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Chapter for the book « Epidermal cells, 3rd edition » (Turksen K., ed)

Reconstruction of normal and pathological human epidermis on polycarbonate filter.

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Summary

This chapter provides methods suitable for the culture of primary human keratinocytes in serum-free culture conditions, starting from very small skin biopsies. It also explains procedures required for reconstruction of a stratified epidermis on polycarbonate filter, starting from keratinocytes cultured in serum-free conditions. Tissues reconstructed according to this method have been proven suitable for characterization of epidermal morphogenesis and for *in vitro* studies of the epidermal barrier. Utilization of the same method for successful isolation of keratinocytes from a patient suffering from Darier's disease and the reconstruction of a pathological epidermis which displays the same histological features as *in vivo* is also presented.

Key Words:

Human keratinocytes; primary culture; epidermis reconstruction; Darier disease; skin biopsy

1. Introduction

Soon after the successful development of cultures of human keratinocytes immersed in liquid medium, limitations in the stages of epidermal differentiation that can be reached under such conditions have led researchers to pursue efforts towards the development of fully differentiated keratinocyte cultures. Already in the early 80s, the requirement for an exposure of differentiating keratinocytes to the air-liquid interface had been clearly identified in order to produce stratified cultures covered by a fully differentiated cornified layer (1). While those initial methods were using serum-containing medium, together with de-epidermized dermis or equivalent dermis (produced by the contraction of a collagen lattice containing cultured fibroblasts) as biological supports for keratinocytes, the possibility to reconstruct the epidermis only, directly over porous substrates and in chemically defined serum-free medium, was later demonstrated (2). Availability of standardized, reproducible models of human epidermis paved the way to valuable alternative methods able to replace laboratory animals. From an experimental point of view and in the perspective of cutaneous toxicological studies, such reconstructed human epidermis (RHE) directly anchored to a filter present the highly valuable advantage that cytokines released by keratinocytes are not trapped into a dermal compartment and can be analyzed in the culture medium, below the porous substrate (3).

In view of their huge potential for basic and applied research, RHE have rapidly become commercially available from several companies. However, for several reasons linked for instance to their relatively elevated price, but also because custom made design of their environment during tissue reconstruction is frequently required for basic investigations, or simply because the reliability of commercial providers is limited for customers seeking for a

guaranteed long-term and stable availability of an epidermal model (4), our laboratory has established a simple protocol for in-house production of RHE (5). For this purpose, we have chosen to rely exclusively on materials available for research worldwide. In addition, this freely available procedure has created conditions for open source tissue production of RHE (see for instance http://www.tissue-factory.com/en/Skin_Model.html May 31, 2013).

Nowadays, our in-house production of RHE has taken benefit from a few refinements of the method, including the possibility for a decreased cell density at the settings of the culture. A detailed study of tissue morphogenesis has also been performed, illustrating elevated basal cell proliferation during the initial phase of the culture and progressive organization of the differentiating suprabasal layers, as evidenced by immunohistochemical localization of various epidermal differentiation markers (6,7). In this chapter, the complete and annotated protocol for RHE production is described starting from keratinocyte isolation and growth, in accordance with the procedure for immersed cultures already published in the second edition of this book (8). Moreover, a new procedure which uses very small punch biopsies from human skin as a source of keratinocytes is explained. Finally, the successful utilization of abnormal keratinocytes for epidermal reconstruction is described. Indeed, keratinocytes from a patient with Darier's disease, a genetic condition that results from a defective calcium transporter in the endoplasmic reticulum, were used for *in vitro* reconstruction of the pathological epidermis. Interestingly, the resulting tissue depicted abnormal features observed *in vivo* in the epidermis of patients, namely some loss of adhesion between epidermal cells (acantholysis) and abnormal keratinisation (9).

2. Materials

2.1 Primary culture of human epidermal keratinocytes from a 3 mm punch biopsy.

1. Medium for culture setting: KBM[®]-2 medium (Clonetics[®] cat. no. CC-3103) is supplemented with SingleQuots[®] KGM-2[®] (Clonetics[®] cat. no. CC-4152) to reach final concentrations of 10 ng/ml human recombinant epidermal growth factor (EGF), 5 µg/ml insulin, 50 µg/ml bovine pituitary extract (BPE), $5 \cdot 10^{-7}$ M hydrocortisone and 5 µg/ml transferrin. For the primary cultures 50 µg/ml gentamycin, 250 ng/ml fungizone and 2.5 µg/ml ampicillin are added to the medium. When thawing the vials for secondary cultures, these antibiotics are replaced by 50 U/ml of penicillin G and 50 µg/ml of streptomycin.
2. Medium for culture growth: after settings of the cultures, a keratinocyte growth medium is used, based on Epilife[®] medium (Cascade Biologics[™] cat. no. M-EPI-500-CA) and supplemented with antibiotics and HKGS (Cascade Biologics[™] cat. no. S-001-5) in order to reach final concentrations of 0.2% BPE, 0.2 ng/ml human recombinant EGF, 0.18 µg/ml hydrocortisone, 5 µg/ml insulin and 5 µg/ml transferrin.
3. Solution A: Washing solution for tissues and cells: 10.0 mM glucose, 3.0 mM KCl, 130.0 mM NaCl, 1.0 mM Na₂HPO₄·7H₂O (or anhydrous), 0.0033 mM phenol red, 30.0mM HEPES. Dissolve HEPES in distilled H₂O and adjust pH at 7.4 with 10 M NaOH. Then, dissolve the other compounds and check pH before adjusting the final volume. Solution A is sterilized through a Sterivex[™] -GP 0.22 µm filter (Millipore cat. no. SVGP01015) and stored refrigerated at 4°C.
4. Dispase II (Roche cat. no. 04942078001): diluted in solution A to reach a concentration of 10 mg/ml.
5. EDTA (Merck cat. no. 108418): diluted in solution A to reach a concentration of 1 mM.
6. Trypsin solution used for the initial dissociation of keratinocytes: trypsin (Sigma cat. No. T-9201) is dissolved at 0.17 % (weight/volume) into ice-cold solution A. The

solution is then sterilized through a MillexTM-GP 0.22 µm filter (Millipore cat. no. SLGP033RB).

7. Trypsin solution used for subculture of keratinocytes: dissolve 0.01% ethylenediaminetetraacetic acid (EDTA) into solution A, adjust the pH to 7.4, chill on ice the solution and then add trypsin (Sigma cat. No. T-9201) to obtain a 0.025% solution. The solution is then sterilized through a MillexTM-GP 0.22 µm filter (Millipore cat. No. SLGP033RB).
8. Dialyzed fetal calf serum: dialysis tubing MWCO-12.000-14.000 Da (Dialysis tubing-Visking Medicell International Ltd) is prepared by boiling in a solution containing 0.1% EDTA and 0.1% Na₂CO₃, followed by two 15 minutes washes in boiling distilled H₂O. Introduce 100 ml of fetal calf serum (Lonza® cat. no. DE14-801F) into the dialysis tubing. Seal the dialysis tubing and stir it at 4°C into 10 liters of phosphate buffered saline (PBS) solution without calcium (PBS: 0.2 g KCl, 0.2 g KH₂PO₄, 8 g NaCl, 1.44g Na₂HPO₄·2H₂O in 1.0 liter of distilled H₂O, pH 7.4). PBS solution is changed four times over a period of 12 hours.
9. Blocking solution: solution A containing 2% dialyzed fetal calf serum.

2.2 Reconstruction of epidermis on polycarbonate filter

1. Medium for epidermis reconstruction at air-liquid interface: medium for culture growth but containing 1.5 mM Ca²⁺ and supplemented with 50 µg/ml vitamin C and 10 ng/ml keratinocyte growth factor (R&D Systems, cat. no 251KG).
2. Insert: polycarbonate culture insert with 12 mm diameter and 0.4 µm diameter pore size (Millipore, cat. no PIPH01250).

2.3 RNA extraction from reconstructed epidermis

1. RNA extraction: To isolate total RNA, RNeasy mini kit (Qiagen cat. no. 74106) and QIAshredder spin columns (Qiagen cat. no. 79656) are used using the spin technology protocol according to the instructions of the manufacturer.

2.4 Protein extraction from reconstructed epidermis

1. Lysis buffer for protein extraction: 0.125 M Tris-HCl pH 6.8, 20 % glycerol, 4% SDS, 0.2 M DTT.

2.5. Histological analysis of reconstructed epidermis

1. Acetic formalin (4% formalin and 1% glacial acetic acid)
2. 100% methanol
3. 100% toluene
5. Embedding cassettes (Simport)
6. Paraffin embedding station (Shandon Histocentre 2)
7. Microtome (Leica RM2245)
8. Hemalun solution : 1 liter of saturated solution of potassium alum containing 3 g standard hematoxylin (Sigma-Aldrich, Fluka) and 20 ml of glacial acetic acid
9. Erythrosine solution: 1 liter of water containing 2 g of erythrosine and 10 drops of 35% formalin solution.

3. Methods

A method for setting up serum-free cultures of keratinocytes from adult human skin samples obtained from excessive tissue removed during abdominoplasty was published in the previous edition of this book (8). The current protocol describes an adaptation of the method to small skin samples with a diameter of 3 mm.

3.1 Primary culture of human epidermal keratinocytes from small skin samples.

1. Collect small skin samples with an area corresponding to 3 mm punch biopsies in Epilife medium supplemented with antibiotics and fungizone and transport on ice to the culture room (*see Note 1*).
2. Transfer the punches into dispase II during 2 h and 30 min at 37°C to allow separation of the dermis from the epidermis.
3. With a sterile pair of tweezers, separate manually the epidermis from the underlying dermis in an EDTA solution at 4°C in order to block the activity of dispase.
4. Transfer the epidermal sheet (*see Note 2*) to a well of a 12-well cell culture plate containing 200 µl of 0.17% trypsin to dissociate the epidermis into single cells.
5. Incubate 1 hour and 30 min at 37°C.
6. Add 500 µl of cold blocking solution and dissociate keratinocytes, using a pair of tweezers. Up and downs may also be performed inside a 5 ml pipet (*see Note 3*).
7. Collect the cell suspension and centrifuge at 335 g during 5 min at 4°C.
8. Re-suspend the pellet in 1 ml medium for culture setting containing 5% dialized fetal calf serum and transfer into a 12-well cell culture plate.
9. Incubate for 3 days at 37°C without moving the plate.
10. Change the medium every other day using medium for culture growth for approximately 10 days.
11. Once cells reach about 60-70% confluence (the culture is then mainly composed of keratinocytes), trypsinize the culture: add 500 µl of trypsin, incubate 1 min at room temperature and aspirate the liquid in order to eliminate contaminating cells such as melanocytes and fibroblasts. Add again 500 µl of trypsin and incubate for 10 to 15 additional minutes at room temperature. When cells are detached from culture plastic substrate (plates can be hit laterally), add 1 ml of cold blocking solution, collect the cell suspension and centrifuge at 335 g for 5 minutes at 4°C. Re-suspend the pellet

into medium for culture growth and seed the cells into 6-well plates or T25 flasks in order to expand the keratinocyte population. At this stage, a total yield between 25000 and 50000 keratinocytes may be expected.

3.2 Reconstruction of epidermis on polycarbonate filter

1. Isolate human keratinocytes from normal adult skin samples from plastic surgery as described previously (8), or from small biopsies as described above. For epidermal reconstruction, third-passage proliferating keratinocytes are used.
2. If keratinocytes are frozen in liquid nitrogen for cryopreservation (8), their proliferation potential must be re-activated by culturing them first in monolayer. Seed approximately $2 \cdot 10^6$ keratinocytes in a 175 cm² culture flask containing 25 ml of medium for culture setting. Incubate cells during 24h at 37°C in a humidified atmosphere containing 5% CO₂. On the next day, renew the culture medium with medium for culture growth in order to remove any trace of DMSO used in the freezing solution of keratinocytes (8) and incubate 24 h at 37°C, 5% CO₂. Then replace medium every other day.
3. When keratinocytes cover approximately 70%-80% of the flask area (*see Note 4*) (usually after 3-4 days of culture), aspirate the culture medium and add 3 ml of trypsin solution. Keep keratinocytes at room temperature during 8-10 minutes and then hit the flask laterally to detach the cells from the flask. Then add 12 ml of ice-cold blocking solution and transfer the cell suspension into a 50 ml centrifugation tube.
4. Centrifuge cells at 335 g and 4°C for 5 min.
5. Aspirate the supernatant and re-suspend keratinocytes from the pellet using 2-3 ml ice-cold medium for culture growth containing 1.5 mM calcium. Keep the cell suspension on ice.

6. Count cells and dilute the suspension with the same medium in order to obtain a minimal cell density of $3 \cdot 10^5$ keratinocytes per ml (*see Note 5*). Keep keratinocyte suspension on ice.
7. Place the polycarbonate culture inserts with a sterile pair of tweezers into a 6-well culture microplate. Add 2.5 ml of medium for culture growth containing 1.5 mM of Ca^{2+} into the wells (*see Note 6*).
8. In the upper chamber of each insert, add 500 μl of keratinocyte suspension ($3 \cdot 10^5$ cells/ml) corresponding to 250,000 cells/cm². Incubate culture at 37°C in a humidified atmosphere containing 5% CO₂.
9. After 24h, cells are exposed to the air-liquid interface by careful aspiration of the culture medium in the upper compartment of the insert (*see Note 7*). Replace the medium from the well under the polycarbonate filter by 1.5 ml medium for epidermis reconstruction at air-liquid interface.
10. Renew this medium every other day. After 11 days of culture at the air-liquid interface, the RHE is morphologically fully differentiated (7) (**Fig. 1**).

3.3. Reconstruction of epidermis from pathological primary keratinocytes: the case of Darier's disease

Primary keratinocytes from a Darier's disease (DD) patient were isolated and used for keratinocyte culture and epidermal reconstruction in accordance with the procedure described above. The pathological biopsy was obtained after informed consent and in accordance with the standards of the relevant ethics committee. DD is a rare dominant genetic skin disorder characterized by warty papules and plaques in seborrheic areas of the skin. Histologically, the pathological epidermis shows acantholysis (loss of intercellular adhesion) and dyskeratotic keratinocytes. After culture as monolayers of keratinocytes from the pathological skin, followed by tissue reconstruction at the air-liquid interface, the Darier RHE showed

morphological features strikingly similar to those observed *in vivo*. This means increased intercellular spaces (acantholysis), abnormal keratinocytes (dyskeratosis) named *corps ronds* or *grains*, and parakeratosis (**Fig. 2**). In other words, the DD RHE presented defective differentiation, as it can be observed in DD lesions *in vivo* (9).

3.4 RNA extraction from reconstructed epidermis

1. Total RNA is extracted from the RHE using the Rneasy mini kit and QIAshredder spin column.
2. Dissect using a sharp surgical blade the circumference of the polycarbonate filter covered by the RHE from the bottom of the insert. Then transfer the dissected disc, using a pair of tweezers, into a 12-well culture plate containing 600 μ l of RLT buffer (*see Note 8*).
3. After 1 or 2 minutes, stratum corneum detaches from the epidermis. Remove it with a pair of tweezers and discard (*see Note 9*).
4. For disrupting keratinocytes, gently scratch the epidermis with a micropipette tip in lysis buffer provided by the kit. Homogenize the lysate by pipetting up and down and transfer into a QIAshredder spin column placed in a 2 ml collection tube.
5. From this point, follow the instructions of the manufacturer. This procedure allows the recovery of enough RNA from the RHE for RT-qPCR analysis of gene expression.

3.5 Protein extraction from reconstructed human epidermis

1. Using a sharp surgical blade, dissect the circumference of the filter holding the RHE as described above for RNA, then transfer the filter and the anchored tissue into 200 μ l of lysis buffer suitable for protein extraction.
2. Boil samples for 5 min at 100°C. This allows the epidermis to detach from the polycarbonate filter.

3. Scratch the filter with a micropipette tip in order to collect the remaining adherent cell material.
4. Boil the lysate during 2 min.
5. Centrifuge at 9300 g during 5 min, in order to pellet the cellular debris not dissolved in lysis buffer, together with the polycarbonate filter.

3.6 Histological analysis of reconstructed epidermis

1. Fix the insert with the RHE in acetic formalin for minimum 24 h at room temperature.
2. Dehydrate in four successive baths of methanol: a quick bath to remove as much water as possible and three other baths for 10 min each.
3. Immerse the insert in toluene, which dissolves the plastic surrounding the polycarbonate filter holding the RHE. The insert is then vigorously stirred in toluene, releasing the disc-shaped RHE (still attached to the filter) (*see Note 10*).
4. Place the sample in an embedding cassette and incubate 4 times for 10 min in pure toluene.
5. The next step is the inclusion of the sample in paraffin. Soak the sample in a large metal mold containing hot paraffin (60°C) in order to replace a maximum of toluene impregnated in the sample by liquid paraffin. Then, place the sample in a smaller mold containing hot paraffin and incubate at 60°C for 1 hour or overnight.
6. The disc-shaped RHE can finally be embedded in paraffin. For this step, it is very important to adequately orient the disc in the mold in order to obtain transversal sections of the RHE (*see Note 11*). Transfer the mold to the cooling plate of the embedding station to allow paraffin solidification. When the paraffin is completely solid, remove the block from the mold.
7. For sectioning, trim the sample by cutting 25 µm-thick sections, before preparing 6 µm-thick sections for histological analysis (*see Note 12*).

8. Separate ribbons of sections into coupons and spread on microscope slides (distilled water is used for spreading), then let dry for 1 hour at 48 °C.
9. Tissue sections are then processed for regular histological staining, using haematoxylin-erythrosin to allow morphological analysis of RHE.

4. Notes

- (1) Avoid disinfection of the skin surface with iodine antiseptic since it will impede subsequent recovery of cells that exhibit enough proliferation potential.
- (2) The epidermal surface can be recognized since it is less viscous, thinner and more transparent than the dermal component.
- (3) Keep cell suspension on ice as much as possible in order to avoid irreversible cell aggregation.
- (4) Keratinocytes should be grown up to no more than 80% confluence as the number of proliferative cells has to be kept at its maximum.
- (5) $3 \cdot 10^5$ cells/ml is an adequate cell density to prepare RHE. 500 μ l from this suspension seeded into the polycarbonate insert correspond to a culture density of 250,000 cells/cm² in each insert. A lower density of keratinocytes does not allow proper reconstruction of the epidermis since, in this case, the culture medium can overlay the seeded keratinocytes for one or two days after the establishment of an air-liquid interface, impeding the formation of stratum corneum.
- (6) Place the insert into the well before adding the culture medium. This will avoid the formation of air bubbles between the polycarbonate filter and the bottom of the culture dish.
- (7) Aspirate delicately the medium above the insert with a micropipette, not with a suction pump.
- (8) Keep 12-well microplates containing the RHE on ice, in order to prevent ribonuclease (RNase) activity.
- (9) Stratum corneum cannot dissolve and does not get through the QIAshredder spin columns, leading to reduced RNA yields.

- (10) Do not leave the insert for too long in toluene dissolvent because the plastic melts rather quickly; the 30 ml toluene solution is discarded after the melting of 5-6 inserts to avoid excessive accumulation of melted plastic in the solvent.
- (11) The disc is immersed vertically to the bottom and in diagonal to the length of the mold filled with hot paraffin. The disc is held in this position (using a pair of tweezers) while laying down the mold on the cooling plate. This particular orientation of the disc ensures transversal histological observation of the RHE after sectioning using a microtome.
- (12) For a consistent morphological analysis of RHE, the block is generally trimmed to 1500 μm , which allows the observation of the RHE along its maximal length.

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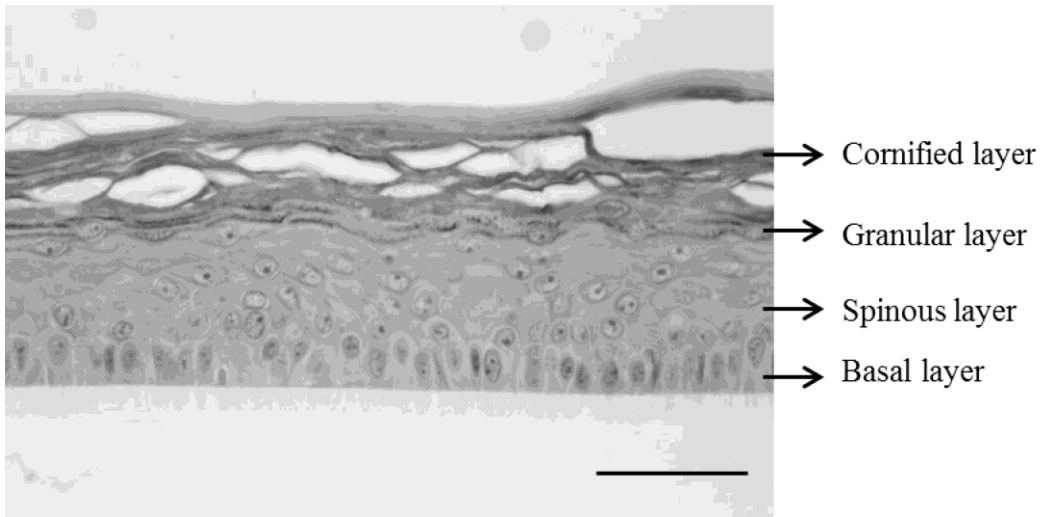


Fig. 1: Histology of reconstructed human epidermis (RHE) at day 11.

After 11 days of culture, RHE are fixed in acetic formalin and embedded in paraffin. Then histological sections perpendicular to the surface of RHE were prepared and stained with hematoxylin-erythrosin to allow the morphological analysis of RHE (bar: 50 μm).

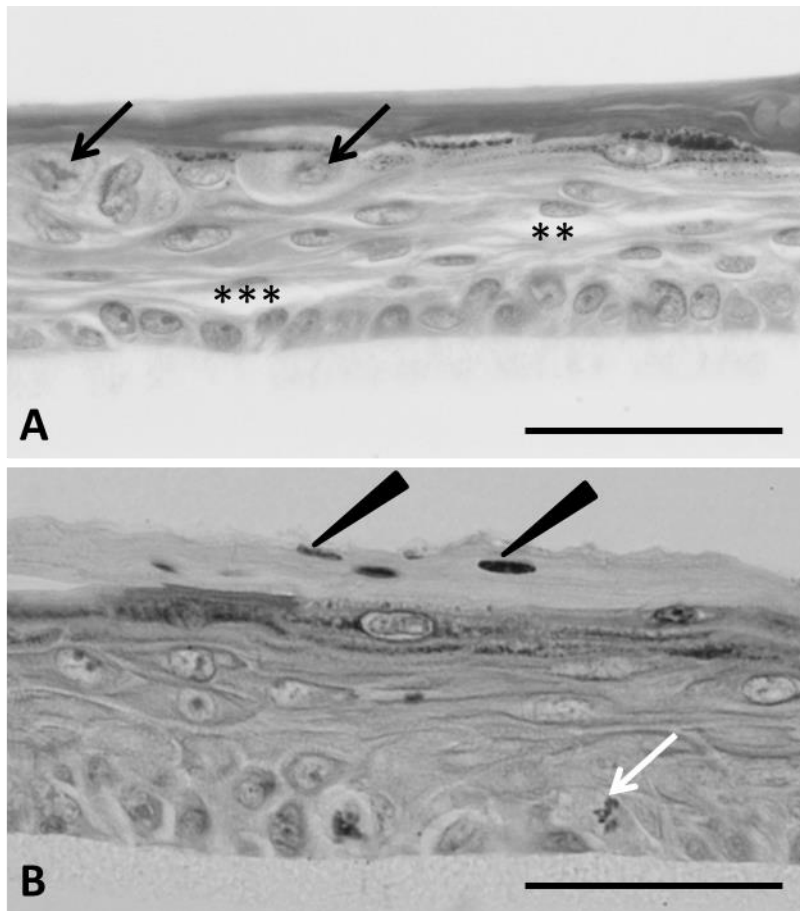


Fig. 2: In vitro reconstruction of epidermis from primary Darier's disease (DD) keratinocytes exhibit similar histopathological features as in DD lesion *in vivo*.

Isolated and amplified DD keratinocytes were seeded on polycarbonate filters and exposed at air-liquid interface in order to obtain DD-RHE. After 11 days of culture, the stratified epidermis exhibits morphological features strikingly similar to those observed *in vivo* with increased intercellular spaces (acantholysis) (A, asterisks), abnormal keratinocytes (dyskeratosis) called « *corps ronds* » (A, black arrows), « *grains* » (B, white arrow) and parakeratosis (B, black arrowheads). DD-RHE present a disruption of the differentiation process as observed in DD lesions *in vivo* (9) (bar: 50 µm).

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