PROTOCOLS FOR FULL THICKNESS SKIN WOUND REPAIR USING PREVASCULARIZED HUMAN MESENCHYMAL STEM CELL SHEET

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Abstract

Split thickness skin grafts (STSGs) are one of the standard treatments available for full thickness wound repair when full thickness grafts are not viable, such as in the case of wounds with large surface areas. The donor sites of STSGs may be harvested repeatedly, but STSG transplants are still limited by insufficient blood supply at the early stages of wound healing. Prevascularized human mesenchymal stem cell (hMSC) sheets may accelerate wound healing and improve regeneration by providing pre-formed vessel structures and angiogenic factors to overcome this limitation. This book chapter provides the protocol of co-culturing hMSCs and endothelial cells to attain a prevascularized hMSC cell sheet. The protocols for implantation of the prevascularized stem cell sheet for full thickness skin wound repair in a rat autologous skin graft model as well as the evaluation of the wound healing effects are also provided.

Key Words: Split thickness skin grafts, human mesenchymal stem cells, prevascularization, full thickness skin wound, wound repair

1. Introduction

Burn injuries represent a leading cause of full thickness skin wounds, accounting for approximately 15,000 – 20,000 hospitalizations per year in the United States [1]. Transplantation of an autologous full thickness graft (FTG) or a skin flap remains the current gold standard of treatment due to immunological acceptance and close match to native skin color and texture [2]. However, the limited supply of donor skin and unavoidable donor site injury restricts the ability to treat extensive wounds with FTGs.

An autologous split thickness skin graft (STSG) on the other hand, in which only the epidermis and a portion of the dermis are harvested rather than the full skin thickness, can be harvested repeatedly to cover larger wounds and can be used under conditions that would cause a FTG to fail, such as low vasculature or moderate infection at the wound site [3]. The disadvantage is that STSG can contract significantly during healing, leading to poor cosmetic outcome, physical disability, and reduced pliability [4]. Including engineered dermal substitutes with the STSG can reduce contraction, but the limited blood supply at early stages of transplantation cause these grafts to experience relatively long hypoxic and ischemic periods after surgery and suffer from degeneration and necrosis [5].

Sufficient blood supply is the primary factor that determines the quality of a transplanted STSG. The graft becomes engorged by plasmatic fluid during the first 48 hours of transplantation, and a poorly vascularized bed hinders plasmatic diffusion [6]. Effective delivery of oxygen, nutrients, and growth factors can be achieved by ensuring that the transplanted graft has sufficient vascular support at these early stages [7]. The inclusion of a prevascularized MSC cell sheet may provide this essential early support while also promoting graft integration to improve wound

healing and enhance therapeutic outcome [8, 9]. Our previous study has shown that a uniform human MSC (hMSC) cell sheet (HCS) can be obtained by growing hMSCs under a physiologically low oxygen concentration (2% O_2), which helps to maintain the stemness of the cells [10]. We have also achieved a prevascularized hMSC cell sheet (PHCS) by co-culturing endothelial cells (ECs) on top of the HCS under a normoxic condition to promote angiogenesis and neovascularization [11]. After implantation of the PHCS in a rat autologous full thickness skin wound model, we have found that the PHCS could significantly reduce skin contraction and improve cosmetic appearance relative to the STSG control group [12]. This book chapter provides the protocol of co-culturing hMSCs and endothelial cells (ECs) to attain a HCS and a PHCS. The protocols for implantation of the prevascularized stem cell sheet for full thickness skin wound repair in a rat autologous skin graft model as well as the evaluation of the wound healing effects are also provided in details.

2. Materials

2.1 Cell Sheet Culture

1. Human mesenchymal stem cells (hMSCs) are expanded in complete α-minimum essential medium (α-MEM) with 20% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin (Life Technologies, Rockville, MD). Passage 3 to 5 hMSCs are used for cell sheet culture (*see* **Note 1**).

2. Endothelial cells (ECs) are expanded in endothelial cell growth medium (EGM-2, BulletKit, Lonza). Passage 3 to 5 ECs are used for cell sheet culture (*see* **Note 2**).

2.2 Animals

1. Sprague Dawley (SD) rats (weighing 180-200g) are used in the animal study under the approval of University Institutional Animal Care and Use Committee (IACUC).

2.3 Solutions for Tissue Fixation and Staining

1. Cell sheet fixation solution: 4% paraformaldehyde (PFA), commercially available.

2. Phosphate-buffered saline (PBS), commercially available.

3. Cell sheet permeabilization solution: 0.2% Triton X-100 in PBS.

4. Blocking buffer: 1.0% bovine serum albumin (BFA) in 0.2% Triton X-100 in PBS.

5. Cell nuclei staining solution: 4',6-diamidino-2-phenylindole (DAPI) solution, commercially available.

6. Hematoxylin solution, commercially available.

7. 1% Eosin Y solution, commercially available.

8. Biebrich Scarlet-Acid Fucshin, commercially available.

9. Working phosphotungstic/phosphomolybdic acid solution: mix 1 volume of phosphotungstic acid solution and 1 volume of phosphomolybdic acid solution with 2 volumes of deionized water (DI H_2O). The solution is discarded after one use.

10. Aniline blue solution, commercially available.

11. Acetic acid, commercially available.

12. Sodium citrate buffer solution (PH 6.0), commercially available.

13. VECTASTAIN® Elite ABC Reagent, commercially available.

14. 3,3'-diaminobenzidine (DAB) substrate kit, commercially available.

15. Antigen unmasking solution: sodium citrate buffer solution (PH 6.0), commercially available.

3. Methods

3.1 Preparation of hMSC Cell Sheet (HSC)

1. Seed hMSCs on coated collagen 1-coated cover glasses at a density of 10,000 cells/cm^2.

2. Culture the cells under 2% O_2 for 4 weeks in complete α-MEM medium. The medium is changed every two days.

3. Harvest hMSC sheets by gently peeling the cell layers off the cover glass. The size of the cell sheet is decided by the size of the glass cover slip (*see* **Note 3**).

4. For evaluation of thickness, fix the detached hMSC sheet in 4% PFA and measure with confocal microscope.

3.2 Preparation of Pre-vascularized hMSC Cell Sheet (PHCS)

1. Seed ECs on top of hMSC sheets at a density of 20,000 cells/cm^2.

2. Culture under 20% O_2 for 1 week in EGM-2. Change medium every other day.

3. Harvest the PHCS by gently peeling the cell layers off the cover glass.

3.3 Microvessel Observation

1. Wash PHCS with PBS for 3 times.

2. Fix the cell sheet with 4% PFA.

3. Permeabilize the sample with 0.2% Triton X-100 in PBS.

4. Block the sample with 1.0% BFA in 0.2% Triton X-100 in PBS.

5. Incubate the sample with primary anti-CD 31 antibody (*see* **Note 4**).

6. Incubate the sample with DyLight 488 secondary antibody (*see* **Note 5**).

7. Stain cell nuclei with DAPI solution.

8. Image with fluorescence microscope (*see* **Note 6**).

3.4 Full Thickness Excision Wound Creation and Graft Transplantation

1. Put the rats in anesthesia box and anesthetize rats with inhaled gas anesthesia (O_2, 2 L/min; isoflurane, 2%).

2. Scrub the dorsal skin of the rat with Betadine Veterinary Surgical Scrub twice and the use a 70% alcohol gauze to remove Betadine, wait for 1 minute to let the operation area dry up.

3. Use iris scissors to create a round full-thickness excisional wound with a diameter of 20 mm on the dorsum. Make sure all procedures are done under sterile conditions.

4. Use iris scissors to remove the deep partial dermis and panniculus carnosus away from the round excised skin to attain the STSG. Protect the dermal side of the STSG with saline gauze (**Figure 1 A**).

5. Use a sharp tipped scalpel to pierce the skin and create evenly spaced fenestrations on the grafts for secretion drainage (the length of every fenestrations should be less than 2 mm).

6. Transplant three cell sheets onto the wound site.

6.1 Discard the culture medium by use of Pap dropper. Make sure the cultured cell sheets are untouched.

6.2 Rinse the cell sheets with PBS twice.

6.3 Use sharp tipped tweezers to pry the glass cover from the bottoms of culture plate. Then set it on the wound bed of the rat, cell sheet side up.

6.4 Use sharp tipped tweezers to strip off the cell sheet.

6.5 Remove the glass cover.

7. Lay the autologous STSG on top of the cell sheets (*see* **Note 7**).

8. Approximate the grafts to the adjacent wound margin and suture with interrupted stitches. The stitches have to be applied 2-3mm apart from each other. After knotting, leave the thread uncut (**Figure 1 B**).

9. Take photos for the grafts in the same exposure setting, from the same distance and angle.

10. Clean the grafts and the skin around by use of saline gauze.

11. Apply a layer of sterilized, transparent, plastic, standard measuring film on the top of each graft.

12. Delineate the shape of the graft by use of fine tipped surgical marker.

13. Clean the grafts and the skin around by use of saline gauze.

14. Apply folded cotton gauze on the grafts and affix the gauze with wrapped elastic bandage.

15. Apply a padded bolster (folded cotton gauze) on top of the skin grafts and use the threads to affix it (knotted the threads that are in a diagonal) to provide appropriate pressure (the bolster is affixed by the knotted threads and cannot be easily lifted from the skin) and prevent scratching.

16. House animals in individual cages post-surgery.

17. Change dressings 3 days after surgery and remove on day 7.

3.4.1 Changing wound dressings (days 3 and 7)

1. On post-operational day 3, put the rats in anesthesia box and anesthetize rats with inhaled gas anesthesia (O_2, 2 L/min; isoflurane, 2%). Use sharp tipped scissors to cut off the threads. Gently remove all the dressing from the graft.

2. Take photos for the grafts in the same exposure setting, from the same distance and angle. Apply a layer of sterilized, transparent, plastic, standard measuring film on the top of each graft.

3. Delineate the shape of the graft by use of fine tipped surgical marker.

4. Clean the grafts and the skin around by use of saline gauze.

5. Apply folded cotton gauze on the grafts and affix the gauze with wrapped elastic bandage.

6. On post-operational day 7, put the rats in anesthesia box and anesthetize rats with inhaled gas anesthesia (O_2, 2 L/min; isoflurane, 2%).

7. Gently remove all the dressing from the graft.

8. Clean the grafts and the skin around by use of saline gauze.

9. Put the rats back in their own cages.

3.4.2 Wound site observation (*see* **Note 8**)

1. At each observation time points, put the rats in anesthesia box and anesthetize rats with inhaled gas anesthesia (O_2, 2 L/min; isoflurane, 2%).

2. Take photos for the grafts in the same exposure setting, from the same distance and angle.

3. Clean the grafts and the skin around by use of saline gauze.

4. Apply a layer of sterilized, transparent, plastic, standard measuring film on the top of each graft.

5. Delineate the shape of the graft by use of fine tipped surgical marker.

6. Clean the grafts and the skin around by use of saline gauze.

7. For post-operational day 0 and 3 rats, apply folded cotton gauze on the grafts and affix the gauze with wrapped elastic bandage.

8. For post-operational day 7, 14, 21 and 28 rats, wait for the rats to regain consciousness and put the rats back to their own cages, or sacrifice the rats.

9. Cut the measuring film along the mark.

10. Weigh the measuring film.

11. Measure skin graft contraction via gravitational planimetry (*see* **Note 9**).

3.4.3 Tissue collection

1. Sacrifice 6 rats in each group for each time point and harvest grafts and surrounding normal tissue (including the skin and subcutaneous tissue).

2. At each time point, put the rats in anesthesia box and anesthetize rats with inhaled gas anesthesia (O_2, 2 L/min; isoflurane, 2%).

3. Apply cervical dislocation method to sacrifice the rats.

4. Use sharp tipped scissors to cut off the graft and its surrounding normal skin (about 0.5 cm wide).

5. Cut samples into two parts, make sure they are of approximate size.

6. Immerse samples in 10% formalin and fix the tissue at room temperature for 24 hours (*see* **Note 10**).

3.4.4. Dehydrate fixed samples (*see* **Note 11**)

1) Put tissues in a pencil labeled cassette.
2) Immerse cassette in 70% ethanol for 1 hour.
3) Immerse cassette in 95% ethanol (95% ethanol/5% methanol) for 1 hour.
4) Immerse cassette in absolute ethanol for 1 hour.
5) Immerse cassette in absolute ethanol for 1½ hours. Perform this procedure twice with fresh solution each time.
6) Immerse cassette in absolute ethanol for 2 hours.

7) Immerse cassette in clearing agent (xylene or substitute) for 1 hour. Perform this procedure twice with fresh solution each time.

8) Immerse cassette in wax (paraplast X-tra) at 58 °C for 1 hour. Perform this procedure twice with fresh solution each time.

9) Open cassette to view the skin sample and choose a mold that best corresponds to the size of the sample. Discard cassette lid.

10) Put small amount of molten paraffin in mold, dispensing from paraffin reservoir. Use warm forceps to transfer skin sample into mold, placing cut side down, as it was placed in the cassette.

11) Transfer mold to cold plate. Add the labeled tissue cassette on top of the mold as a backing when the tissue is in the desired orientation.

12) Add hot paraffin to the mold. Make sure there is enough paraffin to cover the face of the plastic cassette.

13) Fill cassette with paraffin while cooling, keeping the mold full until solid (about 30 minutes).

17) Pry mold when the wax is completely cooled and hardened (**Figure 2 A**).

3.5 Pathology Analysis: Staining, Imaging, and Analysis

3.5.1 Section the paraffin embedded tissue blocks

1) Turn on the water bath and adjust the temperature to 35-37 ^{o}C.

2) Use a microtome to section paraffin embedded tissues.

3) Place the blocks face down on an ice block for 10 minutes.

4) Place a fresh blade on the microtome; Insert the block into the microtome chuck (placing cut side of the sample parallel with the blade (**Figure 2 B**).

5) Set the dial to cut several 10 µM sections to order to plane the block; once it is cutting smoothly, set to 5 µM and cut.

6) Pick tissue sections with 5 µm thickness up with fine paint brush and float them on the surface of the 37ºC water bath.

7) Float the sections onto the surface of clean glass slides.

8) Place the slides with paraffin sections on the warming block in a 65 °C oven for 20 minutes to melt the wax (to bond the tissue to the glass) (**Figure 2 C**).

9) Use for staining.

3.5.2 Dewax and rehydrate samples (*see* **Note 12**)

1) Dry heat the slides under 60 °C for 2 hours.

2) Immerse the slides in xylene for 5 minutes. Perform this procedure thrice with fresh solution each time.

3) Immerse the slides in 100% ethanol for 5 minutes. Perform this procedure twice with fresh solution each time.

4) Immerse the slides in 80% ethanol for 5 minutes.

5) Immerse the slides in DI H_2O for 5 minutes.

3.5.3 Hematoxylin and eosin (HE) staining and Masson's Trichrome staining (*see* **Note 13**)

3.5.3.1 HE Staining (*see* **Note 14**)

1) Put the slides into DI H_2O and leave the slides there for 5 minutes.

2) Put the slides into a staining jar containing 210 mL Hematoxylin solution and leave the slides there for 5 minutes.
3) Put the slides into tap water, then leave the staining jar under running tap water for 10 minutes, make sure the running tap water does not directly hit the slides.
4) Put the slides into DI H_2O and leave the slides there for 2 minutes.
5) Put the slides into a staining jar containing 210 mL 1% Eosin Y solution for 30 seconds.
6) Put the slides into tap water, then leave the staining jar under running tap water for 10 minutes, make sure the running tap water does not directly hit the slides.
7) Dehydrate the slides with 95% ethanol for 1.5 minutes twice, with fresh solution each time.
8) Further dehydrate the slides with absolute ethanol for 1.5 minutes twice, with fresh solution each time.
9) Immerse the slides in xylene for 1.5 minutes three times, with fresh xylene each time.
10) Drop 1 drop of resinous mounting medium and seal the section using glass cover.
11) Observe and image the tissue morphology (*see* **Note 15**).

3.5.3.2 Masson's Trichrome staining (*see* **Note 16**)

1) Immerse the slides in hematoxylin solution for 5 minutes.
2) Put the slides into a staining jar containing tap water, then leave the staining jar under running tap water for 5 minutes, make sure the running tap water does not directly hit the slides.
3) Put the slides into DI H_2O and leave the slides there for 5 minutes.
4) Put the slides into a staining jar containing 210 mL Biebrich Scarlet-Acid Fucshin for 5 minutes.

5) Put the slides into DI H_2O and leave the slides there for 5 minutes. Put the slides into a staining jar containing 210 mL phosphotungstic/phosphomolybdic acid solution for 5 minutes.
6) Place slides in into a staining jar containing 210 mL aniline blue solution 5 minutes.
7) Place slides in into a staining jar containing 210 mL acetic acid for 2 minutes. Discard solution.
8) Put the slides into DI H_2O and leave the slides there for 1 minute twice, with fresh DI H_2O each time.
9) Dehydrate the slides with 95% ethanol for 1.5 minutes twice, with fresh solution each time.
10) Further dehydrate the slides with absolute ethanol for 1.5 minutes twice, with fresh solution each time.
11) Immerse the slides in xylene for 1.5 minutes three times, with fresh xylene each time.
12) Drop 1 drop of resinous mounting medium and seal the section using glass cover.
13) Observe and image the tissue morphology (*see* **Note 15**).

3.5.4 Immunohistochemistry (IHC), Ki67 and NM95 staining (*see* **Note 17**)**.**

1) Antigen unmasking: add 200ml sodium citrate buffer solution (PH 6.0) in plastic staining jar. Place it in Black and Decker vegetable steamer and steam for 30 minutes. Put all sections into the jar when the Sodium citrate buffer solution is 95-98°C, 10 minutes.
2) Take out the staining jar and leave it at room temperature for 60 minutes to slowly cool down the slides. This will prevent tissue destruction caused by drastic temperature change.
3) Incubate the sections for 30 minutes in 0.3% H_2O_2.
4) Wash in 10 mM sodium phosphate (PBS), pH 7.5, for 5 minutes.

5) Apply background buster on slides, incubate 30 minutes at room temperature. Drain excess buster. No wash.
6) Apply rabbit anti-CD68 or diluted rabbit anti-Ki67 and incubate for 45 minutes at room temperature. Immerse slides 2 times in PBS, 1 minute each, drain excess PBS.
7) Apply biotinylated secondary antibody (goat anti-rabbit) and incubate for 30 minutes. Repeat wash as in step 6.
8) Apply Vectastain Elite ABC and incubate for 30 minutes, repeat wash as in step 7.
9) Apply DAB chromagen and incubate for 5 minutes, wash in 3 changes of DI H_2O. Rinse in tap water for 2 minutes.
10) Counterstain in hematoxylin for 30 seconds. Rinse slides in tap water until clear.
11) Blue nuclei by dipping 4 times in ammonium water, rinse in tap water for 2 minutes.
12) Immerse the slides in xylene for 1.5 minutes three times, with fresh xylene each time.
13) Drop 1 drop of resinous mounting medium and seal the section using glass cover.
14) Observe and image the tissue morphology (*see* **Note 15**).

3.6. Image Analysis (*see* **Note 18**)

3.6.1 Count (microvessels, hair follicles, and cells) (*see* **Note 19**)

Open imageJ→hit "File" →hit "Open"→ open one photo→hit "Type"→hit "8-bit"→hit "Image" → hit "Adjust" → hit "Threshold" →hit "Auto" →hit "Apply" →hit "Analyze Particles"——> choose "summarize".

3.6.2 Measure epidermal thickness

Open imageJ→hit "File" →hit "Open"→ open one photo→hit "Straight Line Selection"→measure the scale bar in the photo→hit "Analyze"→hit "Tool" →hit "Scale Bar"

→chose any color you like→hit"OK" →use the mouse to draw a line starts from the epidermal basment membrane and stops at the surface of the epidermis (make sure the line is perpendicular to the epidermal basement membrane).

3.6.3 Measure collagen index

Open imageJ→hit "File" →hit "Open"→ open one photo→hit "Plugins"→hit "Analyze" →hit "Measure RGB"→ write the "means" value from the "Results" window into excel file→ use formula: collagen index= sum (blue mean value, green mean value)/sum(2*red mean value, blue mean value, green mean value).

3.6.4 Vessel area quantification (*see* **Note 20**)

Open imageJ→hit "File" →hit "Open"→ open one photo→hit "Straight Line Selection"→measure the scale bar in the photo→hit "Analyze"→hit "Set Scale" →enter the length of the scale bar in "known distance", enter the unit of the scale bar in unit of length→ hit "Global" →hit "OK" → hit "rectangular" → use the mouse to draw a rectangular frame →hit "Analyze" →hit "Analyze Measurements" →hit "Area" →the result will be the area of the entire photo→enter it in excel→hit "Freehand Selections" → use the mouse to track the shape of every vessels→hit "Analyze" →hit "Analyze Measurements" →hit "Area" →the result will be the area of every vessel→ enter it in excel→ sum up all measured data of vessel area and divided it by the total area of the photo.

4. Notes

1. The hMSCs that we use are from Texas A&M University Health Sciences Center.

2. The ECs that we use are human umbilical vein endothelial cells (HUVECs). The cells are from Lonza (Walkersville, MD).

3. The strong interaction between the cells as well as the cells and the ECM in the cell sheet can overcome the cell adhesion force between the cells and the glass cover slip. Thus, the cell sheet can be easily peeled off from glass cover slip.

4. The primary anti-CD 31 antibody that we use is obtained from Abcam (Cambridge, MA). The other antibodies anti-CD68 and anti-Ki67 mentioned in this protocol are also from Abcam.

5. The DyLight 488 secondary antibody we use is obtained from VECTOR Laboratories (Burlingame, CA). The biotinylated secondary antibody (goat anti-rabbit) mentioned in the text is also from VECTOR Laboratories.

6. A vessel network was defined as a contiguous length of interconnected capillaries. Six low-magnification (20x) images on three individual samples in each group were used for calculation.

7. STSG transplantation without a cell sheet is used as a control.

8. In our study, we observe wound site at days 0, 3, 7, 14, 21, and 28.

9. Since the material density and the thickness of the measuring film are the same, the skin graft contraction can be measured by gravitational planimetry (graft size*thickness*material density=weight of the measuring film) and expressed as a percentage of the remaining skin graft size to its original wound size (relative skin graft size % = (remaining skin graft size/Original skin graft size)*100%).

10. Tissue fixation is a critical step in the preparation of histological sections by which biological tissues are preserved from degradation and putrefaction. Fixation terminates any ongoing

biochemical reactions, and may also increase the mechanical strength or stability of the treated tissues. For skin tissue fixation, neutral buffered Formalin solution is normally used.

11. Dehydrate fixed samples through a graded series of ethanol wash and embed in paraffin. The tissue samples are dehydrated to displace water contained in the samples. Paraffin is used to infiltrate tissues. The paraffin-embedded tissues can be stored for many years.

12. To stain the sections, the wax needs be removed and the samples need to be rehydrated. During the dewax and rehydration processes, the sections need go through multiple changes of xylene, series ethanol solutions (100% to 0%), and finally DI H_2O.

13. The HE and Masson's trichrome staining are conducted according to the manufacturer's standardized protocols.

14. The HE staining is the most commonly used staining method for tissue sections. Hematoxylin stains nuclei of cells and a few other objects, such as keratohyalin granules. The color is blue.

The Eosin Y stains eosinophilic and other structures in various shades of red, pink and orange. This staining technique is essential for recognizing various tissue types and their morphologic changes.

15. At least five stained sections from each group at each time point should be observed. At least three random views should be imaged.

16. Masson's trichrome is suited for distinguishing cells from surrounding connective tissue and for evaluating collagen deposition (collagen index) as well as morphologic change.

17. Ki67 is a nuclear antigen expressed in proliferating cells. It is used to evaluate the proliferative activity of cells in the tissues. NM95 recognizes an antigen associated with the nucleoli in human cells. It is used to determine whether implanted human cells (hMSCs and ECs) actually participated in the process of skin wound healing after grafting.

18. The HE staining images can be used to measure epidermal thickness and count microvessels (**Figure 3 A**), hair follicles (**Figure 3 B**), as well as cells and skin tissues (**Figure 3 C**) in high-power field (HPF) using ImageJ software. The collagen index can be measured using the images from Masson's Trichrome staining (**Figure 3 D**).

19. Microvessels are identified as small circles of endothelial cells with red blood cells in them

20. Quantification is defined as the sum of all vessel segments/HPF, using ImageJ.

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Figures

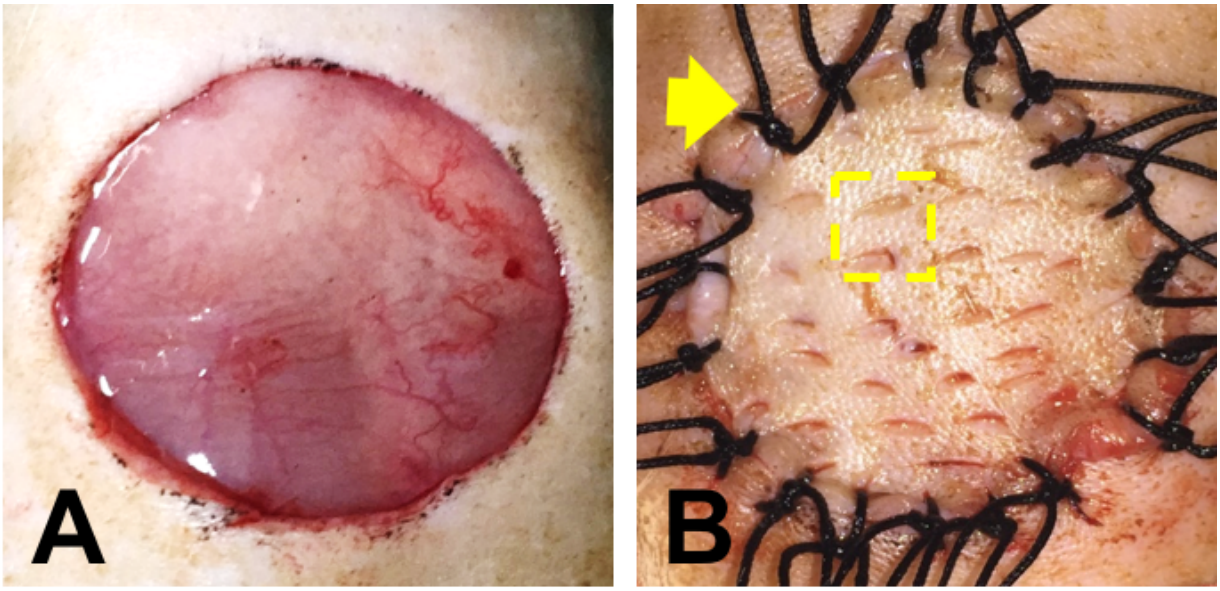

Figure 1. Animal surgery. **A.** A round full-thickness excisional wound with a diameter of 20 mm on the dorsum. **B.** STSG with spaced fenestrations (yellow rectangular box) pierced by a sharp tipped scalpel. The STSG was approximated to the adjacent wound margin and sutured with interrupted stitches (yellow arrow).

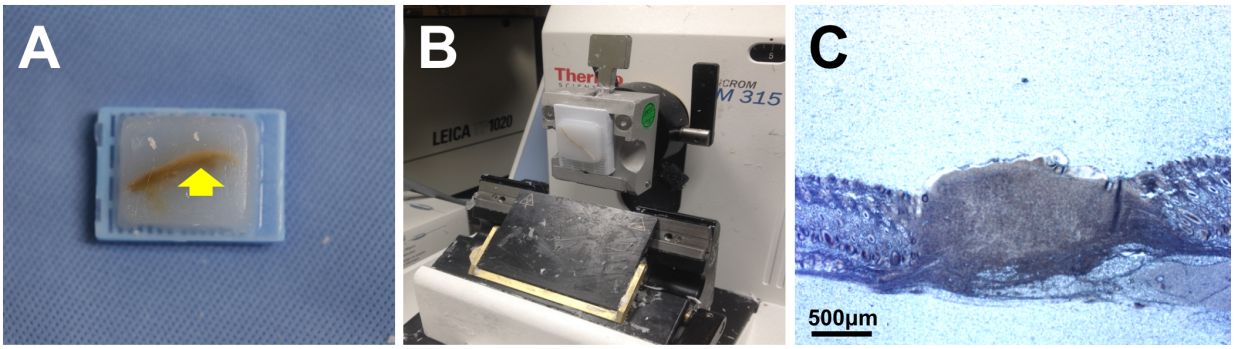

Figure 2. Tissue section. **A.** A paraffin-embedded tissue (yellow arrow) block. **B.** A block inserted into the microtome chuck. **C.** A typical skin tissue section.

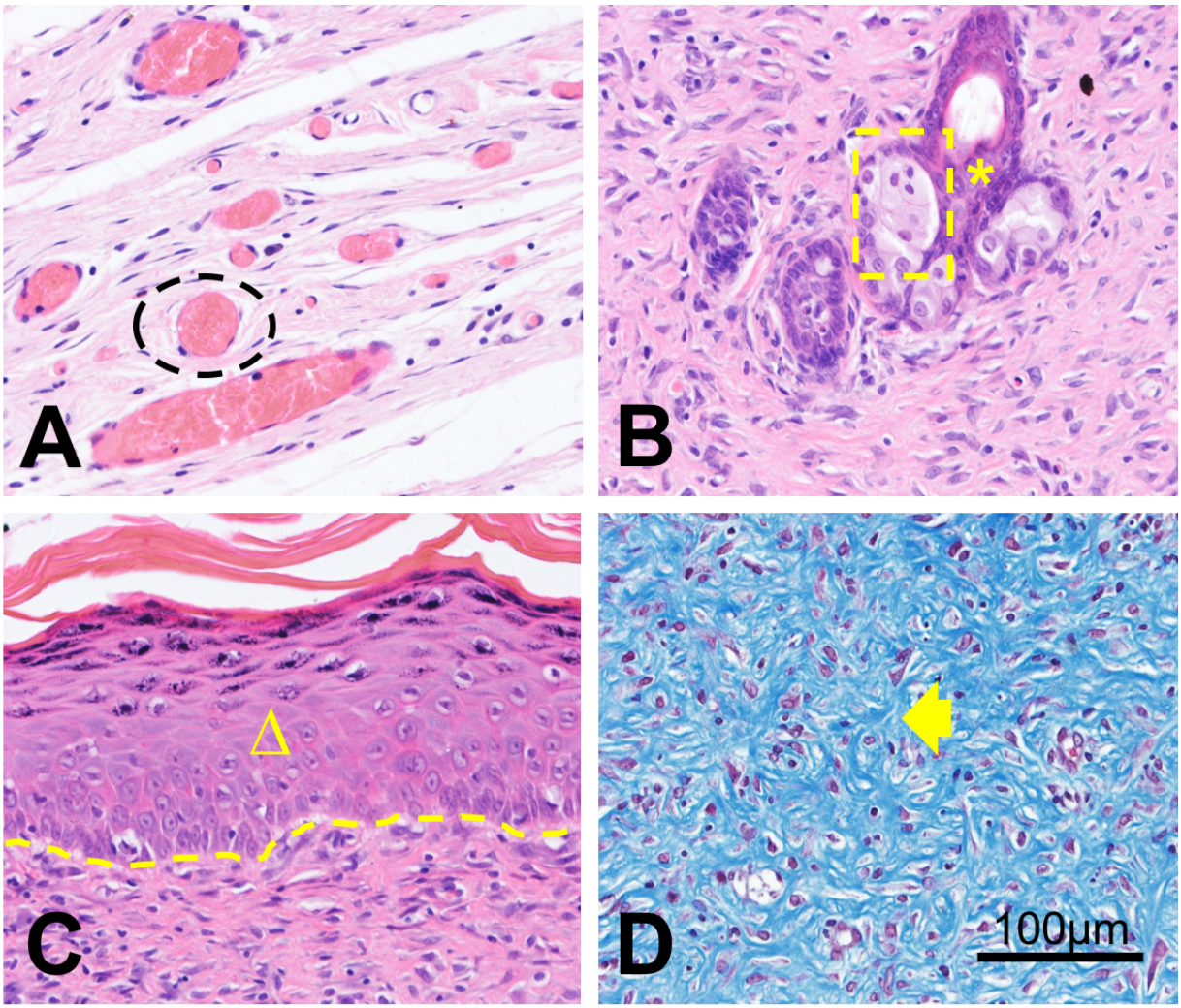

Figure 3. Identified structure in stained tissues. **A.** Blood vessels (black dashed circle). **B.** Hair follicle (yellow star) and sebaceous glands (yellow dashed rectangle). **C.** Epidermis (yellow triangle) and epidermal basement membrane (yellow dashed line). **D.** Collagen fibers (yellow arrow).