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Restructuring skin equivalents with human hair follicle ORSCs and fibroblasts

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Abbreviations

ARK	animal research kit
BSA	Bovine Serum Albumin
C-GAG	collagen glycosaminoglycan
CK	Cytokeratin
DAB	3,3‘- diaminobenzidine
DAPI	4’, 6-diamidino- 2-phenylindole
DK-SFM	defined keratinocytes serum free medium
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl Sulphoxide
EDTA	ethylene diamine tetraacetic acid
FBS	fetal bovin serum
FITC	fluorescein isothiocyanate
HLA-A2	Human Leukocyte Antigen A2
ICF	Immunocytofluorescense
ICH	Immunohistochemistry
IF	intermediate filament
IRS	inner root sheath
MTT	3-(4, 5- dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide
N-LCM	navigated laser capture microdissection
ORS	outer root sheath
ORSCs	outer root sheath cells
PBS	Phosphated buffered saline
SC	stem cells
SE	skin equivalent
TA cells	transient amplifying cells
TBS	Tris Buffered Saline
TD cells	terminally differentiated cells
TDcc	terminally differentiated, cornified cells
TRITC	tetramethylrhodramine isothiocyanate
UF	upper follicle

1. Introduction

1.1. Hair follicle epithelial stem cells and their characteristics

Skin is the body's largest organ. Among many complex functions - including the immune response – skin protects against environmental influences. Acute or chronic loss of this barrier requires the elementary processes of tissue repair for any organism to survive. Within this process reepithelialization of the wounded surface is the primary destination of skin wound healing.

Skin consists of an outer layer, the epidermis, a stratified squamous epithelium derived from the ectoderm, and an inner layer, the dermis of mesodermal origin. The epidermis and dermis are separated by a basement membrane. Skin epidermis and its associated structures arise from two stem cell populations within the interfollicular and the hair follicle regions. One, in the basal layer of skin, normally gives rise to stratified skin layers. A second, the hair follicle stem cell, resides in a region of the outer root sheath (ORS) called the bulge, and it is responsible for the regeneration of hair and sebaceous gland (Alonso and Fuchs, 2003). Recently it was demonstrated that follicular stem cells are not only involved in the formation of the hair follicle but also in the renovation of the epidermis (Fig. 1). Hair follicle epithelial stem cells were shown to give rise to all the epithelia of the follicle as well as potentially contribute to the epidermis (Taylor et al., 2000)**.** Hair follicle stem cells, like other adult stem cells, are thought to be slow-cycling cells or rarely cycling cells, with a superior clonogenicity and proliferative capacity (Lavker and Sun, 2000) and the capacity to proliferate and generate large amounts of tissue for a long time (Lavker and Sun, 1982).

In 1994, Rochat et al (Rochat et al., 1994) examined the growth capacity of keratinocytes isolated from human scalp hair follicles and found out that root sheath cells (ORSCs) isolated from the upper segment of the hair follicle showed

high colon forming efficiency. Rizvi et al noted that the multipotent epidermal stem cell resides in the bulge region associated with the hair follicle and these cells give rise to the epidermis as well as its associated structures such as, sebaceous gland and dermal papilla (Rizvi and Wong MH, 2005). Ohyama et al used navigated laser capture microdissection (N-LCM) and stem cell markers (CD200, PHLDA1, follistatin, and frizzled homolog 1) to precisely identify the human bulge region and collect bulge ORSCs. Bulge ORSCs are enriched in the suspensions trypsinized from the middle portion of hair follicles, representing about 10–20% of all living cells in mid-follicle suspensions (Ohyama et al., 2006).

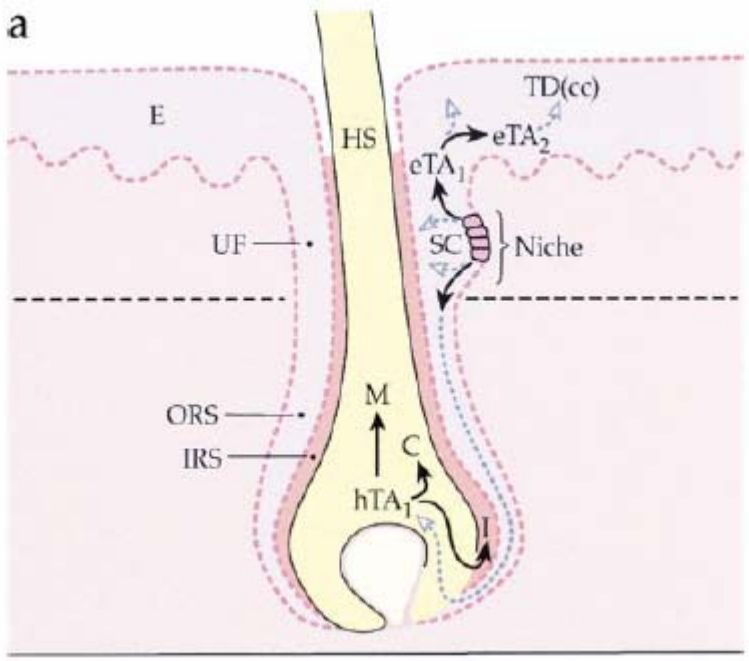


Fig. 1 Keratinocyte Lineages in Skin

A schematic diagram showing the location of stem cells (SC) in the bulge area of the hair follicle in close contact with the arrector pili muscle, which may provide a specialized “niche” known to be crucially important in maintaining the in vivo stem cell features. It is hypothesized that the bulge stem cells are bipotent, as they can undergo two distinct differentiation pathways: the bulge–hair pathway and the bulge–epidermis path way. When the bulge-derived, young transient amplifying cells (TA cells) migrate upward into the epidermis (eTA1, eTA2, eTAn), they may be regarded as the “epidermal stem cells.” These cells are specialized in making various epidermal products culminating in the formation of the terminally differentiated, cornified cells (TDcc). Solid arrows indicate hypothesized (“horizontal”) path of keratinocyte migration along a basement - membrane, while the dashed arrows indicate the (“vertical”) path - of cell migration into the suprabasal layers forming terminally differentiated cells (TD). Other

abbreviations: E (epidermis), HS (hair shaft), IRS (inner root sheath), ORS (outer root sheath), UF (upper follicle). Modified from Taylor et al., (2000)

1.2. Seeding cells of epidermis in tissue engineering skin

As an easily accessible tissue, skin or in particular epidermis has been forwarded for repair and tissue replacement. Cell-scaffold-based tissue engineering of skin or epidermis is rapidly progressing and has already been successfully applied as skin equivalent (SE) (Horch et al., 2005). It was initiated by the pivotal work of Rheinwald and Green who established conditions for the serial cultivation of human epidermal keratinocytes in 1975 (Rheinwald and Green, 1975). Usually isolated from adult skin biopsy specimens or neonatal foreskins by enzymatic dissociation, single keratinocytes are plated for primary culture in appropriate, growth factor-containing media, in the presence either of feeder cells (murine mesenchymal 3T3 cells or human dermal fibroblasts) (Limat et al., 1989) and fetal calf serum or low Calcium concentration and bovine pituitary extract (Boyce and Ham, 1983). Using such media, the expansion of keratinocytes just can be performed in a limited number of subcultures.

As above mentioned, the ORS of human anagen hair follicles is comprised largely of undifferentiated keratinocytes that encompass the cylindrical structures of the hardened inner root sheath and the hair shaft. Human keratinocytes can also be isolated from the ORS of plucked anagen scalp hair follicles or the scalp derived from face-lifting and biopsy (Ohyama et al., 2006; Hoeller et al., 2001). It is an essential advantage that the ORS represents a source of easily and repeatedly available keratinocytes with a high proliferative potential even in old donors and a similar potential for differentiation as interfollicular keratinocytes (Limat and Hunziker, 2002). Therefore, we aimed to isolate ORS cells as the source keratinocytes of SEs.

1.3. Recent progression in the culture method of skin equivalents

Culture conditions mimicking the physiological environment of the epidermis gave rise to multilayered, stratified epidermal equivalents with a well-developed horny layer. Differentiated keratinocyte cultures may be combined with a dermal equivalent, resulting in a reconstructed skin equivalent. Living dermal equivalents can be established by including fibroblasts into collagen (mainly bovine) gels or into synthetic meshes, e.g. of nylon or polyglactin acid (Horch et al., 2005). Nonliving dermal equivalents include deepidermized human dermis and, for example, collagen sponges supplemented with various biological matrix substances (Berthod and Damour, 1997).

There are two ways to combine the keratinocytes with dermis equivalent (DE). Mis combined cultured keratinocyte sheets with DE and covered them with a silicone membrance (Mis et al., 2004). In another approach, sufficient initial amount of keratinocytes were seeded into DE and were cultured till they cover the surface of DE. Both procedures have their disadvantages: The first method requires to either obtain several biopsies from volunteers or patients or to harvest a large piece of skin from the donor in order to guarantee sufficient amount of cells to start with. The second approach is very time consuming till 30 days and needs sufficient cultured keratinocytes. Regarding transplantation purposes, both methods are obviously not perfect and require improvement. Seeding keratinocytes derived from the ORS of human hair follicles into DEs can help to avoid the disadvantages. However, this technique by itself does not accelerate the cultivation of sufficient amount of keratinocytes.

Several studies demonstrated that co-cultivation of keratinocytes and fibroblasts is a more suitable approach to reproduce in vivo conditions of human skin than mono-cultures of certain cell types (Auger et al., 1998; Houdek et al., 1998; Witte

and Kao, 2005) However, if co-cultures are generated by simply adding one cell type to a distinct one, these cultures are mostly unstable when they are cultivated over a longer period of time (Coulomb et al, 1986.). In this case, one cell species sooner or later displaces the other. In systems which remodel human skin, fibroblasts, the predominant dermal cell type, require embedding into three dimensional matrices, e.g. consisting of collagen, to form a dermal equivalent (Mauch et al., 1989). Following, keratinocytes, the major cell type of the epidermis, can be seeded onto the surface of this DE (Sarber et al., 1981). Once embedded, fibroblasts do not leave the collagen network and keratinocytes are promoted to cover the DE. After lifting these constructs to the air–liquid interface, keratinocytes form a multi-layered, fully differentiated epidermis including a basal membrane and a stratum corneum (Schoop et al., 1999; Ponec M et al., 2001;Prunieras et al., 1983). Ojeh and his coworkers researched the interactions of cultured human dermal fibroblasts and epidermal keratinocytes in Integra Artificial Skin. Their results demonstrated that in vitro the collagen glycosaminoglycan (C-GAG) dermal equivalent is biocompatible for cell attachment, migration, proliferation, and differentiation (Ojeh etal., 2000).

1.4 Aim of the study

To overcome the aforementioned disadvantages, on the basis of methods of Jones (Jones et al., 2003) and Geer (Geer et al., 2004), we aim to modify an approach to restructure the three-dimensional SE with isolated human ORSCs and fibroblasts in collagen GAG matrix in vitro and in vivo.

The strategy of this work therefore comprises:

1.) To isolate ORSCs from the middle and low part of hair follicles, which contain some bulge stem cells and to identify them.

2.) To restructure the three-dimensional SE with isolated human ORSCs and fibroblasts in collagen GAG matrix in vitro.

3.) To transplant the restructured SEs into full-thickness skin defect wounds of nude mice and to investigate the characteristics of grafted SEs.

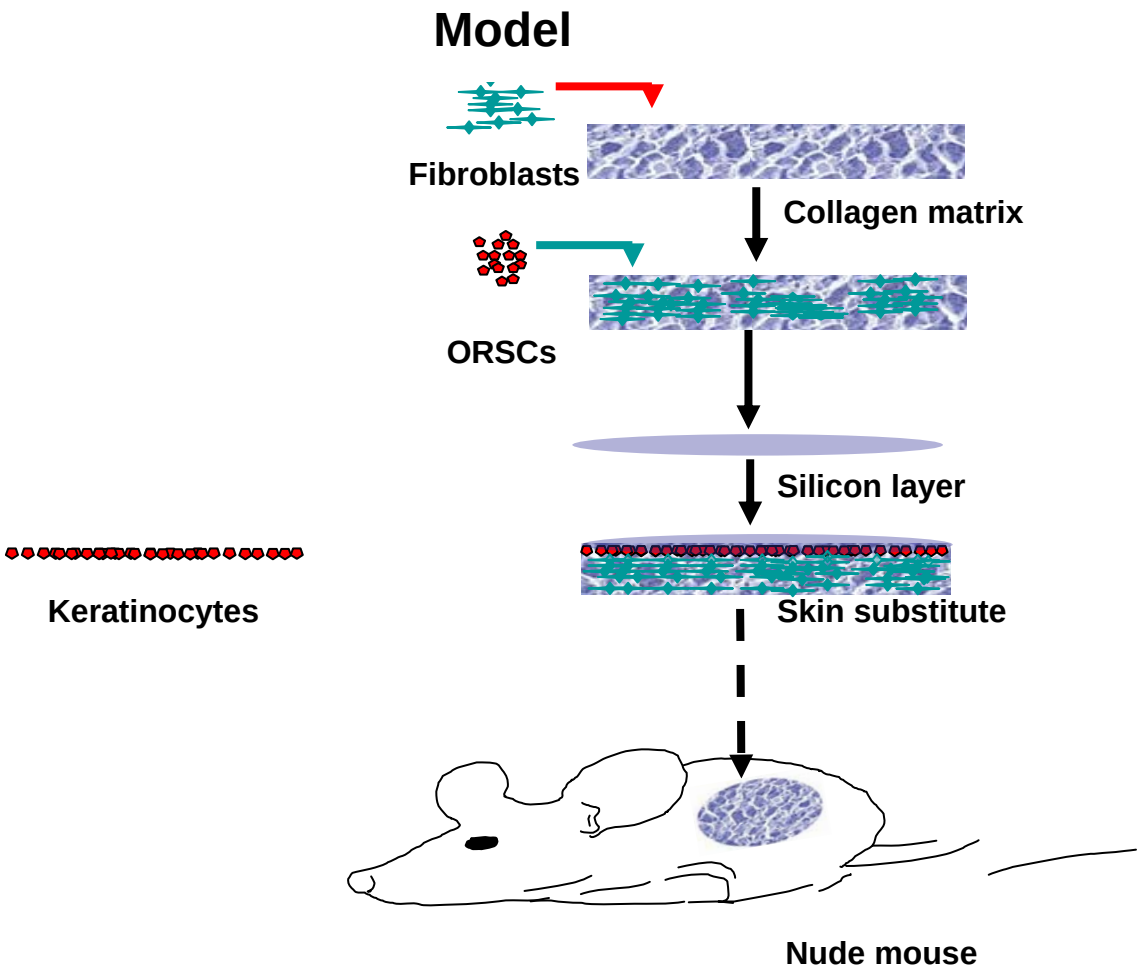


Fig. 2 Schematic show of the main study procedure

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

All chemicals were used in analytic degree of purity

2.1.1.1 Biochemicals

- Reichert-Jung, Nussloch, Germany
- animal research kit, Dako Diagnostics GmbH, Hamburg, Germany
- Avidin/ Biotin Blocking Kit Vector Laboratories, Burlingame, USA
- DAB Substrate Kit Vector Laboratories, Burlingame, USA
- Vectastain Elite ABC-Peroxidase-Kit Vector Laboratories, Burlingame, USA
- Faramount mounting medium, Dako, Carpinteria, USA
- Mayer's haematoxylin Merck, Darmstadt, Germany
- Citric acid monohydrate Sigma, Steinheim, Germany
- Triton X-100 Sigma, Steinheim, Germany
- 4', 6-diamidino- 2-phenylindole (DAPI), Vector Laboratories Inc., Burlingame, CA, USA
- Albumin bovine Sigma, Steinheim, Germany
- Hydrochloric acid Merck, Darmstadt, Germany
- Sodium phosphate dibasic Merck, Darmstadt, Germany
- Sodium phosphate monobasic Merck, Darmstadt, Germany
- Sodium chlorate Merck, Darmstadt, Germany
- Hydrogen Peroxide,30% Sigma, Steinheim, Germany
- Xylene Schuchardt, Hohenbrun. Germany
- Ethanol Apotheke University Luebeck

2.1.1.2 Reagents

- fetal bovine serum, HyClone, Logan, UT

- penicillin-streptomycin, Boehringer Manheim Co, Germany
- DK-SFM, Gibco BRL, Germany
- DK-SFM Supplement, Gibco BRL, Germany
- 0.05% trypsin -0.53Mm EDTA, Gibco BRL, Germany
- Collagen type Ⅳ, Sigma, USA
- phosphated buffered saline (PBS), Gibco BRL, Germany
- Dispase, Gibco BRL, Germany
- Dulbecco's Modified Eagle's Medium (DMEM), Gibco BRL, Germany
- Xylazine, Rompun®, Bayer Leverkusen, Germany
- Ketamine, Ketanest®, Pziser. Kalsruhe，Germany
- Collagen-GAG matrix, Integra®, UK

2.1.1.3 Antibodies and characteristics of these antibodies

- **Anti-ß1 integrin (clone MAB 13)** *Gift from Sue Craig Company*

  The antibody used for the detection of ß1 integrin expression is the mouse monoclonal MAB 13. ß1 integrin is one of candidate hair follicle stem cells markers (Ma 2004). Integrins were upregulated in the stem cells at the transcriptional level, compared with their transiently amplifying progeny (Ivanova, 2002; Ramalho -Santos, 2002).

- **Anti-CK15 (clone CBL272)** *Lab Research Products, Chemicon*

  Clone CBL272 monoclonal is specific for Keratin 15, and can be used in immunofluorescense detection of a variety of cytoslides and cryoslides. It was reported that expression of keratin 15 is restricted to the cells at the bulge region of the human hair follicle, and keratin 15 was suggested to be a stem cell marker (Ma et al., 2004).

- **Anti-CK19 (clone MAB 1608)** *Lab Research Products, Chemicon*

  The antibody used for the detection of keratin19 expression is the mouse monoclonal MAB 1608. Keratin 19 is expressed at low levels in the skin. It is expressed in the bulge region (Lane et al, 1991). There was more keratin 19 expression in newborn than older foreskin and this correlated with keratinocyte culture lifespan. Keratin 19 is a valuable marker, or comarker,

for cells early in the keratinocyte cell lineage, i.e., stem cells (Michel et al, 1996).

- **Anti-Ki-67 (clone MIB-1)** *Lab Research Products, DAKO*

  The Ki-67 antigen is a nuclear protein, which is defined by its reactivity with monoclonal antibody from the Ki-67 clone (Gerdes, 1984). Two isoforms of 345 and 395 kDa have been identified (Gerdes, 1991). The Ki-67 antigen is preferentially expressed during all active phases of the cell cycle (G1, S, G2 and M-phases), but it is absent in resting cells (G0-phase) (Gerdes, 1984). During interphase, the antigen can be exclusively detected within the nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. The antigen is rapidly degraded as the cell enters the non-proliferative state (Scholzen, 2000), and there appears to be no expression of Ki-67 during DNA repair processes (Key, 1994). It has been broadly used in immunohistochemical detection of frozen sections and immunocytohistochemistry.

- **Anti-vimentin (Clone V9)** *Dako Diagnostics GmbH, Hamburg, Germany*

  Monoclonal Mouse Anti-Vimentin, Clone V9, is intended for use in immunocytochemistry. The antibody labels primarily cells of mesenchymal origin in normal and neoplastic tissues. Vimentin is a 57 kDa intermediate filament (IF) protein, which forms part of the cytoskeleton of vertebrate cells. Among the five classes of IFs, comprising nine groups, vimentin belongs to class III, showing a high degree of specificity for cells of mesenchymal origin. The coexpression of intermediate filaments, particularly vimentin and cytokeratin, has now been demonstrated in a variety of normal cells/tissues (Herrmann, 2000).

- **Anti-CK (clone MNF 116)** *Dako Diagnostics GmbH, Hamburg, Germany*

  Monoclonal Mouse Anti-Human Cytokeratin, Clone MNF116, can label epithelial tissues from simple glandular to stratified squamous epithelium, and is a useful tool for the identification of normal and neoplastic cells of epithelial origin (Prieto,1996).

- **Anti-HLA (clone A18)** *Santa Cruz Biotechnology, Santa Cruz, CA*

  Human Leukocyte Antigen A2 (HLA-A2) is a human class I histocompatibility molecule (MHC I). MHC I molecules are integral parts of the immune response and present nonself peptides on the cell surface for recognition by cytotoxic T-lymphocytes (CTLs). HLA-A (A-18) is used for detection of broadly reactive with HLA antigens of human origin by immunohistochemistry.

- **Biotinylated secondary anti-mouse or anti-rabbit IgG antibody**

  Vector Laboratories, Burlingame, USA

- **Secondary goat anti mouse FITC fluorochrome conjugated antibodie**

  Jackson Immuno Research, Germany

- **Secondary goat anti mouse TRITC fluorochrome conjugated antibodie**

  Jackson Immuno Research, Germany

2.1.1.4 Solutions and buffers

- **ABC Reagent**

  The ABC working solution was prepared according to manufacturer's instructions (from Vectastain Elite ABC-Kit: Vector Laboratories, Inc., Burlingame, CA). Briefly, two drops of „Regent A" and two drops of „Regent B" were added into 5 ml PBS and incubated at room temperature for 30 minutes before use. This solution can be stored at 2-8 °C for 3 days.

- **DAB (3,3'- diaminobenzidine) Reagent**

  The working substrate solution was prepared according to manufacturer's instructions (from DAB Substrate Kit: Vector Laboratories, Inc., Burlingame, CA) before use. Firstly, 2 drops of Buffer Stock Solution were added to 5.0 ml of distilled water and mixed well; and then 4 drops of Stock Solution were added and mixed well; finally, 2 drops of the Hydrogen Peroxide Solution were added and mixed well. Afterwards, the tissue sections were incubated with this substrate solution at room temperature until suitable staining develops

- **Sodium Citrate Buffer: 10 mM, pH 6,0**

  To prepare 1 liter, add 2.94 g sodium citrate to 1 liter dH_2O. Adjust pH to 6.0.

- **Alcoholic-hydrochloric acid solution for differentiation of haematoxylin**

  Three ml hydrochloric acid was added to 97 ml of 100% ethanol to prepare the solution for differentiation of over-stained tissues by haematoxylin. This solution can be stored and used for two to three weeks.

- **10X Phosphate Buffered Saline (PBS)**

  To prepare 1 liter of 10X PBS, use 82.33 g Na2HPO4, 23.45 g NaH2PO4•H2O and 40 g NaCl. Adjust pH to 7.4.

- **Triton X-100:, 0.1%**

  Prepare stock of 10% Triton in PBS. Rotate tube overnight to dissolve. Dilute to 0.1% in PBS.

- **Hydrogen Peroxide: 3%**

  To prepare, add 10 ml 30% H_2O_2 to 90 ml PBS.

- **5% Bovine Serum Albumin (BSA)**

  For preparation of 5% BSA, 5 gram albumin bovine was dissolved into PBS, and adjusted to the final volume to 100 ml.

2.1.2 Instruments and equipments

- Microtome: *Vibratome 3000 Plus Automated, CE*
- Centrifugater:
- Light microscope: *Olympus, BX40*
- Humidity CO_2 incubator
- Camera: *Olympus, C5060*
- Cytospin 3: *SHANDON, Zeist, Netherlands*
- poly-L-lysin covered microscopic slides: Dako, Carpinteria, USA
- Pressure cooker
- Humidity chamber

- Wash bottles
- Absorbent wipes
- Coverslips
- Chamber slides
- 75 cm^2 flasks
- 6 cm dishes
- Inverse microscope: *Olympus CKX41*

2.1.3 Samples

This study was a cooperation project between Dermatology Department and Plastic Surgery Department in the University Luebeck.
We used normal temporal scalp tissues from face-lift surgery, with the permission from patients.

2. 2. Animal housing

Female nu/nu mice six to eight weeks of age (body weight 18-20 g were purchased from Charles River (Taconic, Copenhagen, Denmark). All animals had free access to water and food and were maintained with a 12-hr day/night cycle. Animals were fed a regular chow diet (Altromin 1324-pellet, Altromin, Lage, Germany) containing 19 % proteins, 4 % fat, 6 % fiber, 7 % ash, 13.5 % moisture, 0.9 % calcium, 0,7 % phosphorus, 15,000 IE vitamin A, 600 IE vitamin D3, 75 mg Vitamin E and 5 mg Copper.

2.3. Methods

2.3.1. Isolating ORSCs from human scalp

2.3.1.1 Isolating hair follicles with ORS from scalp

Temporal scalp tissues from face-lift surgery (approximately 2-3 cm^2) were washed with low calcium DMEM high glucose with L-glutamine 3 times to remove clots. Hairs were shaved, and the epidermis and subcutaneous tissue were removed

using a scalpel. The remaining dermal tissue contained the down-portion of hair follicles below the isthmus. The tissue was cut into several pieces and incubated overnight in 0.5% dispase in PBS at 4°C. After washing with PBS, individual follicles were gently pushed out from the scalp with fine forceps (Fig.3).

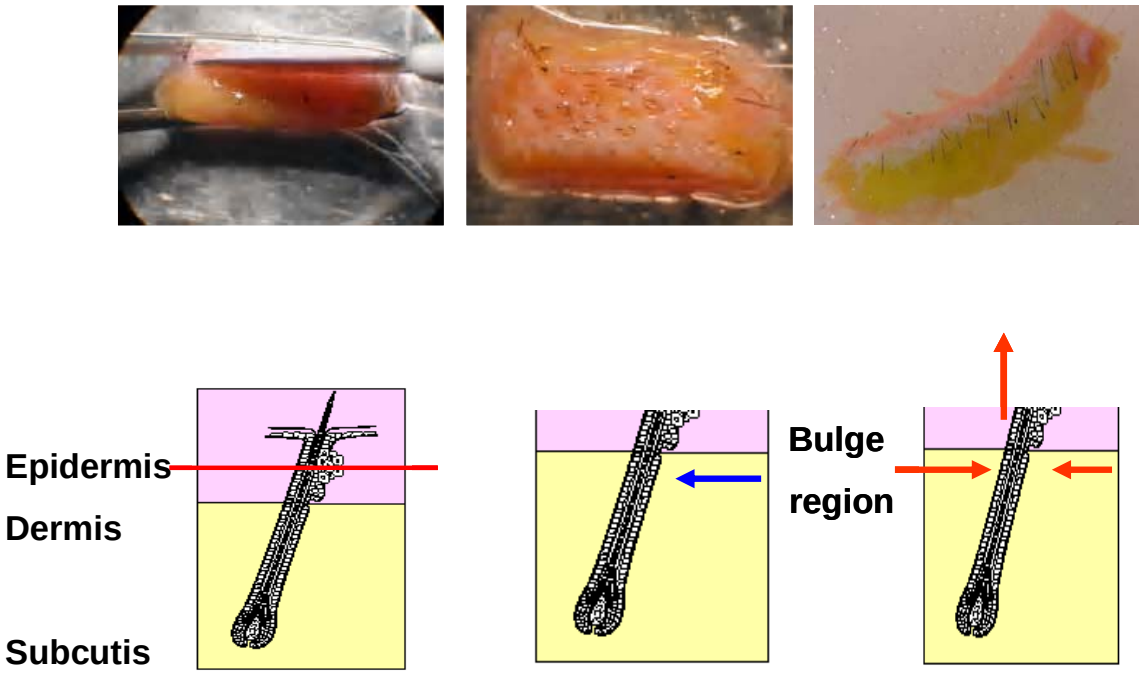


Fig.3 Procedure of isolating hair follicles with ORS from scalp

2.3.1.2 Isolation and culturing of ORSCs

The isolated follicles were incubated with 0.05% trypsin -0.53Mm EDTA at 37°C for 5 minutes (Fig.4). After filtering the suspension through 2 layers of gauze, middle and low follicle cells were collected by centrifuging at 200 g for 5 minutes and resuspended in DK-SFM with the DK-SFM supplement and penicillin-streptomycin. The cells were planted in DK-SFM at the density of 2×10^6 cells/75cm^2 flasks which were coated by Collagen type I (50ug/ml in PBS). The medium was changed 15 minutes later to remove the unstuck cells. The medium was regularily changed three times a week. ORSCs at passage one or two were used for seeding of skin equivalents and growth curve test.

Fig.4 Processing of ORSCs trypsinized from ORS of hair follicles
(a) Hair follicle without dermal sheath was put into 0.05% trypsin -0.53Mm EDTA. (b) ORSCs were removed from ORSs of hair follicles during the period of digest. (c) Plenty of ORSCs in 0.05% trypsin -0.53Mm EDTA after 5 minutes.

2.3.2. Culturing of keratinocytes and fibroblasts

Keratinocytes and fibroblasts were isolated from the same human skin, as described previously (Normand,1992). The skins were treated with 2% Dispase in PBS overnight at 4°C. The epidermis was then stripped off with subsequent enzymatic digestion of the separated and retained epidermal sheet with 0.05% trypsin–0.53 mM EDTA at 37°C for 20 min. single-cell suspensions were obtained and grown in defined human keratinocyte-SFM (all obtained from Gibco BRL) at a concentration of 2×10^6 cells/75cm^2 flask, primarily cultured Keratinocytes from passage 0 to two were used for cytospin.
Fibroblasts were isolated by treating the dermis with collagenase (0.25mg/ml) (Sigma) for 2 hours at 37°C and cultured in high glucose DMEM, supplemented with 10 % fetal bovine serum and penicillin-streptomycin at 37°C with 5% CO2. Passages three to nine were used for experiments.

2.3.3. Growth curve of ORSCs assay

ORSCs of passage one were seeded into the 24 wells plate coated with collagen I at the density of 2.5 x 10^4 in each well and cultured for 8 days. ORSCs in triplicate wells were trypsinized and counted respectively each day. The growth curve was made from the average number of ORSCs at each day

2.3.4. Generating a bioactivated matrix

To generate a bioactivated matrix, commercially available Integra® Artificial Skin, C-GAG matrix, was prepared according to the manufacturer instruction, The C-GAG dermal equivalent was cut into 15mm diameter round shape and put face up in 24 well plates for cell culture prior to seeding. Human fibroblasts were seeded into matrix in 200ul DMEM with 10% FBS with a pipette at density of 1×10^5/cm. 3 hours later, 1 ml DMEM with 10% FBS was added and incubated at 37^0C in a humidified atmosphere with 5% CO_2. On the following day, the medium were removed from 24 well plates and the rest in matrix were soaked by the gauze in the laminar flow bench. ORSCs were trypsinized and distributed into the matrix in 200ul DK-SFM with a pipette at the same density. The seeded matrix was put face up in 24 well plates, added 1.5ml DMEM with the supplement of DK-SFM per well 3 hours later. 4 days later, the surface of the SEs was lifted to the air–liquid interface. One week after the air-liquid co-culture, matrices were transplanted into full-thickness skin wounds on athymic mice. The medium was changed on each second day in vitro. The growth states of ORSCs and fibroblasts was observed under phase contrast microscope with MTT and DAPI staining.

2.3.5. MTT assay in matrix

As described above, we seeded different cells into 15mm diameter round matrices to test the proliferation ability. The details are shown in the table (Table 1.). At day 2, day 4, day 6, day 8, day 10 and day 12, the medium were removed. 10% MTT in each medium was added into wells ready to test and incubated overnight. After MTT solution was removed, DMSO was added and gently shaken for 10 minutes. Then the purple solution was collected respectively into 96 wells plate for ELISA assay at wave length of 470 nm.

Table.1 Groups for MTT assay in Matirx

	number	Culture medium	cells Density	Seeding time
Group ORSCs and fibroblast	18	DMEM with supplement and 10% FBS	both at 5×10^4/cm²	Day0:fibroblast Day1: ORSCs
Group ORSCs	18	DK-SFM with supplement	1×10^5/cm²	Day 1
Group fibroblast	18	DMEM with 10% FBS	1×10^5/cm²	Day 0
Group control	18	DMEM with 10% FBS	0	Day 0

2.3.6. Grafting of composite-grafts

All procedures were performed with approval of the University of Lübeck review committee for experimental work on laboratory animals. 3 female nu/nu mice six to eight weeks of age were grafted in a laminar-flow bench under sterile conditions. The animals were anaethetized with ketamine (10 mg/kg) and xylazine (2.4 mg/kg) via intraperitoneal injection. Two round full-thickness skin defects with 15mm diameter were created on symmetric sides of the nude mice back. The grafts were brought to the wound, trimmed exactly to the wound size and fixed with multiple single stitches of 5-0 nylon. The wound was covered with an occlusive dressing consisting of a sterile gauze and Tegaderm and secured with a tape dressing. The wound dressing was checked once every two days. Animals were sacrificed and analysed after 2 and 4 weeks for histology.

2.3.7. Identification of cells

Preparation of cytospins

The cytospins were made from ORSCs, keratinocytes and fibroblasts in the Cytospin 3 (SHANDON, Zeist, Netherlands) by centrifugation at 300g for 5 minutes. After air-dried at room temperature, the cytospins were fixed by 10% buffered formaldehyde and stored at 4^0 C for immunofluorescence.

Procedure of immunofluorescense stain

- Before immunofluorescence staining, the cryostat slides for control were fixed in 4^0 C for 10 minutes and air-dried ready to use.

- All slides were labeled clearly with a pencil, noting antibody and dilution.
- The slides were rinsed two times for 5 minutes each time in TBS.
- The cytospins were incubated with 100ul 0.1% Triton-100 for 10 minutes.
- The slides were rinsed three times for 5 minutes each time in TBS.
- The slides were incubated in 10% goat normal serum in PBS for 20 minutes.
- The slides were incubated in **Primary antibody** diluted in TBS overnight at 4^0 C.
- The slides were rinsed three times for 5 minutes each time in TBS.
- The slides were incubated in second secondary fluorochrome conjugated antibody (goat anti mouse) **antibody** diluted in TBS, containing 2 % goat normal serum in the dark for 45 min.
- The slides were rinsed three times for 5 minutes each time in the dark in TBS.
- The slides were covered by 1 ug/ml DAPI in the dark for 5 minutes.
- The slides were rinsed three times for 5 minutes each time in TBS in the dark.
- The slides were mounted with fluorescence mount medium.
- slides were stored at -20^0C in the dark until examination with green or red fluorescent light.

Table 2. Concentration of antibodies and kinds of conjugated fluorochrome

Antibody/clone	Dilution	conjugated fluorochrome
ß1 Integrin(MAB 13)	1:500	FITC
CK15 (CBL272)	1:200	FITC
CK19 (MAB 1608)	1:200	FITC
Ki 67 (MIB-1)	1:20	TRITC

Control of immunoflourescense staining

In the present study, positive and negative controls were set up to control the quality of Immunocytofluorescense (ICF). Normal human scalp or skin for specific markers was used as a positive control. As negative control, cytospins of human keratinocyte and fibroblast were used as internal negative control. Known positive tissues stained with normal serum (the same species as primary antibody) replacing the primary antibody was used as external negative control.

Assessment of ICF

Stained ICF slides were watched under a flourescense microscope. Pictures were captured respectively using Prog Res C10 Plus system at magnification of 10x, 20x and 40x.

2.3.8. Histology

2.3.8.1 Slide preparation

For morphological analysis, one part of the grafted tissue samples taken from the mice were embedded in Jung tissue freezing medium (Reichert-Jung, Nussloch, Germany) and frozen in liquid nitrogen vapor. The other part of samples was fixed in 10% buffered formaldehyde, embedded in paraffin and cut by microtome to a thickness of 5 µm. For immunohistological staining, paraffin-embedded slices were mounted on poly-L-lysin covered microscopic slides. Preparations were stained with hematoxylin and eosin. The cryostat sections made at 6 mm settings were mounted on superfrost slides (MENZEL, Germany), fixed in acetone at 4^0 C and air-dried, then processed for hematoxylin and eosin staining and immunohistochemistry (ICH) staining.

2.3.8.2 Immunohistochemistry

To identify the human origin of the reconstituted skin, we used an animal research kit in this study.

Principles of animal research kit (ARK**)**

The ARK is designed for immunohistochemical staining with mouse primary antibodies on formalin-fixed, paraffin-embedded tissues, cryostat tissues or cell preparations from any species, including mouse. The techniques used in this system are based on the avidin-biotin and peroxidase methodologies. This system is formulated to minimize reactivity of secondary anti-mouse antibody with endogenous immunoglobulin that may be present in the specimen. Prior to

application of the primary antibody to the specimen, the antibody is labeled using the Biotinylation Reagent, a modified biotinylated anti-mouse immunoglobulin. The primary antibody and the Biotinylation Reagent are mixed in solution, resulting in binding of biotinylated secondary antibody to the primary antibody (Gu, 1995; Tuson et al., 1990). The blocking reagent, containing normal mouse serum, is then added to the mixture. The mouse immunoglobulin present in the blocking reagent binds residual Biotinylation reagent not bound to the primary antibody, minimizing potential interaction with immunoglobulin endogenous in the specimen (see Figure 4) (Hierck et al., 1994; Fung et al., 1992). The biotin-labelled primary antibody is then applied to the specimen. The specimen is next incubated with streptavidin-peroxidase, followed by reaction with diaminobenzidine/hydrogen peroxide as substrate-chromogen.

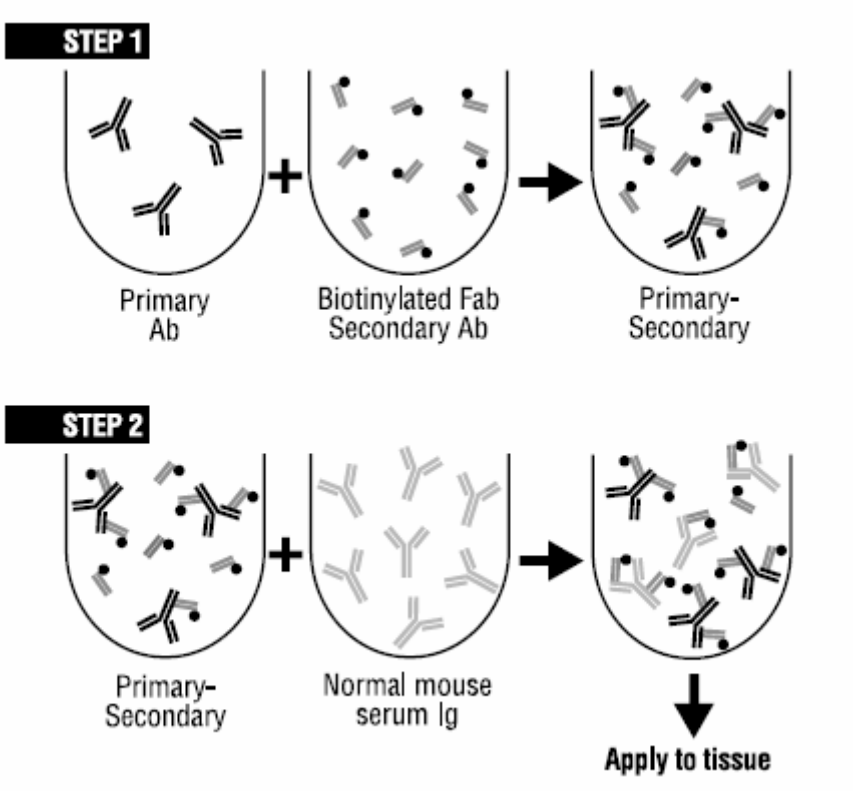


Fig. 5 Principles of animal research kit

Immunohistochemical staining procedure

Deparaffinization and antigen retrieval

- All slides were labeled clearly with a pencil, noting antibody and dilution.

- Deparaffinization and rehydration were performed as follows: Two times for 10 minutes in xylene; two times for 1 minute in 100% ethanol; two times for 1 minutes in 96% ethanol; two times for 1 minute in 70% ethanol; and two times for 1 minute in 50% ethanol.
- To facilitate the immunological reaction of antibodies with antigens in fixed tissue, it is necessary to "retrieve" the antigens through pretreatment of the specimens. Antigen retrieval has been shown to increase reactivity of the majority of antigens in tissues. In the present study, sections were immersed in antigen retrieval solution (10 mM citrate acid, pH 6.0) and boiled for 15 minutes in a pressure cooker. The pressure cooker was brought to full pressure as quickly as possible and the heating times measured exactly from this point.
- At the end of the heating time, the pressure was released. As soon as possible the hot buffer was flushed out with cold water. The specimens were then washed and cooled for 10 minutes.
- The sections were rinsed in PBS for seconds.
- The sections were immersed in 0.1% Triton for 10 minutes.
- Then, the sections were placed in modified endogenous oxidation blocking solution (3% hydrogen peroxide).
- At last, the slides were rinsed once for 5 minutes in PBS.

Blocking and staining

- The sections were incubated with one drop of Avidin reagent for 15 minutes.
- The slides were rinsed three times for 5 minutes each time in PBS.
- The sections were incubated with one drop of Biotin reagent for 15 minutes.
- The slides were rinsed three times for 5 minutes each time in PBS.
- The sections were incubated in 5% normal serum diluted in 5% BSA for one hour at room temperature to reduce non-specific binding of antibody. The incubation was performed in a sealed humidity chamber to prevent air-drying of the tissue sections. (The choice of blocking serum is the same as the species of the biotinylated secondary antibody).
- The slides were rinsed once for 5 minutes in PBS.
- The sections were covered with prepared biotinylated primary antibody diluted in 5% BSA, and incubated at room temperature for 15 minutes.
- The sections were rinsed three times for 5 minutes in PBS, shaking gently.

- The sections were covered with Streptavidin-Peroxidase for 15 minutes at room temperature in the humidity chamber.
- The sections were rinsed once for 5 minutes in PBS, shaking gently.

Development and counterstaining

• The sections were incubated for approximately 4-5 minutes in prepared DAB+ substrate-chromogen solution.

• The slides were rinsed well once for 5 minutes in distilled water.

• The sections were counterstained with Mayer's haematoxylin for 1 minutes.

• The slides were rinsed for 10 minutes in running water.

• The slides were differentiated in alcoholic-hydrochloric acid solution for 1 to 3 seconds.

• Then, the slides were rinsed for 10 minutes in running water.

• Finally, the slides were covered with coverslips.

Concentration of antibodies and DAB substrate incubation time

Initially, common slides of normal human skin were tested to determine the antibody dilution and DAB substrate incubation time. The optimal concentrations for the specific antibodies were obtained and successfully used in the paraffin slides of the reconstituted skin. The antibody dilution and developing times are shown in Table 3.

Table 3. Antibody dilution and DAB developing time of involved antibodies

Antibody	Dilution	DAB developing time (minute)
HLA-A (A18)	1:100	4
CK (MNF116)	1:100	5
Vimentin (V9)	1:50	3

Control of IHC staining

In the present study, positive and negative controls were set up to control the quality of IHC. Section of normal human skin was used as a positive control. As negative control, section of nude mouse skin was used as internal negative control.

Section of normal human skin stained with normal serum (the same species as primary antibody) replacing the primary antibody was used as external negative control.

Assessment of IHC

Stained tissue slides were watched under a light microscope. Pictures were captured respectively using Prog Res C10 Plus system at magnification of10x, 20x and 40x.

3. Results

3.1 Identification of cells

3.1.1 Identification of the isolated ORSCs

3.1.1.1 Characteristics of cell morphology

Seeded into the coated flasks, most of the isolated ORSCs could adhere to the flask in 15 minutes. They were small cells with a bright large nucleus and obvious nucleoli, while with few cytoplasm (Fig.6).

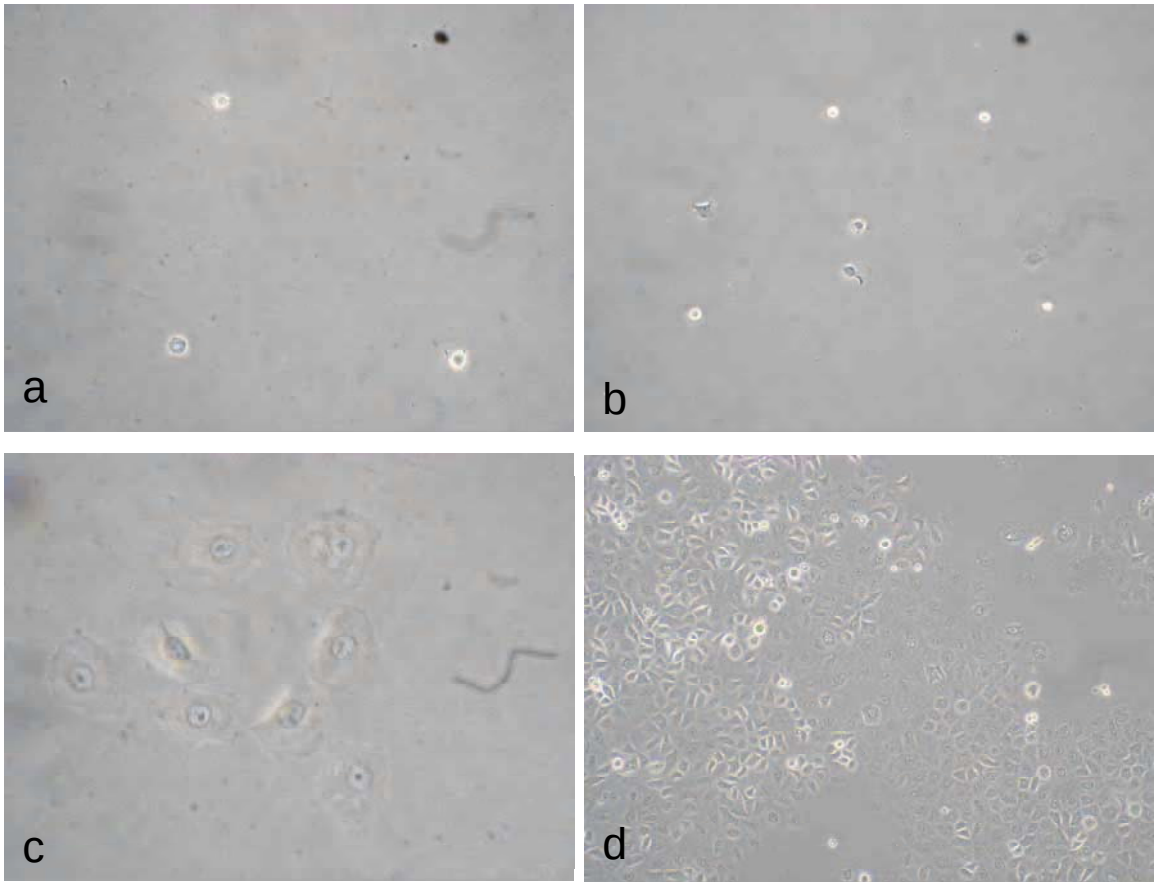


Fig. 6 Morphology of cultured ORSCs in the flask

The cultured ORSCs were round and small with a large nucleus and obvious nucleoli at day 1 after isolation (a). 2 days after seeding, the cells stretched and became larger (b). 3 days after seeding, ORSCs showed typical pavement organization (c) and (d). The ORSC colons were formed at day 6 (d). (a), (b) and (c) at magnification of 40X, (d) 10X.

3.1.1.2 Immunophenotypical characterization of ORSCs

3.1.1.2.1 Expression of CK15 in ORSCs

CK15 staining was present in the ORS of the normal human scalp and strong in the bulge region (Fig.7). The positive staining of CK15, which showed green fluorescence in the cytoplasm, could be found in some of our cultured ORSCs during passage 0 and one. The positive stain rate of CK15 was 57.32% in passage 0, and 26.47% in passage one (Fig.8). But in passage two, it disappeared. In keratinocytes of the same donor, no positive staining was observed then.

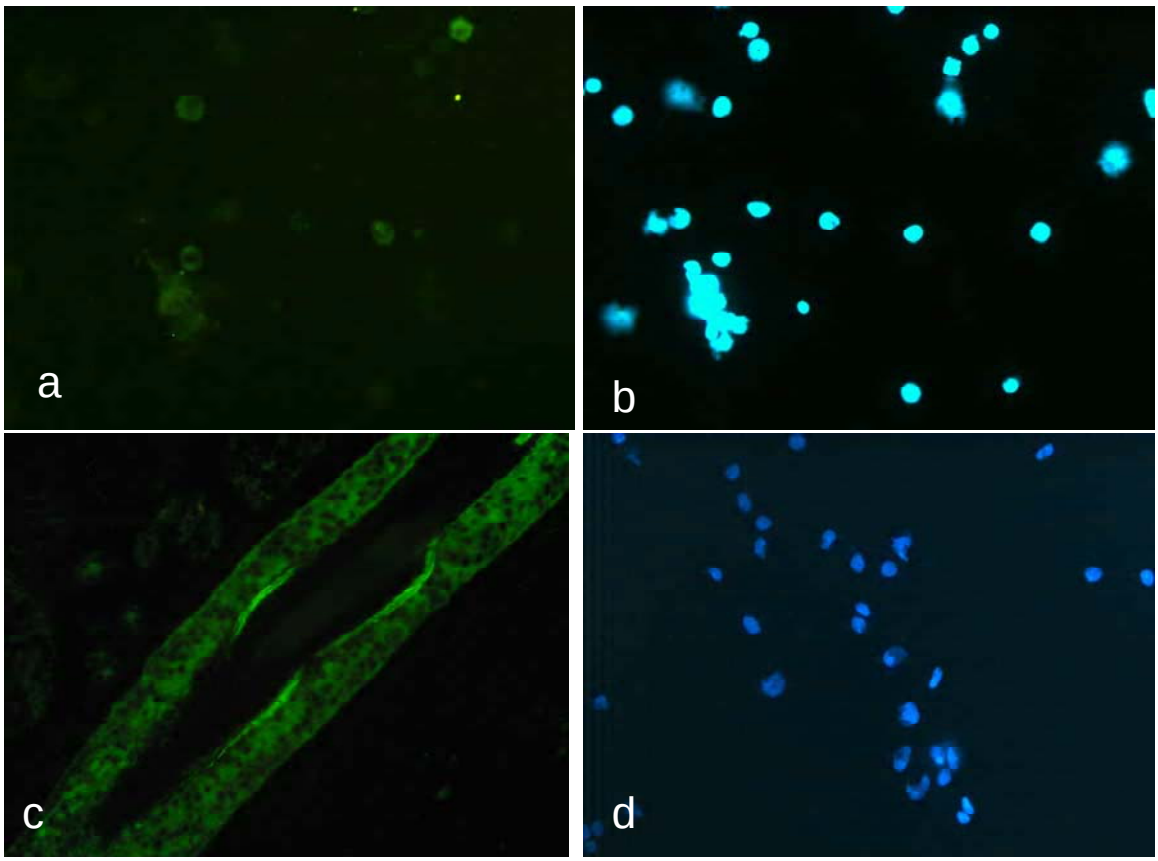
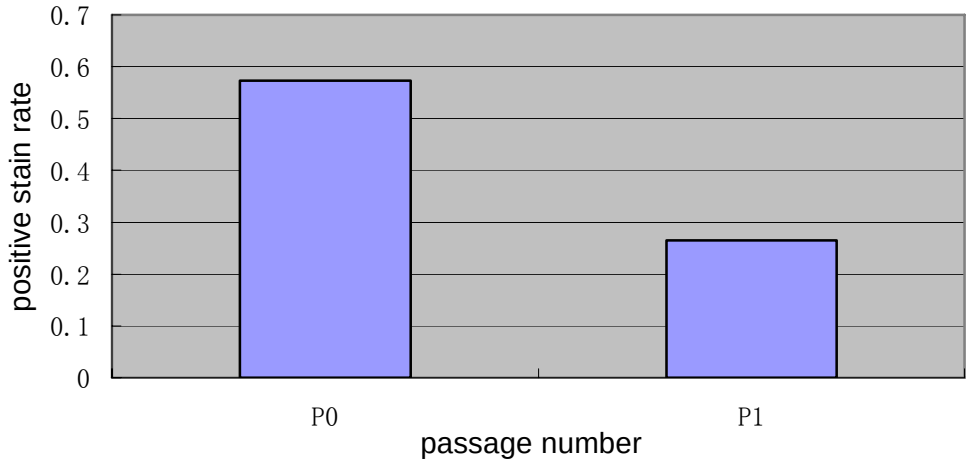


Fig.7 CK15 staining

(a) CK 15 positive stained ORSCs in passage 1. (b) DAPI countstaining of (a). (c) CK 15 positive stained in ORS, strongly positive in bugle region. (d) Negative control in keratinocytes. (a), (b) and (d) at magnification of 40X, (c) at 20X.

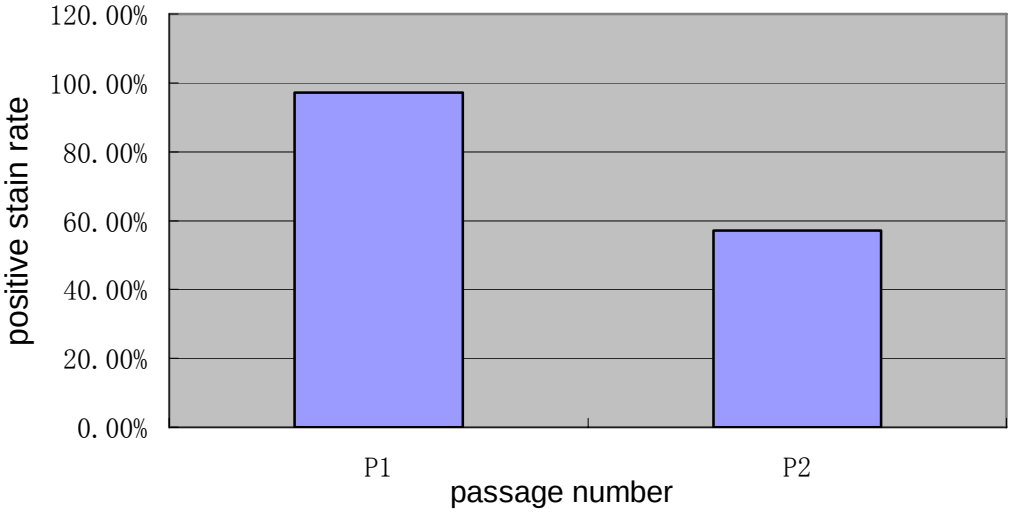
Fig. 8 Positive stain rate of CK15 between passages



3.1.1.2.2 Expression of CK19 in ORSCs

In the normal scalp, the basal keratinocyte cell marker CK19 was found in the most peripheral cell layer of the ORS and basal layer. The strong staining was present in the bulge region (Fig.10). In our cultured ORSCs, positive staining of CK 19 could be detected from passage 0 to passage 2, which was most strongly positive in passage 0, weak in passage 2. The positive stain rate in passage one and passage two were 97.10% and 57.20% respectively (Fig. 9). But in passage 3, the staining disappeared.

Fig. 9 Positive stain rate of CK 19 between passages



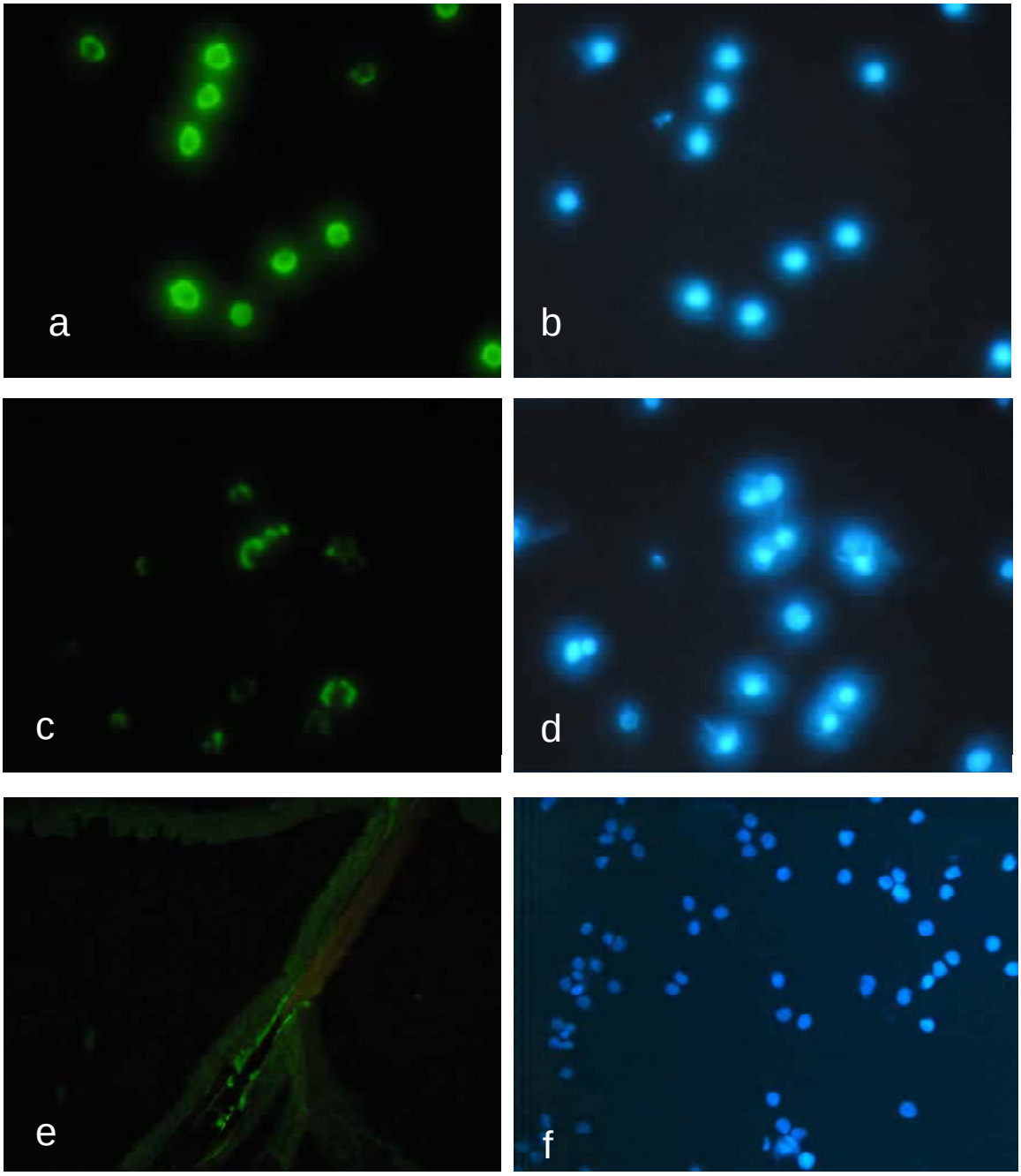


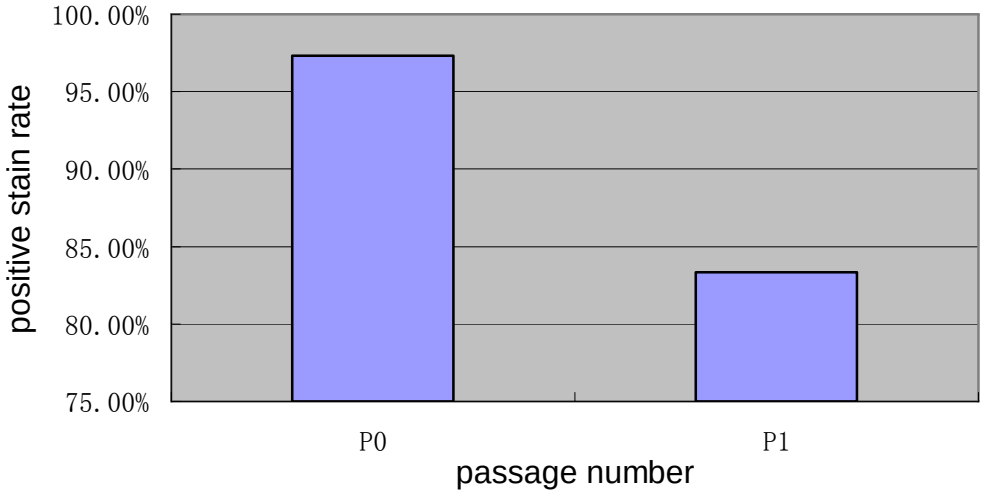
Fig. 10 CK19 staining

CK19 was positively stained in ORSCs passage 0 (a) and passage two (c). DAPI counterstaining passage 0 (b) and passage two (d). CK19 was positively stained in ORS and basal layer, strongly in bulge region (e). No positive stain was observed in keratinocytes (f). (a), (b), (c), (d) and (f) at magnification of 40X, (e) at 10X.

3.1.1.2.3 Expression of ß1 Integrin in ORSCs

Taken both of the normal skin cryoslides and cytospins of keratinocytes as control, the stem marker Integrin ß1 just were positively stained in basal membrane and negatively stained in keratinocytes. In cultured ORSCs passage 0 and passage 1, positive staining of ß1 Integrin could be found and the positive stain rate were 97.3% and 83.33% respectively (Fig.11), while it disappeared in passage 2 (Fig.12).

Fig.11 Positive stain rate of ß1 Integrin between the passages



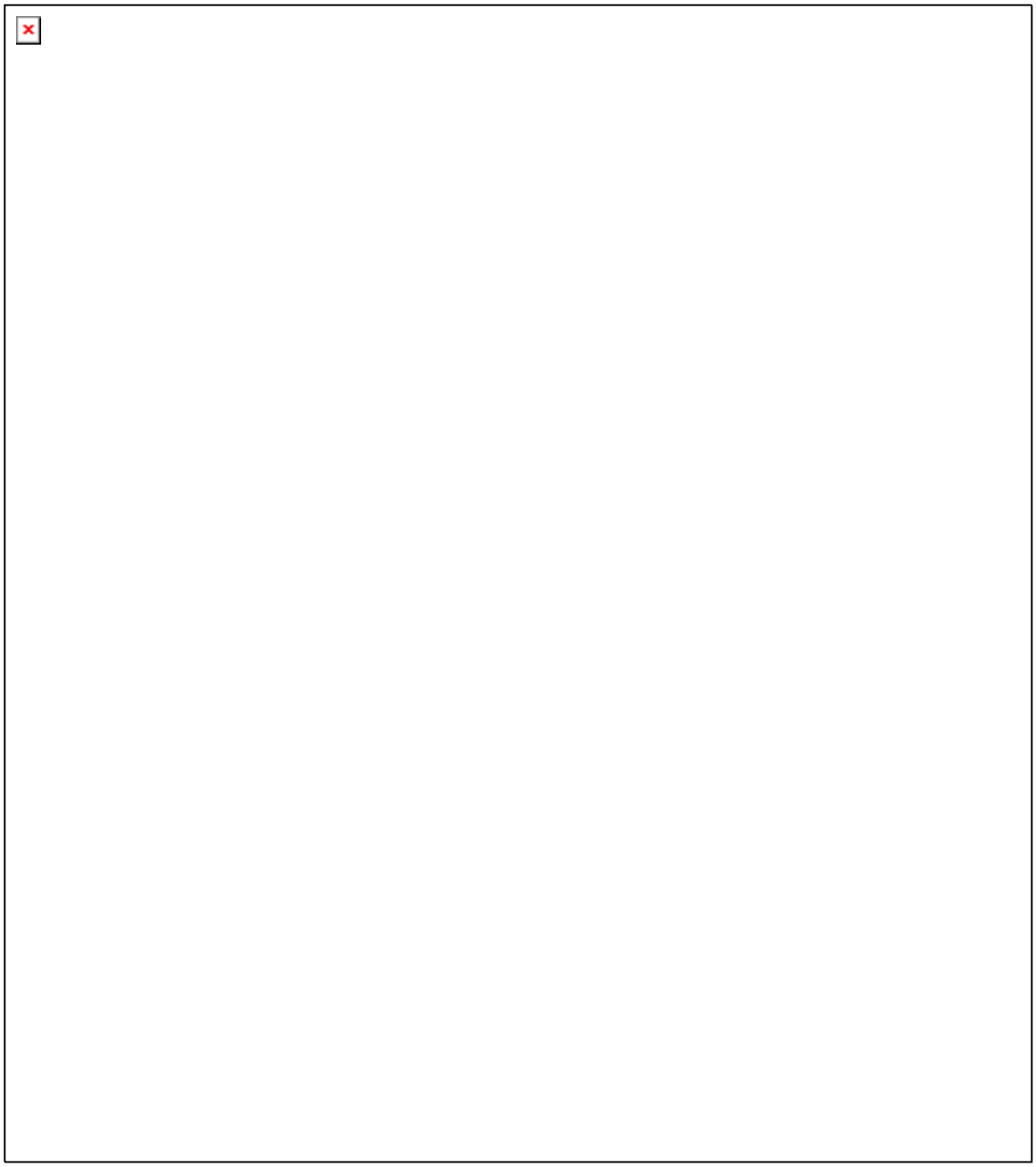


Fig.12 ß1 Integrin staining

ß1 Integrin was positively stained in ORSCs passage 0 (a) and passage one (c). DAPI counterstaining in passage 0 (b) and passage one (d). ß1 Integrin was positively stained in basal layer (e). No positive stain was observed in keratinocytes (f). All at magnification of 40X, (e) at 10X.

3.1.2 Identification of keratinocytes

We used Cytokeratin (MNF116) stain to identify human keratinocytes. Keratinocytes in the cytospins were positive in MNF116 stain, as Fig.13 shown.

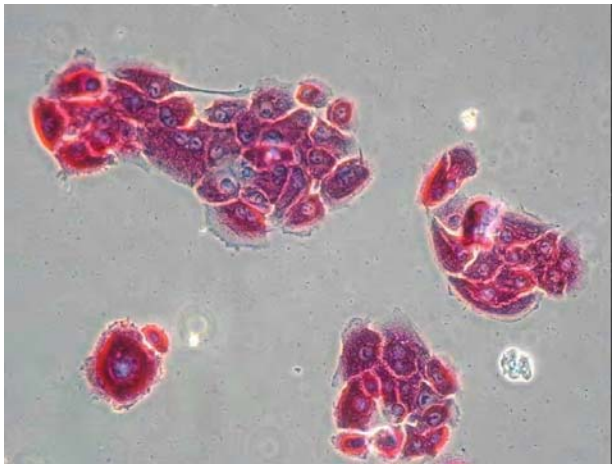


Fig.13 Cytokeratin (MNF116) was positively stained in cytoplasm of keratinocytes. At magnification of 40X.

3.1.3 Identification of fibroblast

Human fibroblasts were identified by Vimentin (V9) stain. The positive red stain was shown in the cytoplasm of fibroblasts (Fig.14).

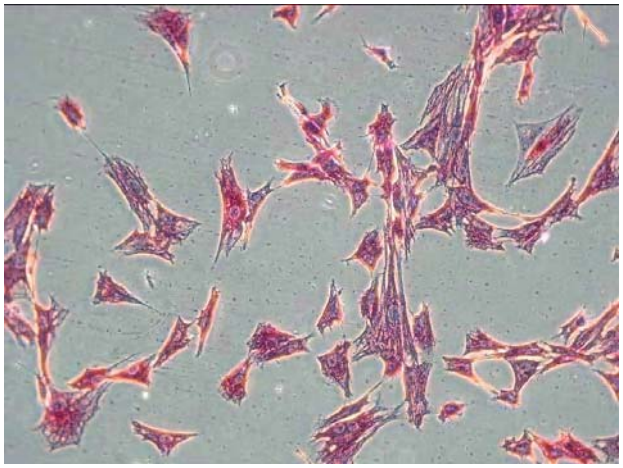


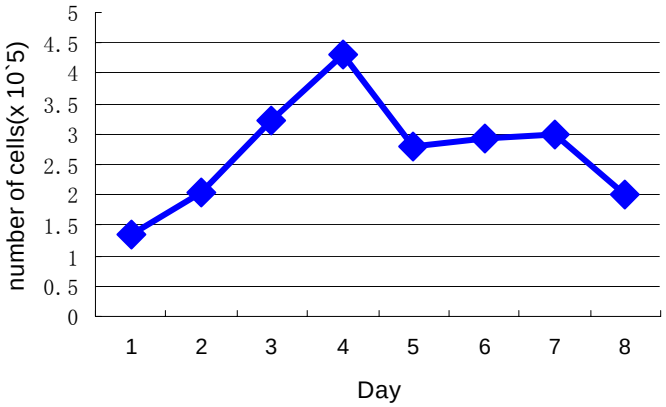
Fig.14 Vimentin (V9) was positively stained in cytoplasm of fibroblasts. At magnification of 40X.

3.2 Proliferation ability of the isolated ORSCs

3.2.1 Cell growth curve

During day 1 to day 4, the cell number of ORSCs passage one increased quickly and doubled after two days. ORSCs passage one was confluent at day 4. So the cells stopped to grow and kept in period of platform from day 5 to day 7. If they were not subcultured in time, the cells number decreased from day 8 (Fig.15). The results show that cultured ORSCs have a high proliferation ability.

Fig.2 Growth curve of ORSCs P1



3.2.2 Ki-67 stain

As the proliferation marker, Ki-67 was used to check the proliferation ability of the isolated ORSCs. In the ORS of same donor's scalp, Ki-67 positive stain could be sporadically observed (Fig.16). One location was near the external low part of the ORS, and the other was in the bulb region. But most of ORSCs passage one were Ki-67 positive stained. These results demonstrated that ORSCs were proliferating after being isolated and cultured.

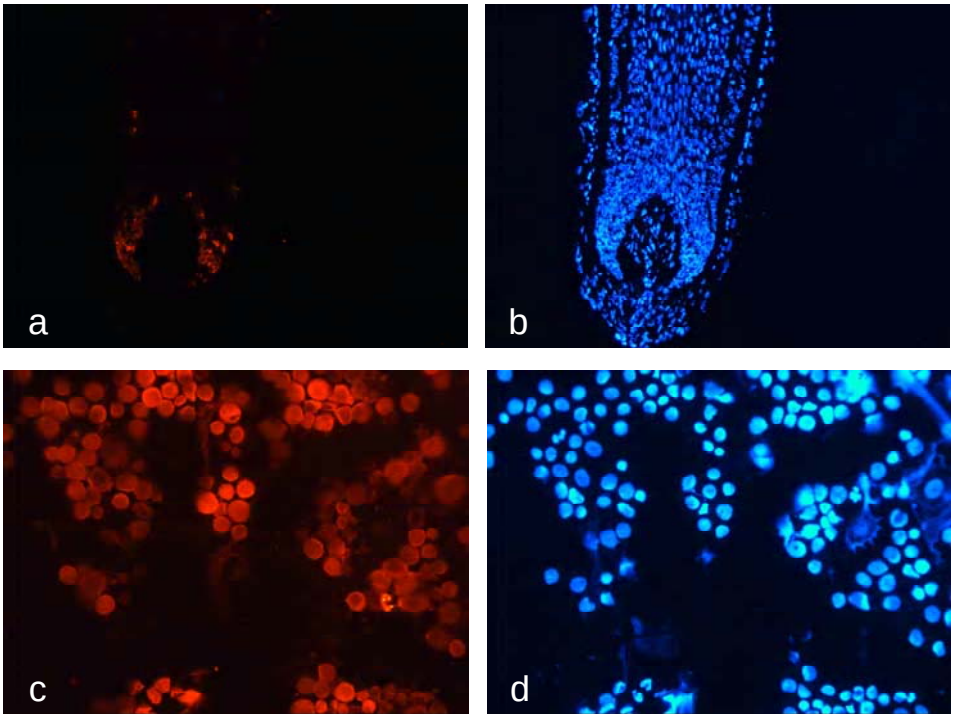


Fig.16 Ki-67 staining

(a) Ki-67 positive stain was sporadically shown in the external low part of the ORS and the bulb region. Most of ORSCs passage one (c) were Ki-67 positive stain. (b) and (d) represent parallel DAPI staining. All at magnification of 40X.

3.3 Proliferation of cells in matrix

MTT assay results

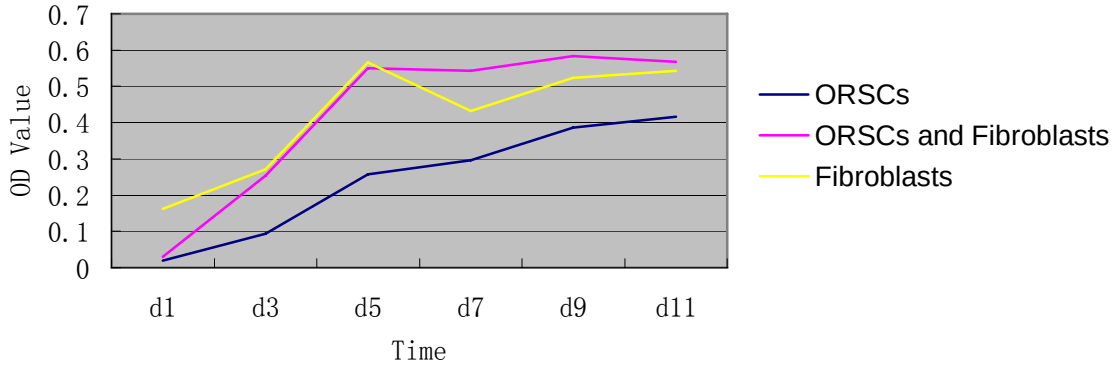
We seeded ORSCs, fibroblasts and ORSCs with fibroblasts into matrices respectively at same density and test the cells vitality in matrix by MTT assay. We calculated the cell growth curves of different groups. ORSCs grew quickly during the first 4-5 days, as shown in the 24 well plates (Fig.17). After day 9, they stopped to proliferate and the curve kept like a platform. This result demonstrates that ORSCs can proliferate in the matrix. Human fibroblasts grew fast in the first 5 days and then kept in the period of platform. But half density of ORSCs with half density fibroblasts showed more fast proliferation ability during the first 5 days and then could keep the cell vitality more stable, compared to fibroblasts alone.

MTT stain and DAPI stain results

Our MTT stain and DAPI stain results also show that combining two kinds of cells

resulted in quicker proliferation abilities (Fig.18).

Fig.17 Growth curves of different cells in Matrix



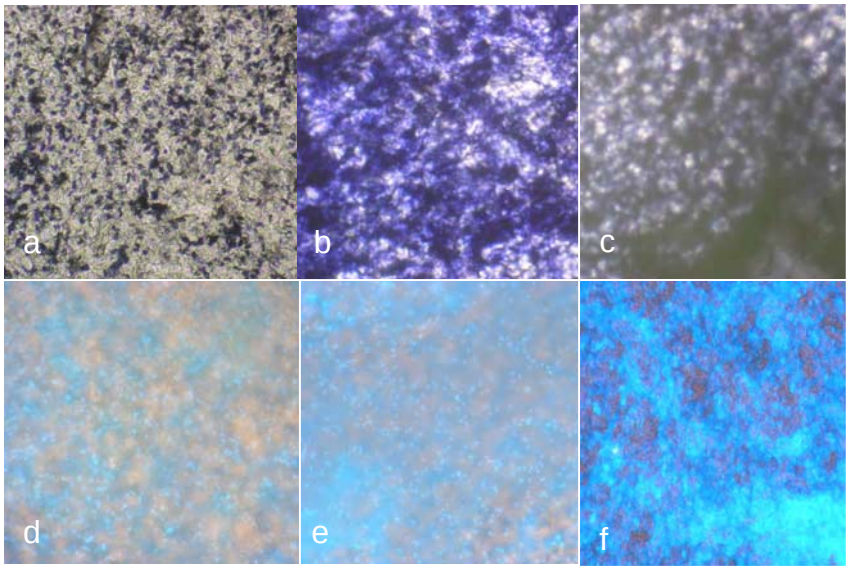


Fig.18 MTT (a-c)and DAPI (d-f) stain of cocultured human ORSCs and fibroblasts in matrix (a) and (d) at day 1; (b) and (e) at day 7; (c) and (f) at day 12. All at magnification of 10X.

3.4 Characterization of grafted SEs

3.4.1 Structure characteristics of grafted SEs

In Haematoxylin and Eosin staining of the grafts, by 2 weeks, the layer of what appeared to be epidermal tissue was apparent on the surface of the matrix, while some cells were accompanied with parakeratosis. The regenerated epidermal tissue closely resembled that of normal human skin tissue comprised of a basal layer, five to ten granular layers and abundant cornified layer. But there were some

small nests constituted with keratinocytes in the matrix (figure. 19).

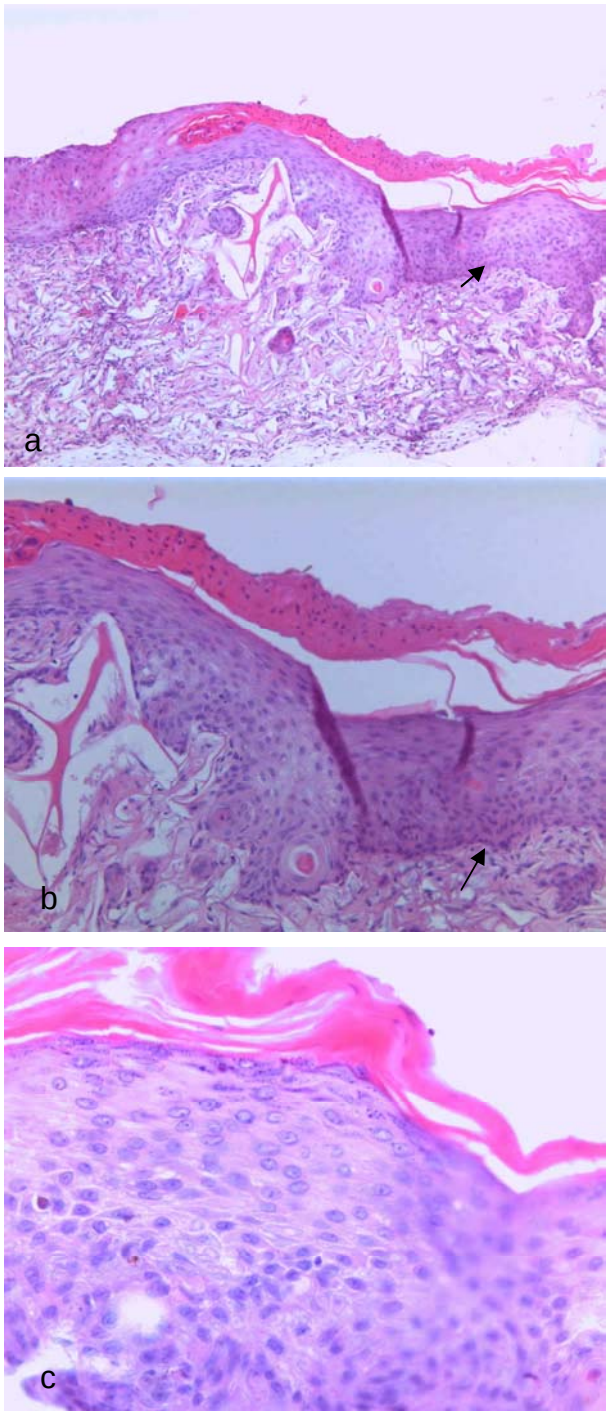


Fig.19. Histological appearance of regenerated skin 2 weeks after grafting on nude mice. (a) Structure with epidermis and dermis, the arrow points at the basal layer (10X). (b) There was some parakeratosis in the neoepidermis. Interface between epidermis and dermis was very tightly (20X). (c) Neoepidermis with a basal layer, granular layers below and abundant cornified layers above the basal layer (40X).

3.4.2 Identification the origin of the grafted SEs

3.4.2.1 Results of HLA-A stain

We used HLA-A (A18) to identify the harvested tissue 2 weeks after transplantation. In both epidermis and dermis, the positively staining cells (human fibroblasts and keratinocytes/OSRCs) were found, while it was negatively stained in mouse skin as controls(Fig.20). These results showed that the cells contributing to new skin originated from human.

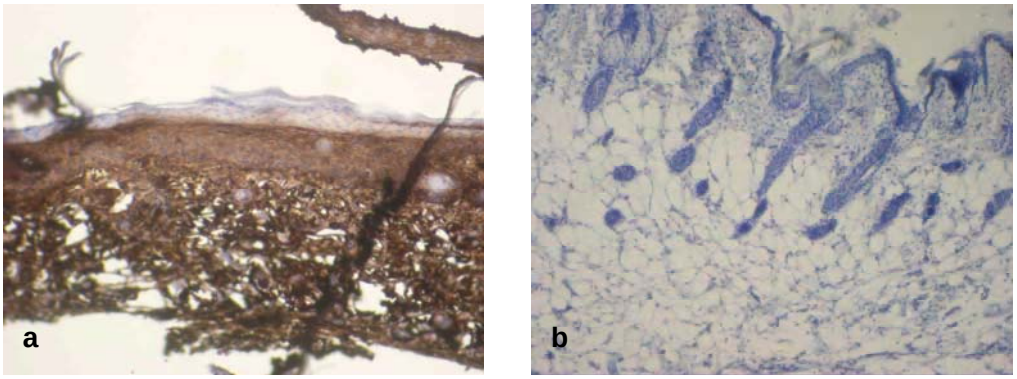


Fig.20 HLA-A staining in graft tissue after 2 weeks. The epidermis and dermis in grafted tissue were positive stained for HLA-A (a), while the mouse skin was negative stained (b). All at magnification of 10X.

3.4.2.2 Results of human CK stain

As the marker of keratinocytes, we used human CK (MNF116) to identify the origin of neoepidermis. For the positive control slide of normal human skin, human CK was only positive stained in the basal layer epidermis. But for the slides from grafted tissue after 2 weeks, the positive stain could be found in most layer of epidermis, such as basal layer and granular layers, except cornified layer. This documents that this kind of structure formed in the nude mice originated from the human ORSCs.

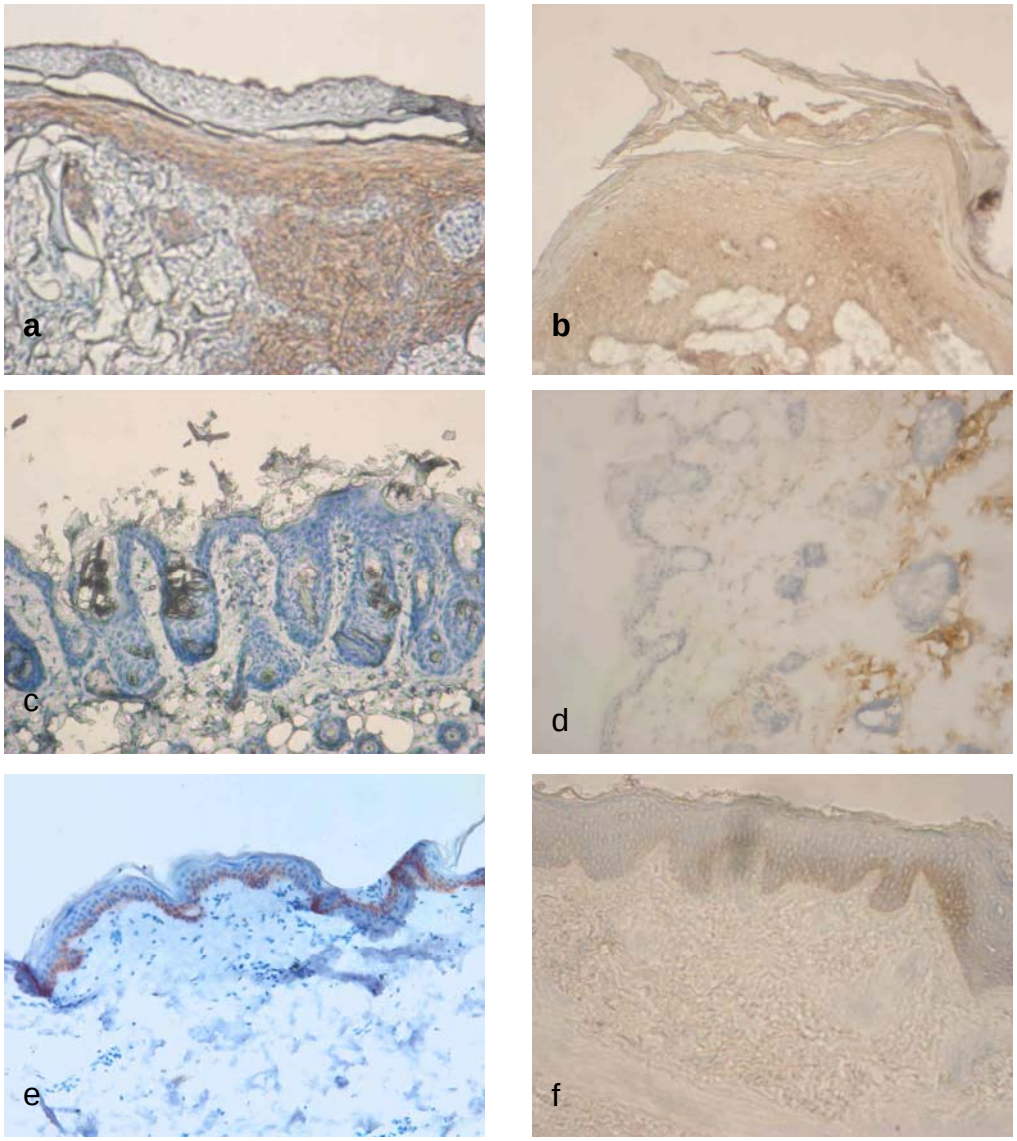


Fig.21 Human CK staining in graft tissue. Human CK was positively stained in the epidermis of grafts 2 weeks after transplantation (a) and in 4 weeks grafts (b). But the mouse skin was negative stained (c) and (d). (e) and (f) were normal human skin as positive control. (a), (c) and (e) were paraffin slides, rests cryoslides. All at magnification of 20X.

3.4.2.3 Results of Vimentin stain

We used an animal research kit and mouse monoclonal anti-Vimentin to identify the dermal grafts origin from human fibroblasts 2 weeks after transplantation. The positive stain could be found in the new dermal layer and in the junction with the epidermis of the graft. The dermis constructed with fibroblasts and matrix was thick and tightly, as shown in the picture (Fig.18). This result gave evidence that the dermis resulted from human fibroblasts which we seeded into the matrix in vitro.

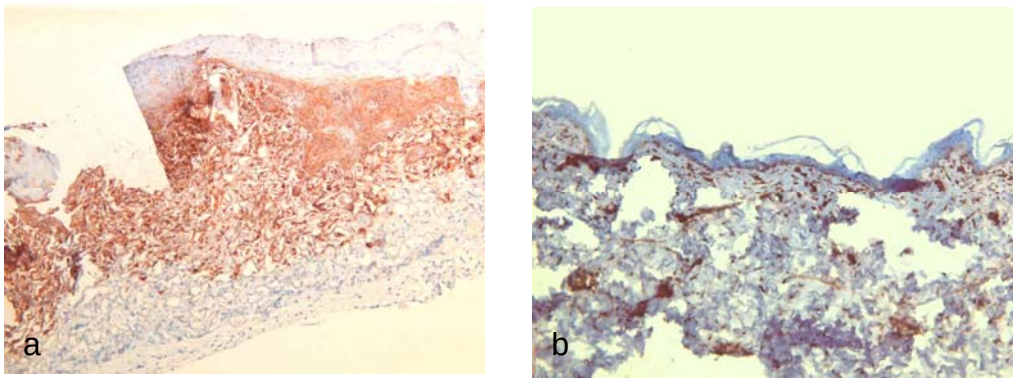


Fig.22 Human Vimentin (V9) staining in graft tissue. Human Vimentin was positively stained in the dermis of graft 2 weeks after transplantation (a). Normal human skin served as positive control (b). All were paraffin slides and at magnification of 10X.

4. Discussion

4.1 Studies on ORSCs

In our studies, the isolated ORSCs could express some epidermal stem cell markers. An important finding in this study is that phenotypical characteristics of the hair follicle from scalp section are preserved in the isolated ORSCs, including the expression of CK 15, CK 19 and ß1 Integrin.

CK 15 was positive stained in some of our isolated ORSCs from passage 0 to passage one and I all ORS of anagen hair follicles. These results correspond well with the data of Lyle (Lyle et al., 1998) and Porter (Porter et al., 2000). The bulge ORSCs only account for a little part of the total ORSCs. This fact may explain why the positive stain rate of CK15 in the isolated ORSCs was not very high. Stem cells after culture in vitro could differentiate into TA cells. Therefore, terminally differentiated cells could be the reason why the positive expression disappeared till passage two.

Michel (Michel, 1996) and Stasiak (Stasiak et al., 1989) reported, CK19 stained positively in the bulge ORS scalp. They isolated ORSCs from passage 0 to passage two and identified these isolated cells as originating from ORS and positive for CK 19. The fact that these cells in the scalp were positive for CK19, but Ki-67 negative, is a strong indication that they represent stem cells in the hair follicle. That the isolated ORSCs in passage two were positive for both CK19 and Ki-67 indicated that they were transient amplifying cells (TA cells) and proliferating in vitro.

ß1 integrin is expressed in all basal keratinocytes It has been shown that as keratinocytes leave the basal layer, they down-regulate the expression of ß 1 integrins (Watt and Hertle, 1994). Ivanova et al (Ivanova et al., 2002) and Ramalho-Santos et al (Ramalho-Santos et al., 2002) found integrins to be upregulated in human hair follicle stem cells compared to their transiently amplifying progeny. In our studies, ß1 integrin were positive stained in the basal layer of human scalp and the culture ORSCs. After the passage one, our culture ORSCs became negative for ß1 integrin. This indicated that the cells probably

differentiate into TA cells or terminal differential cells.

Our data about cell vitality and proliferation showed that cultured ORSCs grew fast and confluent at day 5, which documented that they hada high proliferation ability (Fig.11) after isolation in vitro.

All the markers found in the hair follicle of the scalp were also preserved in our isolated ORSCs, including CK15, CK19 and ß1 Integrin. We therefore conclude that our isolated ORSCs contained some follicular stem cells. Ki-67-positive cells indicated that differentiation may have occurred in these cell cultures.

4.2 Improved techniques in our study

4.2.1 ORSCS instead of keratinocytes

Cell growth dynamics are essential to generate skin equivalents (SE) using keratinocytes. To speed up the keratinocyte proliferation process, in a different study our group has reconstructed skin equivalents with human fibroblasts and keratinocytes from foreskin of newborns, which were genetically modified to express PDGF-BB temporarily. Theoretically, the method is feasible in laboratory. But clinically, this technique is hindered due to donor shortage and the clinical problems with handling genetically modified cells with concomitant biohazards. As our results on ORSCs have shown, human keratinocytes can also be quickly isolated from the ORS of plucked anagen scalp hair follicles or the scalp derived from face-lifting and biopsy, as other researchers also have reported (Ohyama et al., 2006; Hoeller et al., 2001). It is an essential advantage that the ORS represents a source of easily and repeatedly available keratinocytes with a high proliferation potential even in older donors and that a similar potential for differentiation as interfollicular keratinocytes (Limat and Hunziker, 2002) can be achieved. Therefore, we took isolated ORS cells instead of neonatal foreskin keratinocyes as source keratinocytes of SEs.

4.2.2 Technique of cell isolation

On the basis of methods described by Ohyama (Ohyama, 2006) and Geng (Geng, 2006), we improved the method of isolating ORSCs. We used the scalp-only derived cells from face-lifting, not the plucked hair follicles. So the age of donors was in average over 50 years old. But our isolated ORSCs still kept their high proliferation ability, as our results have shown. Especially, we dissected and isolated ORSCs only from low and middle parts of hair follicles, which contain the bulge region. Also, we selected ORSCs for reconstructing SEs by removing the unstuck cells 15 minutes after planting, which guaranteed to contain more stem cells in the flasks and contributed to the high quality of our reconstructed SEs.

4.2.3 Culture technique of artificial skin

Three-dimensional SEs composed from C-GAG matrix, fibroblasts and keratinocytes can improve experiments requiring co-cultivation of different skin cell types (Moll et al., 1998) and can be served as transplant (Auger et al., 1998). An obvious advantage of three-dimensional SEs is their morphological similarity to *in vivo* skin when compared with any other available artificial skin systems such as keratinocytes on a carrier or keratinocytes in matrix. In a three dimensional SE, other cell types of the skin can be integrated to better mimic the *in vivo* situation. Recently, the successful integration of cell types like Langerhans cells (Fransson et al., 1998), melanocytes (Archambault et al., 1995; Sanquer et al., 1998), endothelial cells (Black et al., 1998) and pilosebaceous units (Michel et al., 1999) has been reported to improve the different characters of the SE.

Upon starting to design our SEs, we first referred to the method described by Erdag (Erdag and Sheridan, 2004). However, in the course of our experiments we modified several aspects which we believe are important to improve the generation of these SEs. First, as Limat (Limat and Hunziker, 2002) and Gho (Gho et al., 2004) reported, human keratinocytes can be isolated from the ORS of plucked scalp hair follicles. We isolated amounts of ORSCs efficiently from hair follicles derived from the face-lifting scalp, used for restructuring SE. Our isolated ORSCs contained some hair follicle stem cells, which can differentiate into TA and terminally differentiated cells to help to restructure SE quickly after in vitro culture. Secondly, according to the method described by Geng (Geng et al.,2006), we modified to

restructure SE with human fibroblasts and human ORSCs in C- GAG matrix (Integra^{TM}) by one-step method. Thirdly, we altered the composition of the culture medium and added the DK-SFM supplement in medium as described in our Materials and Methods. Lastly, we transplanted the SEs into the full-thick defect wound in vivo, where they formed the epidermal and dermal structure within 2 weeks.

4.2.3.1 3-Dimension culture method in C-GAG

We used a 3- dimension culture system to co-culture human ORSCs and fibroblasts in C-GAG matrix and reconstructed SEs within in 12 days in vitro. After 3 days of co-culturing in the medium, we lifted the matrix to the air-liquid interface to induce the keratinocytes to form epidermal structures at the air-liquid interface. Usefully, at that time, the matrix with silicon layer was hard enough to lie on the stainless steel ring. This could be due to the proliferation of fibroblasts in matrix. Thus, unlike other 3-dimension culture method (Witte et al., 2005), we needed not to use the insert, which was complex to operate and might increase the culture cost if the method will be used for patients in the future.

The well-differentiated epidermis of grafts at 2 weeks identified our 3-D culture model in vitro as successful. In Medalie's study, employed SEs cultured under submerged conditions showed that the transplanted tissues matured 8 weeks after grafting. The SEs were grown at the air-liquid surface to form a well-stratified epithelium containing stratum corneum and exhibited a considerable barrier function (Geer et al.,2002; Andreadis et al., 2001). Using keratinocytes isolated from the outer root sheaths of hair follicles, Limat (Limat et al., 1996) obtained a sheet ready for transplantation after more than 3 weeks. Hoeller used plucked human hair follicles implanted into DEs and restructured SEs wthin 14 days on average (Hoeller et al., 2001), while their restructured SEs did not achieve in vivo transplantation quality. Compared to them, we decreased the total culture time of our restructured SEs in vitro to 12 days, including seeding fibroblasts and ORSCs into matrix at day 1 and day 2 respectively, co-culture in medium for 4 days and culture at air-liquid interface for 7days. This implies that our procedure could shorten the time to prepare the SEs for acute wounds if it is proven efficient for

clinical use in the future.

4.2.3.2 Improved medium ingredients

We altered the composition of the culture medium and added the KD-supplement in medium as described in Materials and Methods. For the isolated ORSCs, we cultured them in DK-SFM with the supplement. But for the restructured SEs, it is the first time to use DMEM high glucose with L-glutamine, 10% FBS and DK-SFM Supplement, in contrast to the previous studies (Limat et al., 2002; Hoeller et al., 2001). The calcium condition and DK-SFM supplement in DMEM with 10% FBS could contribute to TA cells to differentiate into epidermal keratinocytes and construct epidermal structures in the matrix (Savignan et al., 2004).

4.2.3.3 Co-culturing ORSCS with fibroblasts

As many studies identified, an intact dermis is necessary to promote the renovation of epidermis in several skin diseases, in particular chronic leg ulcer (Coulomb et al., 1998; Limat et al., 1996; Limat et al., 2002; Lamme et al., 2000; Leary et al., 1992). Thus, a suitable DE should be the basis of any approach to generate SEs *in vitro* for transplantation purposes *in vivo.* We suggest to culture ORSCs with fibroblasts together in collagen GAG matrix by a one-step method to overcome putative problems arising from either just using DEs or keratinocyte grafts, respectively. This notion is supported by a recent observation, showing that fibroblasts integrated in collagen synthesize significantly higher amounts of vascular endothelial growth factor than in monolayer cultures, thus promoting the angiogenic potency of the transplants and resulting in a better survival rate (Pinney et al., 2000). After transplantation of three dimensional SEs generated on the basis of a DE, the cosmetic results, mechanical strength, and hemostasis are improved when compared to epidermal equivalents composed from keratinocytes exclusively (Coulomb et al., 1998). Thus, we improved to take Integra as a matrix frame to seed fibroblasts and ORSCs and restructure SEs by one-step method.

4.3 Characteristic advantages of reconstructed skin

To investigate the similarity of the transplanted SEs to the human skin, histology and immunohistochemistry were usually performed (Schoop et al., 1983; Lenoir et al., 1988; Lenoir et al., 1990). Here we studied the transplanted SEs' compartments like dermis and epidermis by immunohistochemistry with human CK (MNF116) and Vimentin (V9) as primary antibodies.

Two weeks after grafting, the human epidermis was fully differentiated. The regenerated graft comprised a basal layer, five to ten granular layers and abundant cornified layer. Human CK positive staining could be expressed in both stratum spinosum and stratum basal layer. While in the normal skin, it was only stained in the stratum basal layer. This result implies that the transplanted SEs preserved their high proliferation ability.

Plenty of positive stained fibroblasts and matrices constituted the neodermis and endothelial cells could be found within dermis. These results demonstrate the high degree of similarity between our reconstructed SEs and the structure in normal human skin as delineated in the Results section both after 2 weeks and 4 weeks. Interestingly, among the structure of grafts after 2 weeks, some nest-like structures, which were CK positively stained, were found under the basal layer and in the new dermis. But in the grafts after 4 weeks, this kind of structure disappeared and the new dermal structure looked more similar to that of normal human skin. This appearance implied that the nests might move towards and reach the surface of SEs, which contributed to regenerate a robust epidermal layer (Jones et al., 2003).

4.4 One step method of transplantation in vivo

We used a one step method to transplant the reconstructed SEs into the full-thickness skin defect model of nude mice after 3-D culture in vitro. The nude mouse model is an economic and convenient model for transplantation. Also, since human cells were employed, we had to employ an immunodeficient animal model to exclude host versus graft reactions. Apart from immunology, the pig is an excellent model to engineer skin equivalents in transplantation experiments

because of its physiologic similarities to that of human skin. In our grafts of 4 weeks, only part of human epidermis is left due to wound contraction, compared to two weeks. Our 2 weeks data, however, have already shown that within this relatively short time frame, we were able to generate epidermo-dermal constructs within a one step procedure due to the fast developing epidermal regeneration capacity of OSRCs.

4.5 Prospect

The technology of autologous epidermal equivalents prepared with ORS cells to treat chronic wounds has been made commercially available (EpiDex™). The clinical outcome of EpiDex is currently compared to autologous split-thickness meshed grafts in a controlled clinical study. For the hard-to-heal chronic ulcers, it is difficult to use EpiDex to repair wounds in one time because it doesn't contain dermal substitute and human fibroblasts.

Our reconstructed SEs contain well-differential epidermis from human ORSCs, which also can be easily and quickly isolated from autologous plucked hair follicle (Hoeller et al., 2001) and fibroblasts. The main indication for the grafting of SEs generated from autologous ORSCs in acellular dermis will be as an alternative to surgical autografting to avoid an additional wound at the donor site. A major obstacle still represents the vascularisation of the dermal layer. This problem has not yet been adequately solved and still represents the main clinical barrier to be overcome.

5 Summary

The bulge region, a portion of outer root sheath, contains the hair follicle stem cells, which show multipotency to differentiate into almost all epithelial cell types. To produce an improved quality skin equivalent, we established a rapid, easy and effective three-dimensional skin equivalent model on the basis of human dermal fibroblasts, collagen-GAG matrix (dermal equivalent) and outer root sheath cells (ORSCs).

We dissected hair follicles from temporal scalp tissue after face-lift surgery and isolated ORSCs from the middle and lower parts of hair follicles. The ORSCs were primary cultured and subcultured in KD-SFM for cell studies and seeded into collagen-GAG matrices. The matrices were seeded with human fibroblasts, cultured in DMEM with 10% FBS to form a dermal equivalent (DE). 24 h later, passage 1 ORSCs were seeded into the DE and cultured. 4 days later, the surface of the SEs was lifted to the air–liquid interface. One week after the air-liquid co-culture, matrices were transplanted into full-thickness skin wounds on athymic nu/nu mice. Wound tissue samples were harvested and examined by means of histology and immunohistochemistry.

The isolated ORSCs had high proliferation ability. The expression of the immunofluorescence antibodies of CK15, CK19 and ß1 Integrin were all positive in the primary ORSCs. After passage 2, the expression of Integrin ß1 and CK15 decreased, while expression of CK19 was still positive. 2 weeks after transplantation, a fully developed, multi-layered and cornified epidermis layer could be observed in the matrices. After 4 weeks, the epidermal layers were still stained positive both for antibody human cytokeratin and HLA-A.

This study gives evidence that our isolation technique allows culturing of ORSCs, which contain some bulge stem cells. The skin equivalent which we restructured with fibroblasts and OSRCs in vivo is able to form histological structures similar to human dermis and epidermis within 2 weeks.

5. Zusammenfassung

Die Wulstregion ist ein Teil der äußeren Haarwurzelscheide. Sie beinhaltet die Stammzellen des Haarfollikels, welche sich als multipotente Zellen in fast alle epitheliale Zelltypen differenzieren können. Um ein verbessertes Hautäquivalent zu erhalten entwickelten wir ein einfach zu handhabendes, effektives dreidimensionales Model. Die Vergleichbarkeit zur Haut wurde durch die Verwendung von humanen dermalen Fibroblasten, Kollagen-GAG-Matrix (der dermalen kollagenen Matrix entsprechend) sowie Zellen aus der äußeren Haarwurzelscheide (outer root sheath cells, ORSCs) erreicht.

Wir präparierten Haarfollikel aus der temporal Region (das Gewebe wurde im Rahmen von face-lift Operationen gewonnen) und isolierten ORSCs aus dem mittleren und unteren Anteil der Haarfollikel. Die ORSCs wurden primär kultiviert um Subkulturen in KD-SFM für Zellstudien anzulegen und um sie auf Kollagen-GAG Matrizes auszusähen.

Um ein Lederhautäquivalent (dermal equivalent, DE) zu schaffen wurden in die Matrizes zunächst menschliche Fibroblasten eingebracht. Daraufhin wurden die Matrizes in DMEM sowie 10%igem FBS kultiviert. Nach 24h wurden dann ORSCs (Passage 1) in das DE eingebracht und kultiviert. Nach vier weiteren Tagen wurde die Oberfläche des Hautäquivalents (skin equivalent, SE) auf die Höhe der Luft-Flüssigkeitsgrenze gebracht. Eine Woche später wurden die Matrizes in transdermale Wunden von thymuslosen nu/nu Mäusen transplantiert. Das Gewebe aus den Wundarealen wurde schließlich histologisch und immunhistologisch untersucht.

Die isolierten ORSCs zeigten eine hohe Proliferationsfähigkeit. In der Immunfluoreszenz zeigte sich eine Positivität für die Antikörper auf CK 15, CK 19 und ß-Integrin in den primären ORSCs. Nach der zweiten Passage nahm die Expression von Integrin ß1 und CK 15 ab wohingegen die Expression von CK 19 weiterhin positiv blieb. Zwei Wochen nach der Transplantation konnte eine voll entwickelte, mehrschichtige und verhornende epidermale Zellschicht in den Matrizes nachgewiesen werden. Nach vier Wochen konnten die epidermalen Zellschichten

weiterhin sowohl mit Antikörpern gegen humanes Zytokeratin als auch HLA-A angefärbt werden.

Die Ergebnisse dieser Studie legen nahe, dass mit der beschriebenen Technik ORSCs isoliert und kultiviert werden können, die Stammzellen der Wulstregion enthalten. Das Hautäquivalent (SE), welches in vivo mit Fibroblasten und ORSCs restrukturiert wurde kann innerhalb von zwei Wochen histologische Strukturen ausbilden, die Ähnlichkeit mit menschlicher Dermis und Epidermis aufweisen.

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8. Thesis-related publications and abstracts

1. Shuhua Liu, Tomas Egana, Insa Wilcke, John Lohmeyer, Daniel Thome, Eniko Bodo, Zhongfa Lu, Stefan Kruger, Hans-Günther Machens. Restructuring skin equivalents with human hair follicle ORSCs and fibroblasts. 124th Congress of the German Society for Surgery / Surgical Forum (oral presentation; 4th May 2007).

2. Shuhua Liu, Tomas Egana, Insa Wilcke, John Lohmeyer, Daniel Thome, Eniko Bodo, Zhongfa Lu, Stefan Kruger, Hans-Günther Machens. Restructuring skin equivalents with human hair follicle ORSCs and fibroblasts. 1st Congress of the German Society for Stem Cell Research in Cologne (abstract; 4th November 2006).

3. Shuhua Liu, Tomas Egana, Insa Wilcke, John Lohmeyer, Daniel Thome, Eniko Bodo, Zhongfa Lu, Stefan Kruger, Hans-Günther Machens. Restructuring skin equivalents with human hair follicle ORSCs and fibroblasts. 10th annual meeting of the European Conference of Scientists and Plastic Surgeons in London (oral presentation; 22nd September 2006)

4. Shuhua Liu, Tomas Egana, Insa Wilcke, John Lohmeyer, Daniel Thome, Eniko Bodo, Zhongfa Lu, Stefan Kruger, Hans-Günther Machens. Restructuring skin equivalents with human hair follicle ORSCs and fibroblasts. Chirurgisches Forum 2007 für experimentelle und klinische Forschung, Band 36: in press (2007)

9 Curriculum Vitae

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