The Influence of Stromal Cells on the Pigmentation of Tissue-Engineered Dermo-Epidermal Skin Grafts

Thomas Biedermann, PhD,^{1,2,*} Sophie Böttcher-Haberzeth, MD,^{1–3,*} Agnieszka S. Klar, PhD,^{1,2} Daniel S. Widmer, PhD,⁴ Luca Pontiggia, PhD,^{1,2} Andreas D. Weber, MD,^{2,3} Daniel M. Weber, MD,^{2,3} Clemens Schiestl, MD,^{2,3} Martin Meuli, MD,^{2,3} and Ernst Reichmann, PhD^{1,2}

It has been shown *in vitro* that melanocyte proliferation and function in palmoplantar skin is regulated by mesenchymal factors derived from fibroblasts. In this study, we investigated *in vivo* the influence of mesenchymal–epithelial interactions in human tissue-engineered skin substitutes reconstructed from palmar- and nonpalmoplantar-derived fibroblasts. Tissue-engineered dermo-epidermal analogs based on collagen type I hydrogels were populated with either human palmar or nonpalmoplantar fibroblasts and seeded with human nonpalmoplantar-derived melanocytes and keratinocytes. These skin substitutes were transplanted onto full-thickness skin wounds of immunoincompetent rats. Four weeks after transplantation the development of skin color was measured and grafts were excised and analyzed with regard to epidermal characteristics, in particular melanocyte number and function. Skin substitutes containing palmar-derived fibroblasts in comparison to nonpalmoplantar-derived fibroblasts showed (a) a significantly lighter pigmentation; (b) a reduced amount of epidermal melanin granules; and (c) a distinct melanosome expression. However, the number of melanocytes in the basal layer remained similar in both transplantation groups. These findings demonstrate that human palmar fibroblasts regulate the function of melanocytes in human pigmented dermo-epidermal skin substitutes after transplantation, whereas the number of melanocytes remains constant. This underscores the influence of site-specific stromal cells and their importance when constructing skin substitutes for clinical application.

Introduction

THE HUMAN PALMOPLANTAR epidermis exhibits distinct differences compared with interfollicular epidermis, most importantly with regard to epidermal marker expression and melanocyte function.¹ In this respect, Cytokeratin 9 (CK9) is a marker expressed exclusively by palmoplantar suprabasal keratinocytes.² It has been shown that its expression pattern is regulated by fibroblasts of the palms and soles through an epithelial–mesenchymal interaction.^{3,4} This influence could be demonstrated when pure nonpalmoplantar epidermal sheets were grafted to wounds of the human sole: keratinocytes adapted phenotypical characteristics of palmoplantar skin and expressed the specific marker Cytokeratin 9.⁵

This site-specific epithelial–mesenchymal interaction in palmoplantar skin also has an influence on the melanocyte proliferation and differentiation. Yamaguchi *et al.*⁶ showed *in vitro* that palmoplantar fibroblasts suppressed prolifera-

tion and pigmentation of melanocytes through Dickkopf 1 (DKK1), an inhibitor of the Wnt signaling pathway in melanocytes, obviously leading to the lighter skin color of the palms and soles.^{7–9} This influence was seen when nonpalmoplantar split-thickness skin grafts containing nonpalmoplantar fibroblasts were transplanted to the sole. The graft remained darker than the surrounding skin. In contrast, nonpalmoplantar epidermal sheets containing melanocytes and no fibroblasts, showed a clear hypopigmentation when transplanted to palmoplantar wounds in human patients.¹⁰

Interestingly, opinions differ regarding melanocyte density in palmoplantar human skin. According to Yamaguchi *et al.*, melanocyte density in palmoplantar skin is significantly lower than in interfollicular skin, thereby impacting skin color. In contrast, according to Hasegawa *et al.*, melanocytes exist in a similar density, but with a reduced melanogenic activity in palmoplantar skin. This leads to a decreased number of mature melanized melanosomes in melanocytes accounting for the lighter skin color in palmoplantar skin.

¹Tissue Biology Research Unit, University Children's Hospital Zurich, Zurich, Switzerland.

²Children's Research Center, University Children's Hospital Zurich, Zurich, Switzerland.

³Department of Surgery, University Children's Hospital Zurich, Zurich, Switzerland.

⁴Department of Dermatology, University Hospital Zurich, Zurich, Switzerland.

*These two authors contributed equally to this article.

Although many components regarding palmoplantar epithelial–mesenchymal interactions have been studied *in vitro* or observed clinically,^{3–8} little is known about the direct and objective *in vivo* results showing a difference between palmoplantar and nonpalmoplantar fibroblast with respect to their influence on skin pigmentation. Since several years, we are investigating diverse characteristics and properties of dermo-epidermal skin substitutes (DESS) for future clinical application to full-thickness skin defects.^{11–15} The importance of site-specific cellular epidermal and dermal components has been shown in the context of reproducing the original donor skin color in our pigmented skin substitutes.¹⁶ Moreover, the applied preclinical model enables us to analyze the influence of different fibroblast populations in an *in vivo* setting.

In this study, we investigated the influence of two different human dermal fibroblast populations on pigmentation in human tissue-engineered DESS. We used human palmar and nonpalmoplantar fibroblasts in combination with different ratios of nonpalmoplantar melanocytes and keratinocytes in DESS for an *in vivo* assay on immunoincompetent rats. Graft color, pigmentation, and histological characteristics, in particular melanocyte number and function, were analyzed 4 weeks after transplantation.

Materials and Methods

Human skin samples

The study was performed according to the Declaration of Helsinki Principles and after permission by the ethics commission of the Canton Zurich. For the use of foreskins or skin of accessory digits removed in cases of polydactyly parents and/or patients gave informed consent. The age of the children ranged from 1 to 16 years. The foreskins were used for the isolation of keratinocytes, melanocytes, and dermal fibroblasts, and the digits for the isolation of dermal fibroblasts from palmar sites. Foreskins and digits were obtained from different patients. Tissue samples for histological examinations were prepared for paraffin sections or embedded in OCT compound (Sakura Finetek, Stauf, Germany) and stored at -80°C .

Isolation and culturing of primary cells

The skin cells were isolated and cultured as described in Böttcher-Haberzeth *et al.*¹⁷ In brief, small skin pieces (about 3 mm^3) from three different dark foreskins (similar *L*-values) and from three different digits were digested overnight in 12 U/mL dispase (BD Biosciences, Allschwil, Switzerland) and Hank's balanced salt solution containing 5 mg/mL gentamicin (all from Invitrogen, Basel, Switzerland) at 4°C . The epidermis and dermis were subsequently separated by forceps. The epidermis was digested in 1% trypsin and 5 mM EDTA (all from Invitrogen) for 10 min at 37°C . Keratinocytes were harvested and cultured in serum-free keratinocyte medium containing 25 mg/mL bovine pituitary extract, 0.2 ng/mL epidermal growth factor, and 5 mg/mL gentamicin (all from Invitrogen). Melanocytes were harvested and cultured in melanocyte growth medium, containing 0.4% bovine pituitary extract, 1 ng/mL basic fibroblast growth factor, 5 mg/mL insulin, 0.5 mg/mL hydrocortisone, 10 ng/mL phorbol myristate acetate (all from PromoCell, Heidelberg, Germany),

and 5 mg/mL gentamicin (Invitrogen). The dermal tissue was further digested in 2 mg/mL collagenase blend F (Sigma, Buchs, Switzerland) for 4 h at 37°C . Dermal fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 4 mM L-alanyl-L-glutamine, 1 mM sodium pyruvate, and 5 mg/mL gentamicin (all from Invitrogen).

Preparation of DESS

The human DESS were prepared as described by Braziulis *et al.*¹³ Briefly, 3 mL of bovine collagen type I (5 mg/mL, containing Neutral Red pH indicator; Symatase Biomaterials, Chaponost, France) was mixed with 1×10^5 human fibroblasts (from palmar skins or from foreskins, passage 1–2) and chilled neutralization buffer containing 0.5 M NaOH (Sigma), and casted into six-well cell culture inserts ($3.0\text{ }\mu\text{m}$ pore size) (BD Biosciences). Dermal equivalents were grown in DMEM containing 10% FCS, 4 mM L-alanyl-L-glutamine, 1 mM sodium pyruvate, and 5 mg/mL gentamicin for 6 days. On each dermal equivalent 5×10^5 keratinocytes (passage 1–2) and melanocytes (passage 1–2) isolated and cultured from foreskins were seeded in a ratio of melanocytes:keratinocytes 1:5 or 1:10. Skin analogs were prepared with palmar ($n=9$, with 1:5 melanocytes:keratinocytes), palmar ($n=9$, with 1:10 melanocytes:keratinocytes), and nonpalmar ($n=9$, with 1:5 melanocytes:keratinocytes) fibroblast (from the nine skin analogs with different seeding ratios three analogs were prepared from each of the three different donors). DESS were cultured for 1 week in a 5:1 mix of keratinocyte medium and melanocyte growth medium (PromoCell), and subsequently transplanted.

Transplantation of DESS

The surgical protocol was approved by the local Committee for Experimental Animal Research (permission number 115/2012). Immunoincompetent female nu/nu rats, 8–10 weeks old (Harlan Laboratories, Venray, Netherlands), were prepared and anesthetized as described by Schneider *et al.*¹⁸ Skin analogs [containing palmar ($n=18$) and nonpalmar ($n=9$) fibroblast] were transplanted onto full-thickness skin defects created surgically on the backs of animals. Wound closure from surrounding rat skin was prevented and the skin substitutes were protected by surgical steel rings (26 mm in diameter) sutured to the skin defects using non-absorbable polyester sutures (Ethibond®; Ethicon, Zug, Zurich). DESS were covered with a silicone foil (Silon-SES; BMS, Allentown, PA), a polyurethane sponge (Ligasano; Ligamed, Ötztal, Austria), and a tape as wound dressing. Dressing changes were done once per week. After 4 weeks photographic documentations and chromameter measurements were performed, the transplanted skin substitutes were excised *in toto* and processed for cryo- and paraffin sections and transmission electron microscopy (TEM).

Chromameter measurements

In 1976, the color vision is described as trichromatic with uniform three-dimensional color space ($L^*a^*b^*$) by the International Commission on Illumination (Commission Internationale de l'Eclairage, CIE). In the color space, the *L*-value correlates to perceived lightness. It ranges from

absolute black (0) to absolute white (+100). The visible spectrum of light reflected from foreskins and skin analogs was measured, and a color change was recorded by the *L*-value (mean $\pm$ SD) as it is the most sensitive of the trichromatic values of the reflectance spectroscopy. For the statistical significance test an unpaired two-sided Student's *t*-test was performed. The spectroscopy was conducted by employing a Chromameter CR-200 (Minolta, Osaka, Japan).

Histological analysis

Paraffin sections (5 μ m) were stained with Hematoxylin and Eosin (Sigma) to assess the histological morphology. The Fontana-Masson technique was performed to visualize melanin.

Immunohistochemical staining

Immunofluorescence staining was performed as described in Pontiggia *et al.*¹¹: The following antibodies were used for immunofluorescence: HMB45 (1:50), E-cadherin (clone NCH-38, 1:30), all from Dako (Baar, Switzerland); Laminin-5 (clone P3H9-2, 1:100), Tyr (M-19, polyclonal, 1:50), Tyrp-1 (H-90, polyclonal, 1:50) all from Santa Cruz (Heidelberg, Germany); CK1 (clone LHK1, 1:200; Chemicon, Zug, Switzerland); CD90/Thy-1 (clone AS02, 1:100; Calbiochem, Dietikon, Switzerland). Photos of immunofluorescence stainings were taken with a DXM1200F digital camera connected to a Nikon Eclipse TE2000-U inverted microscope. The device was equipped with the Hoechst 33342-, FITC-, and TRITC-filter sets (Nikon AG, Switzerland; Software: Nikon ACT-1 version 2.70). Images were processed with the Photoshop 10.0 (Adobe Systems, Inc., Basel, Switzerland).

Immunohistochemical analysis was performed as described above.¹⁹ Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were deparaffinized in xylene and rehydrated. For antigen retrieval, slides were heated with the cell conditioner 1 (Ventana Medical Systems, Tucson, AZ). Sections were incubated with microphthalmia-associated transcription factor (MITF) antibody (clone 5pD5, 1:50; Abcam, Cambridge, United Kingdom). The antibody binding was visualized using the detection kit by Ventana Medical Systems (iVIEW DAB detection kit; Ventana Medical Systems). Slides were counterstained with Hematoxylin.

Electron microscopy

Samples (~ 1 mm³) were prefixed for 2 h in 0.1 M cacodylate buffer (Merck, Darmstadt, Germany), pH 7.3 containing 2.5% glutaraldehyde and washed in cacodylate buffer for TEM analysis. Tissue blocs were postfixed for 1 h with an aqueous solution of 1% OsO₄ and 1.5% K₄Fe(CN)₆, dehydrated, and finally embedded in EPON 812 (Promega AG, Dübendorf, Switzerland). Ultrathin sections (50–70 nm) were collected on copper grids, contrasted with 4% uranyl acetate, and 3% lead citrate. Examination of sections was performed with a CM 100 transmission electron microscope (Philips, Zurich, Switzerland). All reagents were from Sigma unless mentioned otherwise.

Statistical analysis

Melanocytes in the basal layer of the skin analogs were evaluated by counting MITF-positive cells four times in

each of the five representative sections from the skin samples. The results were calculated to display the mean $\pm$ SD graphically (Microsoft Excel).

Results

Human palmar skin shows distinctive macroscopic and microscopic characteristics

Macroscopically, color discrepancy between palmar (as well as plantar) and interfollicular skin are known, although, it is more obvious in skin type V–VI (Fig. 1a) than in skin type I–II (Fig. 1b) according to the Fitzpatrick scale of skin type pigmentation.

Microscopically, palmar skin shows several unique characteristics: a very thick stratum corneum (Fig. 1c, white two-sided arrow), a distinctive stratum lucidum, no hair follicles, but abundant eccrine sweat glands, and pronounced rete ridges (Fig. 1c, black arrows). Highlighting melanocytes with an HMB45 antibody, sporadic melanocytes contacting the basement membrane (stained with an anti-Laminin 5 antibody) can be seen (Fig. 1d). Regarding the epidermal marker expression, Cytokeratin 9, expressed exclusively by palmoplantar suprabasal keratinocytes, is superimposed with CK1 (Fig. 1e). As in interfollicular skin, all epidermal cells express E-cadherin and dermal fibroblasts are positive for a CD90 staining (Fig. 1f).

Pigmentation of human DESS prepared with human dermal fibroblasts from different body sites

To easily survey and analyze the influence of fibroblasts on the pigmentary system, DESS were produced with keratinocytes and melanocytes from dark nonpalmoplantar skin in the epidermal compartment. The dermal compartment was produced either with human palmar fibroblasts or with human dermal fibroblasts from the same dark skin biopsy as the epidermal cells. Four weeks after transplantation onto immunoincompetent rats, there was a clear-cut color difference between the two groups of grafts (Fig. 2a, b). When fibroblasts from palmar skin were used, the skin substitutes showed a very light color (Fig. 2a). In contrast, the substitutes with the same epidermal cells, but produced with dermal fibroblasts from nonpalmoplantar skin showed a dark skin similar to the color of the donor biopsy.

These results could be confirmed by objective color measurements using a chromameter (Fig. 2c). Transplants with all three cell types (keratinocytes, melanocytes, fibroblasts) from the dark nonpalmar skins ($n=9$, $L=43.4$, SD: 0.98) showed the same measurements as the donor skin biopsies ($L=44.8$, SD: 0.82). However, measurements from the transplants constructed with palmar fibroblasts differed significantly ($p<0.0001$) from transplants with nonpalmar fibroblasts or from the original dark foreskins.

To additionally investigate a possible influence of various epidermal cell seeding ratios, the palmar fibroblast-derived DESS were transplanted with different initial melanocyte: keratinocyte seeding ratio of 1:5 ($n=9$) and 1:10 ($n=9$). The *L*-values obtained were similar, namely with a ratio of 1:5 the *L*-value was 61.5 (SD: 0.51), with a ratio of 1:10 the *L*-value was 58.0 (SD: 3.29). Hence, the different seeding number of melanocytes did not influence skin color.

Histologically, all transplants showed the development of a similar, stratified, and cornified epidermis 4 weeks after

FIG. 1. Palmar skin of children of two different skin types and histological appearance of palmar skin. **(a)** Palmar and dorsal skin of Fitzpatrick skin type V displaying the hypopigmented palmar skin compared with the dark pigmented dorsal skin. **(b)** Palmar and dorsal skin of Fitzpatrick skin type I displaying no subjective difference in pigmentation between the palmar and dorsal side. **(c)** Hematoxylin and eosin (H/E) staining of Fitzpatrick skin type I palmar skin. Note the thick stratum corneum (white two-sided arrow), pronounced rete ridges (black arrows) and the absence of hair follicles. **(d)** HMB45-positive melanocytes (green, white arrows) in the basal layer of the palmar skin. Laminin 5 (Lam5, red) represents a marker for the basement membrane. **(e)** Cytokeratin 1 (green) and cytokeratin 9 (red) are expressed in the suprabasal keratinocytes in the palmar skin in an overlapping manner. **(f)** E-cadherin (green) expression of keratinocytes and CD90 (red) expression in dermal cells of palmar skin. Counterstaining (**d–f**) for cell nuclei (blue) with Hoechst 33342. Scale bars (**c–f**) = 50 μ m. Color images available online at www.liebertpub.com/tea

transplantation (Fig. 3a, b, higher magnifications thereof in Fig. 3c, d). No stratum lucidum or thick stratum corneum could be detected in the DESS produced with palmar fibroblasts.

Melanin distribution in the transplanted DESS was revealed by a Fontana-Masson staining. All transplants showed melanin throughout their entire epidermis (Fig. 3e–h), located mainly as supranuclear caps in the keratinocytes (arrows, Fig. 3g, h). However, a clear difference regarding melanin quantity between transplants with palmar (Fig. 3e, g) and nonpalmar fibroblasts (Fig. 3f, h) was evident. Substitutes produced with nonpalmar fibroblasts showed a higher concentration of melanin in the basal layer (keratinocytes and melanocytes) as well as in the suprabasal keratinocytes.

To demonstrate melanin uptake by keratinocytes and physiological melanocyte position, TEM was performed on DESS produced with palmar fibroblasts. Melanosome-containing melanocytes were detected in the basal layer of the epidermis (M in Fig. 4a) with dendrites reaching into suprabasal layers (Fig. 4a, black arrowhead). Adjacent keratinocytes (K in Fig. 4a), affirmed by their possession of

desmosomes (Fig. 4a, white arrows), showed melanin inclusions in a physiological supranuclear position (Fig. 4a, black arrows).

TEM photographs were used to determine the number of melanocytes in the basal layer of all DESS (Fig. 4b). Thereby, a similar percentage of melanocytes in the basal layer in the different groups was revealed: 20.2% (± 3.89) in DESS with palmar fibroblasts and a seeded melanocyte:keratinocyte ratio of 1:5; 19.7% (± 4.85) in DESS with palmar fibroblasts and a seeded melanocyte:keratinocyte ratio of 1:10. The different initial seeding ratio of melanocytes:keratinocytes (1:5 or 1:10) *in vitro* did not have an influence on the final melanocyte:keratinocytes ratio of about 1:5 observed after 4 weeks *in vivo*.

DESS with nonpalmar fibroblasts and a seeded melanocyte:keratinocyte ratio of 1:5 displayed a percentage of 23.6% (± 1.91) melanocytes in the basal layer of the epidermis.

In addition, a quantification of MITF-positive melanocytes using immunohistochemistry stainings resulted in the same percentages of basal melanocytes in all transplants

FIG. 2. Macroscopic appearance of transplanted skin analogs and chromameter measurements. **(a, b)** Dermo-epidermal skin substitutes (DESS) containing dark foreskin-derived keratinocytes, melanocytes, and palmar-derived fibroblasts **(a)**, or nonpalmar-derived fibroblasts **(b)**, 4 weeks after transplantation into created full-thickness skin wounds on immunoincompetent rats (melanocyte to keratinocyte ratio in DESS 1:5). **(c)** Chromameter measurements of dark foreskins (as control), DESS containing nonpalmar-derived fibroblasts and DESS (melanocyte to keratinocyte ratio 1:5 or 1:10) containing palmar-derived fibroblasts 4 weeks after transplantation. The *L*-value represents a range between 0 (*absolute black*) and 100 (*absolute white*). Five measurements for nine different nonpalmar and palmar containing fibroblast DESS were performed. Error bars represent mean $\pm$ SD. The rings in **(a, b)** are 2.6 cm in diameter. Color images available online at www.liebertpub.com/tea

(Supplementary Fig. S1a, b, higher magnifications in Supplementary Fig. S1c, d; Supplementary Data are available online at www.liebertpub.com/tea).

Skin substitutes produced with either palmar or nonpalmar fibroblasts differ in melanosome maturation

Melanosome maturation in human skin differs depending on body site and skin pigmentation type. Melanocytes of palmar skin produce early melanosome stages I–II (shown in Fig. 5 by an HMB45 staining), whereas stages III–IV (shown by a Tyrp-1 staining) are not produced (Fig. 5a–c). Nonpalmo-plantar dark pigmented skin express more mature melanosome stages III–IV (Fig. 5d–f). However, nonpalmo-plantar light pigmented skin shows predominantly early stages I–II (Fig. 5g–i). Our DESS constructed with

palmar fibroblasts were only positive for HMB45 (stages I–II) (Fig. 5j–l), in accordance to the expression pattern of palmar skin (Fig. 5a–c). In contrast, DESS constructed with nonpalmar fibroblasts showed expression of both, HMB45 and Tyrp-1 (Fig. 5m–o). (Higher magnifications for HMB45 and Tyrp-1 expression pattern in palmar and nonpalmar DESS are shown in Supplementary Fig. S1e–h.)

In addition, we investigated the expression of tyrosinase (Tyr) regarding melanosome maturation (Supplementary Fig. S2). In normal human skin, palmar melanocytes produce no tyrosinase, whereas nonpalmo-plantar melanocytes express tyrosinase (data not shown). Our DESS, constructed with palmar fibroblasts, were only positive for HMB45 (Supplementary Fig. S2a–c, higher magnifications in Supplementary Fig. S2d, e), but not positive for tyrosinase expression, again in accordance to the expression pattern of palmar skin. Moreover, DESS constructed with nonpalmar fibroblasts

FIG. 3. Histological appearance of transplanted skin substitutes 4 weeks after transplantation. (a, b) H/E staining of DESS containing palmar-derived fibroblasts (a) and nonpalmar-derived fibroblasts (b) reveals a stratified, cornified, multilayered epidermis in both types of substitutes. Of note, no thickened stratum corneum could be seen in skin analogs containing palmar fibroblasts. (c, d) Higher magnifications of the H/E staining (a, b). (e, f) Fontana-Masson staining of skin analogs containing palmar-derived fibroblasts (e) and nonpalmar-derived fibroblasts (f) displays cytoplasmic melanin in cells of the epidermis in both types of skin substitutes. (g, h) Higher magnification of the Fontana-Masson staining. Less melanin is visible in skin analogs with palmar fibroblasts (g) compared with nonpalmar fibroblast analogs (h). Melanin stored in supranuclear caps (arrows). Scale bars (a, b, e, f) = 50 μ m, scale bars (c, d, g, h) = 25 μ m. Color images available online at www.liebertpub.com/tea

showed expression of tyrosinase (Supplementary Fig. 2f–h, higher magnifications in Supplementary Fig. 2i, j).

Skin substitutes produced with either palmar or nonpalmar fibroblasts differ in Cytokeratin 9 expression

To analyze the palmoplantar specific marker CK9 in nonpalmoplantar keratinocytes of our DESS, immunofluorescence staining using an anti-CK9 antibody was performed. Skin substitutes produced with palmar fibroblasts revealed a scattered CK9 expression pattern in suprabasal keratinocytes, whereas CK1 was evenly expressed in all suprabasal cells (Supplementary Fig. S3a). In contrast, the same nonpalmoplantar keratinocytes in DESS produced

with nonpalmoplantar fibroblasts were negative for CK9 in keratinocytes, whereas CK1 was expressed by all suprabasal keratinocytes (Supplementary Fig. S3b).

To prove the persistence of human fibroblasts 4 weeks after transplantation, skin substitutes were stained with an anti-CD90 staining, detecting specifically human fibroblasts. In both groups, abundant human fibroblasts were present underneath the human E-cadherin-positive epidermis (Supplementary Fig. S3c, d).

Discussion

Structural differences between human palmoplantar and interfollicular skin, in particular the influence of the mesenchyme on epidermal phenotype development and

FIG. 4. Transmission electron microscopy (TEM) analysis of transplanted skin substitutes 4 weeks after transplantation. **(a)** TEM of DESS containing palmar-derived fibroblasts highlights the presence of melanin granules (black arrows) in melanocytes (M) in the basal layer and in keratinocytes (K) in suprabasal layers. The black arrowhead marks a melanocyte dendrite projecting to suprabasal layers. White arrows denote desmosomes of keratinocytes. Black dashed line demarcates the dermal-epidermal junction. **(b)** The bars in the diagram represent the percentage of positive melanocytes in the basal layer for skin substitutes containing palmar-derived fibroblasts and nonpalmar-derived fibroblasts. Melanocytes are present in the physiological number of about 20% of all basal cells in all types of DESS (Mel-Ker-Ratio = seeded melanocyte to keratinocyte ratio). The bars display the mean $\pm$ SD. Scale bar **(a)** = 5 μ m.

expression pattern of different cytokeratins are known and have been studied previously.^{5,10} It has also been demonstrated that by transplanting interfollicular skin onto the palms or soles, the original phenotype of the epidermis remains as long as fibroblasts from the initial site are included.^{3,4} Yet, little is known about the specific influence of palmoplantar or interfollicular fibroblasts on the melanocyte number and function in an *in vivo* model. To further investigate the hypothesis that palmoplantar fibroblasts act on keratinocytes and melanocytes, that is, specifically on melanogenesis, we compared pigmented DESS constructed with human palmar, respectively, nonpalmoplantar fibroblasts in an *in vivo* assay. Generally, we could show now in an *in vivo* assay that human palmar fibroblasts regulate the melanogenesis of melanocytes in human pigmented DESS after transplantation, whereas the number of melanocytes was identical in all transplants.

Macroscopically, we could detect a striking and significant difference in skin color when palmar and nonpalmar fibroblasts were used in the otherwise same skin substitute. This difference was examined in more detail. In accordance with the previously described findings about the physiological distribution of melanocytes in the basal layer of the human epidermis, we chose an initial seeding ratio of me-

lanocytes to keratinocytes in our DESS of 1:5.^{1,20,21} In addition, we also prepared DESS *in vitro* with a melanocyte to keratinocyte ratio of 1:10 to investigate the influence of palmar fibroblasts on the proliferation of melanocytes. Regarding the number of melanocytes *in vivo*, we found a consistent ratio of about 1:5 of melanocytes to keratinocytes in the basal layer of the epidermis in all DESS 4 weeks after transplantation despite the fact that we used both a 1:5 and a 1:10 ratio of melanocytes to keratinocytes.

This is an important finding, as opinions regarding the influence of palmoplantar fibroblasts on melanocyte number are controversial. Yamaguchi *et al.*⁴ claim that there are less melanocytes in the palmoplantar skin than in the interfollicular skin and that palmar fibroblasts have a suppressive effect on melanocyte proliferation *in vitro*.⁶ However, Hasegawa *et al.*²³ describe that there are equal numbers of melanocytes in the palmoplantar and interfollicular skin. In this study, in contrast to Yamaguchi *et al.*,⁶ we did not observe an inhibitory influence of palmar fibroblasts on melanocyte proliferation in our DESS, as the equal physiological melanocyte to keratinocytes ratio was established. Obviously, in terms of melanocyte number regulation, keratinocytes display a greater influence than fibroblasts as also suggested by Scott and Haake.²² Moreover, we had already used melanocytes and keratinocytes

FIG. 5. Expression pattern of Tyrp-1 and HMB45 in melanocytes. **(a–c)** Example of Tyrp-1 (green) and HMB45 (red) expression in melanocytes of human palmar skin. No expression of Tyrp-1 is present. **(d–i)** Expression of Tyrp-1 (green) and HMB45 (red) in dark pigmented foreskin **(d–f)** and in light pigmented foreskin **(g–i)**. Note the different presence of Tyrp-1 in **(e, h)**. **(j–o)** Expression pattern of Tyrp-1 (green) and HMB45 (red) in DESS containing palmar-derived fibroblasts **(j–l)** and nonpalmar-derived fibroblasts **(m–o)**. No Tyrp-1 expression is detected in **(k)**, resembling the pattern in **(b)**. Counterstaining **(a–o)** for cell nuclei (blue) with Hoechst 33342. Scale bars **(a–o)** = 50 μ m. Color images available online at www.liebertpub.com/tea

in different ratios (1:1, 1:5, and 1:10) with nonpalmoplantar fibroblasts in DESS.¹⁶ There, we also consistently detected a ratio of 1:5 (melanocytes:keratinocytes) after transplantation, indicating the establishment of a physiologically 1:5 melanocyte:keratinocyte ratio under homeostatic conditions.

With regard to melanogenic activity, we could show that the epidermal melanin content in the basal and suprabasal keratinocyte layers was significantly lower in skin substitutes constructed with palmar fibroblasts, resulting in higher chromameter values. This could be confirmed by the presence of less melanin granules shown by Fontana-Masson staining, as well as by TEM analysis. More specifically, we detected a reduced formation of mature melanosomes revealed by an absent expression of Tyrp-1, a marker for mature melanosome stages III–IV. These results are in accordance with the previous studies showing a suppression of melanosome maturation *in vitro* as well as in normal palmoplantar skin.^{6,23}

These results indicate that we can reproduce mesenchymal–epithelial interactions with palmar and nonpalmar fibroblasts acting on the pigmentary system of a nonpalmoplantar epidermis in our transplantation model. We suggest that palmar

fibroblasts act only on melanosome maturation and on melanin transfer to keratinocytes. That is to say, melanosome maturation in melanocytes is suppressed and the transfer of melanin from melanocytes to keratinocytes is decreased. We speculate that this influence could be caused by DKK1, an inhibitor of the canonical Wnt signaling pathway, shown to be expressed in higher amounts by palmoplantar fibroblasts.⁶

Although the epidermis of skin substitutes produced with palmar fibroblasts did not show an extremely thick stratum corneum or a stratum lucidum, keratinocytes started to express CK9, an exclusive differentiation marker of palmoplantar skin. This is somewhat surprising, as keratinocytes used in skin substitutes were derived from nonpalmoplantar skin, and CK9 expression was previously considered to be regulated endogenously.⁵ On the other hand, several studies have demonstrated that mesenchymal factors can modulate the phenotype of epidermal keratinocytes.^{3,24–28}

In conclusion, this study demonstrates a specific influence of human palmoplantar fibroblasts on the pigmentation of nonpalmoplantar epidermis in DESS transplanted in an animal model. It emphasizes the importance of using site-specific stromal cells when constructing pigmented dermo-epidermal

skin grafts for clinical application, especially when aiming to produce skin grafts matching the color of the site to be transplanted.

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Disclosure Statement

The authors declare that they have no conflicts of interest.

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Address correspondence to:
Ernst Reichmann, PhD
Tissue Biology Research Unit
University Children's Hospital Zurich
August Forel Street 7
Zurich 8008
Switzerland

E-mail: ernst.reichmann@kispi.uzh.ch

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