Poor survival	Reduce concentration of serum, or increase neuregulin-1β
		Poor coating of substrate	Coat surface with fresh laminin and poly-D-lysine
		Cell density not high enough	Increase plating density, or serum concentration

ANTICIPATED RESULTS

Generating SKPs

We have found that in postnatal rat back skin, approximately 0.5–1% of cells in the skin will generate SKP spheres. As a result, the majority of cells plated in each flask will either adhere to the flask (these mostly comprise epidermal cells and dermal fibroblasts) and others will die. Primary spherical colonies should be observed after 5–7 d of exposure to FGF2 and EGF (i.e., proliferation medium). After 7–14 d *in vitro*, you will observe large numbers of spheres, of varying size, potentially a result of the heterogeneous capacity for self-renewal between stem cells and more restricted progenitors within the SKP spheres. **Figure 1a** illustrates examples of a SKP culture after 10 d in SKPs proliferation medium. The quality of SKP spheres (and not aggregation) can also be verified by immunocytochemistry for characteristic SKP markers such as nestin, versican (shown in **Fig. 1b**), fibronectin and vimentin. It is noteworthy that both rat and human SKPs show more robust growth than murine cells.

Generating Schwann cells

The appearance of Schwann cell colonies will be apparent following 2–3 weeks of culture. These colonies may vary in number (i.e., 5–100 Schwann cells) and the majority of cells within the culture will consist of other cell types. SKP-derived Schwann cells exhibit stereotypic Schwann cell characteristics such as a phase-bright cell body with thin bipolar extensions. Initially, SKP-derived Schwann cell colonies will look disorganized (shown in **Fig. 2a**), but with increasing numbers and density, cells

will align into swirling parallel arrays, particularly following expansion of an isolated SKP-derived Schwann cell colony (shown in **Fig. 2b**). It is important to note that the presence of other adherent differentiating cells within the culture will inhibit growth and proliferation of Schwann cells. Thus, the Schwann cell colonies generated from primary SKP differentiations will rarely expand into large swirling arrays as can be seen following isolation and replating. We have found that addition of heregulin and forskolin significantly enhances generation of SKP-derived Schwann cells relative to N_2 supplement alone. Indeed, in several of our clonal sphere differentiation experiments we have observed that up to 80% of spheres generate S100β+ or Glial Fibrillary Acidic Protein (GFAP)+ cells as opposed to less than 5% when differentiated in N_2 supplement alone[31]. SKP-derived Schwann cells can be identified *in vitro* by a variety of immunocytochemical markers specific to Schwann cells, including GFAP, S100β, peripheral myelin protein P0, myelin basic protein, p75 neurotrophin receptor, peripheral myelin protein 22 and CNPase. An example of SKP-derived Schwann cells expressing GFAP and P0 is shown in **Figure 2c**.

COMPETING INTERESTS STATEMENT The authors declare competing financial interests. See the HTML version of this article for details.

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