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Melanocytes from the Outer Root Sheath of Hair Follicles Cultivated on Collagen Membrane Improve their Melanotic Properties

Vuk Savkovic[1]*, Katharina Sülflow[1], Katrin Rabe[1], Marie Schneider[1] and Jan-Christoph Simon[2]

[1]*Translational Centre for Regenerative Medicine, Leipzig University, Germany*
[2]*Department of Dermatology, Venereology and Allergology, Leipzig University Medical Center, Leipzig, Germany*

Abstract

Local depigmentation disorders such as Vitiligo are detrimental for both physiological and psychological reasons and demand a causal, non-invasive treatment. The only available treatment of a kind at present is melanocyte transplantation, disadvantageously dependent on an invasive skin biopsy as melanocyte source. We have previously reported our work on an autologous, non-invasive method of differentiating melanocytes from the outer root sheath of human hair follicles for use in transplantation treatment. Choice of a suitable biocompatible, biodegradable carrier as a temporary niche for transplanted melanocytes can be crucial in such applications. Therefore, we tested decellularized bovine collagen type I scaffold membranes as a candidate for a favourable carrier of the hair follicle melanocytes.

This study provides an additional insight to the reported upgraded explant method of cultivating melanocytes from human hair follicle and the advantages of collagen I membrane as a biocompatible carrier for them. We have displayed detailed analysis and detailed interpretation of the methodological improvements, showing that the upgraded explant procedure favoured the release, migration and proliferation of cell content with more cultivating potential and manifold higher melanocyte yield. The three-dimensional culture of melanocytes, seeded on collagen I membranes, displayed regular melanocyte markers, proliferated more rapidly and produced more melanin than the two-dimensional adherent culture. We concluded that the collagen I decellularized scaffolds provided a benevolent niche to hair follicle melanocytes and even upgraded their melanotic properties, which makes collagen I membranes an excellent candidate for a biocompatible carrier in future clinical applications.

Keywords: Outer root sheath; Hair follicle; Melanocyte; Vitiligo; Stem cells; Transplantation; Collagen I

Introduction

Vitiligo

Vitiligo is a skin disorder manifested as local depigmentation. Depigmented spots (so called white patches) severely lack functional melanocytes in the epidermis and therefore remain deprived of melanin, the pigment necessary for protection against solar radiation. The skin of the white spots becomes extremely sensitive to the sun and burns strikingly easily, having no innate protection. Perhaps even more sinister, white patches are often regarded as disfigurement, more than ever in the times of high cosmetic standards, especially if the patches are situated on exposed parts of the body such as face, arms and hands (38% of depigmented surfaces in Vitiligo patients) [1]. All of the mentioned has a tremendous impact on life quality and social life of the affected (52% single patients, [1-7]) and can lead to psychiatric morbidity in 34% of Vitiligo patients [1,2,8], and devastating chronic depression (24%) [1]. The urge to treat Vitiligo is strong in times of intensifying cosmetic awareness.

Therapeutic principles

The existing conservative therapies such as phototherapy, topical immune suppressors or chemical depigmentation are alleviating, short-term and non-causal, which imposes an unmet need of a causal Vitiligo treatment with a long-lasting effect [9-11].

Transplantation therapy

The closest approach to the causal, in terms of addressing the problem of melanocyte loss/absence, is delivering functional melanocytes to the points of demand (i.e., melanocyte transplantation). Melanocyte transplantation has been used as a standard procedure for quite long as reported by several groups [12-18]. For all of the transplantation procedures, melanocytes are harvested from the epidermis upon excision of a piece of skin, differing in the ways of handling the cells after the biopsy. The simplest procedure involved resuspension of the cells from digested epidermis and subsequent application of the entire cell suspension onto previously dermabraded patches [18]. The heterogeneous content of such suspensions has an advantageous ratio of epidermal cells, especially that of keratinocytes vs. melanocytes, but also carries an innate peril of non-sterile or in best case semi-sterile isolation procedure, resulting in roughly one third of graft rejections due to mycosis [19]. Further on, the melanocytes can be isolated and grafted or further cultivated [19], proliferated and grafted. Some of the performed melanocyte transplantations have brought about pigmentation improvement on the depigmented skin patches [12-19]. Nevertheless, all of the procedures involved a surgical removal of up to 6 cm^2 of skin.

***Corresponding author:** Vuk Savkovic, Translational Centre for Regenerative Medicine, Leipzig University, Phillip-Rosenthal-Str. 55, 04103 Leipzig, Germany, Tel: +49 341 9739647; Fax: +49 341 9739609; E-mail: vsavkovic@trm.uni-leipzig.de

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Non-invasive procedures

In times of a strong and persistent trend towards non-invasive or minimally invasive procedures, it is very important that material sampling remains non-invasive. This aspect involves even more sensitivity when it comes to treatment of skin disorders due to a visible cosmetic result of the application. Therefore, difference between sampling and biopsy carries much significance when the starting material for the cell therapy is being harvested. In that sense, sampling hairs with follicles by plucking (completely painless and harmless procedure finalised in matter of minutes) presents us with a non-invasive replacement for an invasive excision biopsy of skin (procedure which involves pain, bleeding, requires post-operative care and leaves a scar).

The melanocytes cultivated from the Outer Root Sheath (ORS) of hair follicles provide a possibility of transplantation onto the prepared depigmented patches of skin. Several application routes are possible in terms of transplantation. The simplest procedure is by all means an application of cell suspension, liquid or in the form of aerosol, stabilised by fibrin and/or thrombin, which works well on even surfaces. Pioneer study of Vanscheidt and Hunziker [20] involved successful transplantation of a whole ORS suspension onto a Vitiligo patch, without prior isolation of melanocytes. Further application variants involve biocompatible carriers that mechanically and biologically stabilize the HM before, in the course of transplantation and post-transplantationally. Candidates for such carriers are of cellular nature (for instance, keratinocyte-based epidermal equivalents), scaffolds based on cellular derivates (such as collagen-based decellularized scaffolds) or scaffolds synthesized from synthetic biocompatible materials. Modifications of the carriers involve a remarkably high number of variants.

Goals

We have initially used the experimental platform of Dieckman et al. [21] to establish a medium-air-interface culture of hair follicles and subsequent adherent culture of ORS melanocytes. Despite the reported high purity, the cultivation timeline of two months left room for more efficiency. Along the way, we introduced several improvements to the initial Dieckmann experimental procedure [22].

In this article, we have provided further analysis of the upgraded method and of the cells obtained. We have additionally compared the two methods, Dieckmann explant method [21] and the upgraded method [22] at each phase of the culture, gaining further data that not only explained the dynamics and differences in the early stages, but also revealed significant accumulative differences in cell numbers at the advanced stages of the culture.

Using the human HM cells obtained from the upgraded method, we have tested decellularized bovine collagen type I-based membranes (Collagen Cell Carrier, CCC, [23]) as biocompatible carriers for HM that were cultivated in improved conditions described in [22] and reflected in this study.

Material and Methods

Cultivation, expansion, differentiation and selection of pure melanocyte culture

Human outer root sheath melanocytes (HM) were cultivated from the ORS of human follicles as described [22]. Briefly, the follicles were shortened at the proximal end, partially digested with collagenase V and cultivated in medium-air-interface conditions until the stem cells and progenitor cells migrated out of the follicle and the progenitors proliferated on a nylon mesh. After harvesting the cells by trypsinization, adherent culture was performed until the cell showed correct melanocyte morphology, expressed Tyrosinase, glycoprotein 100 (gp100) variants and produced melanin using a set of enzymes (Tyrosinase, Tyrosinase-Related Protein 1 and 2). The ORS melanocytes were selected during the adherent culture by the means of differential trypsinization and Geneticine selection, which helped withdraw keratinocytes and fibroblasts as usual contaminants of the ORS melanocyte culture. Basic steps of the method are shown in figure 1. Since the combined improvements of the upgraded method yielded one million pure functional melanocytes in four weeks instead of the initial eight weeks [22], we analyzed the features of all cultivation steps of the explant culture. We performed comparison experiments of the initial (Dieckmann) versus the upgraded explant method, monitoring follicle activity, follicle surface, cell numbers, outcome of Geneticine selection, marker expression and total cell yield.

For further comparison purposes, we performed a parallel cultivation procedure of intact hair follicles, cultivated in medium-air-interface with supplemented M2 Promocell medium [24], as described in Dieckmann et al. [21], with and without Geneticine selection step during adherent culture described in our upgraded procedure [22]. The initial method of selective elimination by Geneticine can be found in [25].

Follicle activity

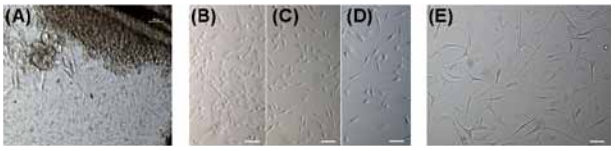
The follicles cultivated in medium-air-interface, according to both the method of Dieckmann and the current method, were monitored for growth activity and regularly documented (Figure 2).

Measurement of the outer root sheath surface changes

Surface of the follicle ORS was measured by the means of Nikon NIS Elements BR (Version: 3.00) software. The ORS surface, which is morphologically clearly distinguishable, was selected from surface micrograph of hair follicle by the means of an adjustable polygonal frame and the surface values were read out by software. The average values of ORS surface of four independently set up cultivation experiments with five technical replicates were compared at day 0, day 7 and day 14 (Figures 3 and 4).

Cell numbers

After harvesting the cells released from the ORS in the course of medium-air-interface cultivation, the cells were further cultivated



(A) Shortened, active follicle pre-treated with collagenase V and cultivated in medium-air-interface conditions with migrating cells on the surrounding mesh forming own colonies. (B) Non-selected cells in the adherent culture (C) Geneticine challenged cells in the adherent culture (D) differentiated ORS melanocytes (HM) (E) Control: Normal Human Epidermal Melanocytes (NHEM).

Figure 1: Current method for cultivating melanocytes from the outer root sheath cell pool of the hair follicle according to Savkovic et al. [22].

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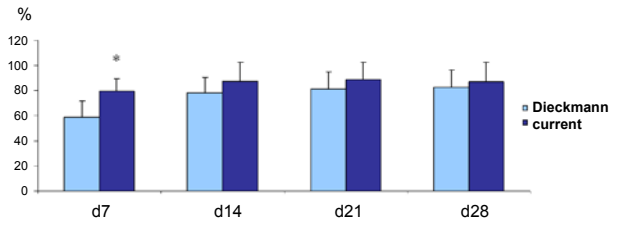
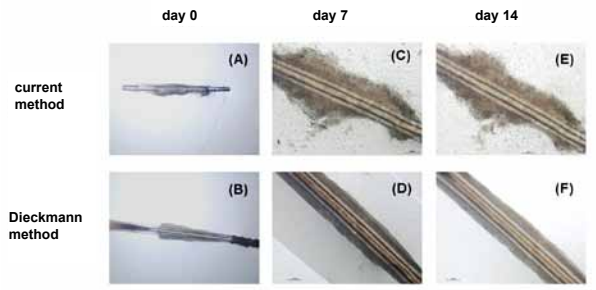


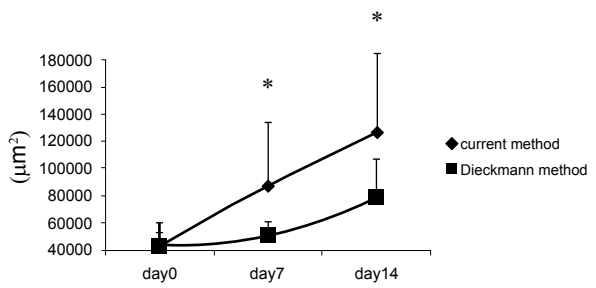
Both the current method and the method of Dieckmann et al. [21] comprised an active outgrowth of hair follicle outer root sheath. The percentage of active follicles cultivated according to the current method was higher than that of follicles cultivated according to the Dieckmann method, as measured at day 7, 14, 21 and 28. At day 7, the difference in percentage of active follicles between the two methods was statistically significant (p=0.038*), whereas on day 14, 21 and 28, the trend persisted on a non-significant level (p>0.05).

Figure 2: Comparative changes in follicle activity.



Example of hair follicles cultivated in the medium-air-interface upon the current method (A, C, E) displaying higher increase in ORS surface than those cultivated according to the Dieckmann method (B, D, F). On day 0, all of the follicles were compact (A, B). On day 7, outgrowth and migration of the cells was evident (C, D), as well as on day 14 (E, F). Stronger outgrowth of the follicles cultivated according to the current method was visible (C, E). (A) Current method, day 0; (B) Dieckmann method, day 0; (C) Current method, day 7; (D) Dieckmann method, day 7; (E) Current method, day 14; (F) Dieckmann method, day 14.

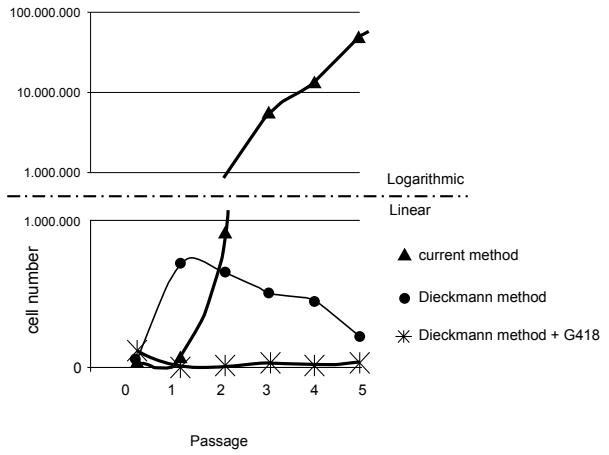
Figure 3: Representative photographs of comparative ORS surface changes.



Both the current method (♦) and the method of Dieckmann et al. (■) comprised an increase of the ORS surface. The surface increase was significantly higher in ORS of the follicles cultivated according to the current protocol in comparison with that of the Dieckmann method, as measured at day 7 (ORS surface ratio 0.69, p=0.01**) and day 14 on day 14 (ORS surface ratio 0.72, p=0.018*).

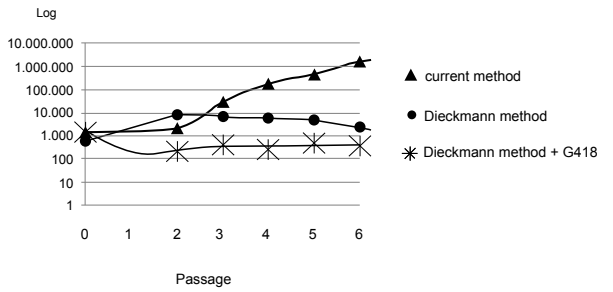
Figure 4: Comparative changes of the Outer Root Sheath surface.

according to the specifications of both methods. The cells were stained with Trypan Blue and counted in Neubauer chamber at each passage in order to determine the total cell number in the culture. The passage at the day of harvesting the cells from the medium-air-interface and their set-up as adherent culture was nominated passage 0 (Figures 5 and 6).



Example of growth curves of ORS cells cultivated according to the current method (▲), Dieckmann explant method (●), Dieckmann explant method with 50 µg/ml Geneticine supplement over 48 h at early passages of adherent culture (*). The lower part of the graph is displayed linearly and the upper part logarithmically. The cells cultivated upon the method described herein reached the number of 1,000,000 cells by the second passage and as much as 80,000,000 cells by the passage 5 of adherent culture (displayed results of single donor using comparative methods).

Figure 5: Comparative cell growth in the adherent culture.



Growth curves of ORS cells per sampled hair follicle cultivated according to the current method (▲), Dieckmann explant method (●), Dieckmann explant method with 50 µg/ml Geneticine supplement over 48 h at early passages of adherent culture (*). The graph is displayed logarithmically. The theoretical average cumulative cultivation potential per single follicle reached as much as 2,700,000 melanocytes.

Figure 6: Comparative growth in the adherent culture per hair follicle.

Comparison of the Dieckmann method and herein provided method with geneticine addition

Additionally, in order to withdraw the contaminant fibroblasts and keratinocytes as competitors against differentiating melanocytes, we have treated the 'Dieckmann' adherent culture with 50 µg/ml geneticine after the p0 as a corresponding selection measure used in the current method. Geneticine affects translation and hereby the quickly-dividing cells with high demand for protein synthesis, therefore the melanocytes survive Geneticine treatment, whereas the fibroblasts and keratinocytes do not [22,25,26].

This part of the treatment was also intended to clarify the effect of Geneticine on the amplified heterogeneous cell pool harvested according to the Dieckmann et al. [21], e.g. to estimate the cellular composition of such cell pool. Working hypothesis predicted a small

number of survivor cells in case of the high number of contaminant fibroblasts and keratinocytes and vice versa–more survivors of the Geneticine treatment in case of the low contaminant cell content (Figures 5 and 6).

Cultivation of HM cells on collagen membranes

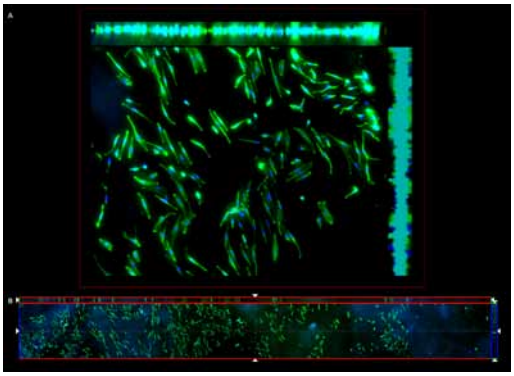
Bovine decellularized collagen I membrane scaffolds, also called CCC (Collagen Cell Carrier, Viscofan Bioengineering, Weinheim, Germany) were tested as a temporary niche for ORS melanocytes for purposes of grafting.

The scaffolds were circle-shaped with a sterile hollow punch, with an 8 mm diameter so that they covered the 10.7 mm wide bottom of the 360 mm^2 wells (96-well-plate) in calibrated condition. The membranes were placed on the bottom of the 96-well and calibrated with sterile PBS and subsequently with DLM medium for 24 hours. The position of the membranes and presence of air bubbles were inspected and they were tightly positioned to the bottom of the wells using sterile forceps. The medium used for calibration was discarded. Resuspended cells were seeded over the membranes and left to adhere, 10,000 cells per scaffold, resuspended in 200 µl medium.

Over the next 5 days, the HM cells were populating the collagen membrane and proliferating (Figure 7).

Immunofluorescence

The cells on scaffolds were fixed with paraformaldehyde (PFA) and labelled with Tyrosinase and NKI-beteb mouse-generated antibodies, followed by the secondary labelling by an Alexa 647 anti-mouse antibody as described [22]. The cells on scaffolds were washed with PBS and fixed with ice-cold Para formaldehyde (PFA, 4%) for 8 minutes, then washed with PBS again. The cells were blocked with 0.5 ml of 2% bovine serum albumin 2% BSA-PBS and permeabilised with 0.1% Triton PBS solution for 1 h. Solutions of primary mouse-generated antibodies against NKI/beteb and Tyrosinase were set at 2% in PBS with 2% BSA/0.1% Triton and incubated with the cells on scaffolds overnight at 4°C. After washing with 2% BSA/0.1% Triton solution, biotinylated anti-mouse secondary antibody was added to the cells and incubated for 1 h, then washed. Solution of 2% BSA-PBS/0.1 % Triton/0.25% IGG was incubated with the cells for one hour, and then washed. Finally, Streptavidin-coupled Alexa594® (red) and Alexa 488® (green) fluorochrome 0.25% solution in 2% BSA/0.1%. Triton were incubated with the cells together with 1/1000 DAPI nuclear dye, for 1h, then washed two times with 2 % BSA/0.1% Triton solution. Scaffolds with the cells were left to dry out for 2 minutes, fixed onto glass with Fluoromount® sealing liquid, and covered with a glass slip.



(A) Maximum Intensity Projection (MPI) z-stacked take of HM on CCC membrane. Side projections display the depth of the scaffold reconstructed from 10 consecutive images taken with a 10 µm progression interval. The cells populated the 100 µm depth of the CCC scaffold. Over 97% of the cells expressed, the gp100 variant NKI-beteb, present in melanosomes of ORS melanocytes (HM). (B) 'cross-census' panoramic image of 7 images consecutively taken along the same axis across the full width of a mounted slide.

Figure 7: HM cells cultivated on bovine collagen membrane (CCC).

Microscopic analysis of the 3D HM cultures

The marker signals were analysed by the means of fluorescence microscopy (Nikon Eclipse Ti-S; Nikon Corporation, Deutschland) and ApoTome Laser-Scanning microscope (LSM 510 Meta, Carl Zeiss Jena GmbH, Germany).

Reconstruction of the three-dimensional structure of the membrane-adherent HM culture was analyzed by the means of 'z-stacking'. Images of the HM cells seeded onto the decellularized bovine collagen I membranes were taken using the ApoTome microscope system (Zeiss) navigated by AxioVision Software. Serial images were taken along the z-axis at different depths of the 100 µm membrane with a progressing interval of circa 10 µm (z-stack function). The resulting 10 images were integrated into a single Maximum Intensity Projection (MIP) image. The incorporated visual information of the MIP images enabled an estimate of the depth along the z-axis and modelling of the three-dimensional structure (Figure 7).

For the purposes of analysis and in order to avoid inconsistencies in choice of a random field for analysis, seven z-stacks in a row were taken along the horizontal axis of the slide and built into a single panoramic image using MosaiX-function of the AxioVision Software, creating a cross-census of each mounted slide. The images were analyzed by the means of public domain ImageJ 1.42q Software [27]. Tyrosinase and NKI-beteb signals were analyzed along with the nuclear DAPI-signals to derive the expression index.

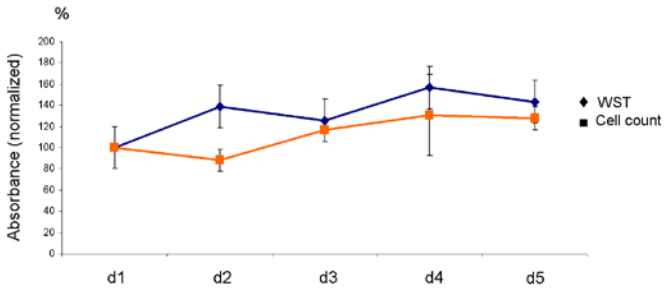
Mitochondrial activity

Viability and further on proliferation of the HM cells were monitored by the means of tetrazolium-reduction-based WST test (Roche Applied Science, Germany) after 5 days as described in [28]. WST dye was diluted 1:10 in DMEM medium. The medium above adherent cells and scaffolds was aspirated. The cultures were washed once with PBS buffer, 100 µl of diluted WST-dye was added over the cells and left at 37°C to incubate. After the incubation time of 120 minutes, the conditioned WST-reagent mixture was transferred into empty 96-wells and the extinction at 450nm was measured by the means of Multiscan® Spectrum Photometer (Thermo Fisher Scientific, Germany). The background extinction values of blank wells filled with DMEM were subtracted from the values of the conditioned WST reagent mixture.

The WST values measured during the five-day-culture were compared to the cell counts determined by Trypan Blue exclusion and counted by the means of Neubauer chamber (Figure 8).

Melanin production

Melanin content was quantified by complete lysis of cell structures, including melanosomes and subsequent measurement of lysate extinction, as described [22].



The seeded 10,000 HM cells proliferate during 5 days of cultivation, reaching 12,500 cells. The increase of 27.5% corresponds to the increase of 43% in the mitochondrial activity measured at day 5.

Figure 8: Mitochondrial activity increase over 5 cultivation days in adherent culture corresponded to the increase in cell count.

Statistical analysis

All the experiments were performed with material of four donors and performed in biological triplicates with five technical replicates. The statistical analysis was performed by the means of a two-tailed t-test, with significance threshold set at 0.05 p value ($p<0.05^*$ for significant, $p<0.01^{**}$ for highly significant, $p<0.001^{***}$ for very highly significant). All the p values higher than P=0.05 were regarded as non-significant.

Results

Cultivation, expansion, differentiation and selection of pure melanocyte culture

The upgraded procedure has yielded a higher-expanded culture of 95-100% pure, almost fully differentiated human melanocytes, which reached the melanotic stage within 3 to 4 weeks post-hair plucking, as early as p3 or p4 of the primary culture [22]. In particular, the cultivation and differentiating procedure from 60 hair follicles yielded 10^6 pure, differentiated human melanocytes in melanotic stage within 3-4 weeks post-epilation, in the second to fifth passage of adherent culture. In comparison to the results of Dieckman et al. [21], the method presented here yielded an output of 10^6 HM in half the time and by far exceeded the yield of protocol by Dieckmann already in very early passages of adherent culture according to our findings.

Furthermore, the improved procedure reached much higher numbers in the further passages of the adherent culture (p3 to p6).

Morphological characteristics

HM and NHEM were morphologically quite similar. Both cell types have a typically large soma that can vary in surface. The soma of HM tends to be somewhat smaller than that of NHEM. Both cell types developed two or more (typically 3-4) dendrites. Both HM and NHEM produced vesicles with melanin-melanosomes transported from the soma towards dendrite tips, visible as dark inclusions (Figure 1).

Follicle activity

Number of follicles that yielded migrating cells at medium-air-interface cultivating system was higher in follicles pre-treated according to the current method (discarding of the proximal part and digesting with collagenase V). At day 7 of the follicle culture, 79.75% of the follicles in our culture were outgrown in comparison to the 58.81% of intact follicles cultivated upon Dieckmann et al. [21] ($p=0.038^*$). In the later stages, this difference persisted on a non-significant level ($p>0.05$, Figure 2).

ORS surface growth

Difference in growth between methods was visible already after day 7 of the medium air interface follicle cultivation on nylon meshes, with clearly displayed enhanced growth of the follicles cultivated upon the method described herein in comparison to the Dieckmann method (Figure 3). The ORS surface of hair follicles digested with collagenase and cultivated according to the current method, increased from 42.480 ± 17.451 μm^2 on day 0 to 87.74 ± 45.4 μm^2 on day 7 and 125.84 ± 58.45μm^2 on day 14, whereas the follicles cultivated according to the Dieckmann method increased from 43.30 ± 9.23 μm^2 on day 0 to 50.04 ± 10.75 μm^2 on day 7 and 77.49 ± 29.57 μm^2 on day 14 (Figures 3 and 4). The ratio of the Outer Root Sheath surface between the Dieckmann method and the current method hereby declined from 0.94 on day 0 to 0.69 on day 7 ($p=0.01^{**}$) and 0.72 on day 14 ($p=0.018^*$), displaying faster growth and migration of cells from shortened, collagenase-treated follicles (Figure 3). At the point of harvesting the cells from the Transwell® nylon meshes in order to take them into adherent culture, the number of harvested cells exceeded that of the Dieckmann method by factor 1.84 when all the sampled hair follicles were taken into account.

Comparative growth in adherent culture

The cells in the adherent culture, cultivated according to the method described herein, continued to proliferate and reached the numbers of 1,000,000 at the passage 2, adherent culture day 11 and as much as 80,875,000 at passage 6, day 44 (Figure 5). This means that each follicle was able to yield as much as 30,666 melanocytes in 5 weeks and upto 2,695,833 melanocytes within 9 weeks (Figure 6). In contrast to our method, the cells in the adherent culture cultivated according to the Dieckmann et al. [21] protocol reached 710,000 cells at passage 1 of the adherent culture as the highest cell number and 650,000 cells by passage 2 at day 11 of the adherent culture (reaching theoretical values of 7,717 cells per follicle at p1 and 7,065 cells per follicle at p2) (Figures 5 and 6). Unlike the cells cultivated upon the method described herein, the cells cultivated according to the Dieckmann method decreased in numbers with further passages (Figures 5 and 6).

Geneticine treatment

Geneticine treatment very efficiently helped in removing keratinocytes and fibroblasts from the culture, leaving large numbers of melanocytes for further cultivation (Figures 1, 5 and 6). Additional Geneticine treatment of the adherent Dieckmann culture as a corresponding selection measure used in our current method resulted with extremely low numbers of survivor cells (<2% confluence) (Figures 5 and 6).

3D collagen I scaffold cultivation

The HM cells seeded on bovine collagen I membranes populated the scaffold, adhering well, interfacing with the scaffold and migrating the depth of 100 µm. The cells expressed melanocyte markers Tyrosinase and gp100 variant NKI-beteb (Figure 7). 98.17% of the seeded cells expressed Tyrosinase and 97.13% expressed NKI-beteb variant of gp100, which fully corresponded to the reported 96% of marker-expressing cells in the adherent culture ([22], Figure 7).

Mitochondrial activity

The 10,000 seeded HM cells proliferated both on polystyrene and CCC membrane over 5 cultivation days. The comparison of percentual growth in the adherent culture of HM shows that the extinction values of the WST test, as a measure of mitochondrial activity, corresponds to cell counts determined by Trypan Blue exclusion in the course of the five-day culture (Figure 8). Mitochondrial activity of HM cells seeded on CCC membranes and measured on day 5 exceeded that of the polystyrene-adherent HM culture by 28% (p=0.026*) (Figure 9).

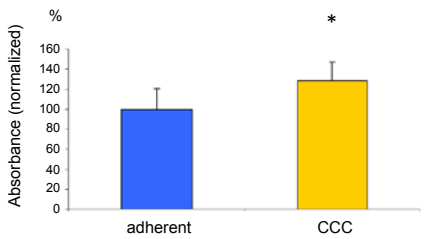
Melanin production

Melanin content of the HM cells cultivated on CCC membranes exceeded that of the polystyrene-cultivated HM by 19% (1.62 ± 0.37 ng/cell on CCC membrane, 1.36 ± 0.22 ng/cell polystyrene-adherent HM, p=0.038*) (Figure 10).

Discussion

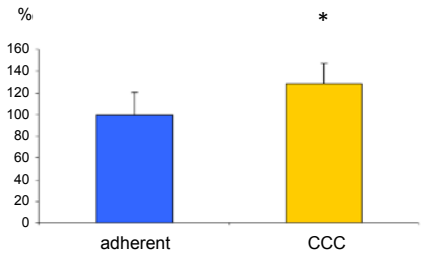
The goal of the upgraded explants method was to differentiate stem cells and progenitor cells from ORS of hair follicle and obtain high amounts of differentiated cells that would, both in morphology and function, represent a peer to epidermal melanocytes.

As already shown in [22], combined upgrades of the Dieckmann method [21] brought about exceedingly high improvements in terms of cell yield. Discarding of fibroblasts from the cut-off follicle proximal part, loosening of the extracellular matrix by collagenase, favourable cultivation conditions with a low-serum and non-toxic DermaLife medium, along with combined selection methods of isolating melanocytes by differential trypsinization and eliminating keratinocytes and fibroblasts by Geneticine challenge, resulted in a very reliable and reproducible method of cultivating melanocytes from the ORS. The upgraded method brought about not only one million of ORS melanocytes in four weeks, but also as much as 80 million cells within nine weeks, which represents a substantial cultivating potential.



Proliferation of HM cells cultivated on CCC membranes, estimated by measuring the mitochondrial activity (WST test), exceeded that of polystyrene-adherent HM by 28% (p=0.026*).

Figure 9: Comparative mitochondrial activity of polystyrene-adherent and CCC-cultivated HM culture.



Melanin content of HM cells cultivated on CCC membranes, exceeded that of polystyrene-adherent HM by 19% (p=0.038*).

Figure 10: Melanin content of the HM in adherent culture and 3D culture on CCC membrane.

Follicle activity

Outgrowth of stem cells and progenitors from the ORS during medium-air-interface cultivation was more rapid in our upgraded method than in those cultivated according to Dieckmann et al. [21]. For the purposes of this comparison, we have followed the number of outgrown follicles during medium-air-interface cultivation for 28 days (Figure 2). Whereas the difference between the current and Dieckmann method was more pronounced and statistically significant on day 7 of the follicle culture (p=0.038*), it decreased with time, upholding the trend on a non-significant level. Our interpretation of this decrease is that both methods involved follicle activation, but with qualitatively different cell content being released onto the nylon mesh. The Dieckmann method kept yielding substantial numbers of cells at this stage (visible as active follicles with cells leaving ORS), however those cells were prevalently keratinocytes and fibroblasts. This interpretation was corroborated by the low numbers of cells that remained after Geneticine selection of primary cell yield, which indicated that the melanocyte amount in the output adherent culture of the Dieckmann method was modest and by all means lower than according to the upgraded method (Figures 5 and 6).

ORS surface changes

Collagenase effect on loosening the extracellular matrix through collagen proteolysis had obviously enabled easier migration of the cells out of the ORS. This effect was visible by the enlarged surface of the ORS and by a more intensive migration of cells from ORS onto the nylon mesh (Figure 3). The initial growth of ORS in follicles digested with collagenase V as per the current method exceeded that of the non-digested follicles according to the Dieckmann method in all stages of adherent culture following day 0 (Figures 3 and 4). That gave the current method the advantage of harvesting more cells from the medium-air-interface follicle culture and having more cells for the set-up of the adherent culture to start with. The number of cells harvested from the follicles cultivated by the current method on day 0 of the adherent culture exceeded that of Dieckmann method by factor 1.84 (Figure 4).

Morphology

It is easy to comprehend that HM and NHEM are remarkably similar [21,22]. They have the same developmental origin [21,22,29,30]. ORS stem cells and progenitors regularly give rise to epidermal melanocytes that subsequently migrate out of hair follicle and home to the *stratum basale* for the purposes of epidermal regeneration [30-42].

Both HM and NHEM can be morphologically quite heterogeneous in appearance, depending on the time of adherence and passage.

HM and NHEM both displayed a typically large soma and several dendrites (in adherent culture, they are in average tripolar or tetrapolar) [21,22] (Figure 1). Cell size and number of dendrites often differed between donors and between passages in both NHEM and HM.

Comparative growth in the adherent culture

The cells in the adherent culture were continuously dividing in

cultivation experiments according to the method described herein, reaching 1,000,000 cells at the passage 2 of the adherent culture, (half the time needed for the same number of cells cultivated according to Dieckmann et al. [21]) and as much as 80,875,000 by the passage 6 (Figure 5). The cells cultivated according to the Dieckmann method did not reach the mentioned numbers; in fact, their numbers persistently declined after the passage 2 of the adherent culture.

The fact alone that a single hair follicle could in average produce almost 2.7 million cells in favourable culture conditions (Figure 6) speaks very much in favour of the ORS as a very promising source of stem cells and progenitor cells for regenerative therapies. As for the cultivation conditions, it is clear that the combined upgrades enabled quicker release and proliferation, and hereby accumulation of stem cells and progenitors at the early stages of cultivation before yielding them from the medium-air-interface cultivation set-up, which assured more cells for the adherent culture. Non-toxic and non-tumour-promoting medium DLM was evidently favourable enough to ensure further selection and amplification of melanocytes in the hypoxic culture. Elimination of fibroblasts and keratinocytes relieved the melanocyte culture of competitive pressure, which also helped the increase of the yield.

Geneticine treatment

Clearly, it was not only the number of cells, but also the cell content that proved favourable within the yield obtained from primary culture (medium-air-interface follicle cultivation). The portion of fibroblasts and keratinocytes in the material harvested from the medium-air-interface follicle primary culture was much higher in the preparation upon the Dieckmann method, judging by their massive cell death during and after the Geneticine selection. An extremely low number of cells that survived the Geneticine treatment indicated that the Dieckmann method did not provide enough stem cells and progenitor cells to quickly cultivate larger amounts of melanocytes in the adherent culture (Figures 5 and 6). In contrast to the outcome of Geneticine selection of the cells cultivated according to Dieckmann et al. [21], large number of cells cultivated as described in our upgraded method survived this selection step and those cells gave rise to very high amounts of melanocytes.

Altogether, the cells cultivated according to the current method reached higher numbers than the cells cultivated according to Dieckmann method, for several reasons. Upon the current method, the initial release and migration of stem cells and progenitor cells out of ORS onto the nylon mesh occurred in more follicles (Figure 2) and with higher intensity yielding more stem cells and progenitors from the primary culture to start with. Our interpretation of the primary culture yield includes a higher stem cell content vs. a lower fibroblast and keratinocyte content in the current preparation. This was deduced from the outcome of the subsequent selection steps (Geneticine challenge and regular differential trypsinization), which left much more living melanocytes behind than in the identical selection procedure of the adherent cells yielded from the primary culture according to the Dieckmann method (Figures 5 and 6).

Synergy of the higher start number of stem cells and progenitors in adherent culture and more favourable culture conditions given by the current method significantly advanced the total yield of HM.

3D membrane cultivation

Main concerns by the choice of material for a graft scaffold is its biocompatibility, biodegradability and the kind of environment it can provide for the grafted cells. Collagen, being a major structural component of the body and present in the matrix of most natural tissues, has been widely utilized as a biocompatible, resorbable carrier material. Of all collagen types, collagen type I has been the most commonly used in regenerative medical applications, purified as well as additionally technologically manipulated into matrix variants with even higher biocompatibility. Xenogeneic (bovine or porcine) collagen I display low immunogenicity and can be applied in human tissues without risk of rejection [43]. Ectopic application of this primarily dermal protein appears to function without problems in skin grafting. Collagen type I as a physiological component of the skin is often used as a sole or combined component of dressings and biological membranes for wound healing [44,45], as an epithelial graft [46,47] and for delivery of epidermal or dermal cells [48]. As the most common protein of dermis, it has been plentifully used for dermal grafting [48], nevertheless it was just as efficient in epidermal grafting applications.

We have considered collagen type I as a carrier in melanocyte grafting applications for several reasons. General features of collagen I and its biocompatibility were certainly some of them. In addition, collagen I has an apparent temperate effect on melanocytes. Such favourable effects of collagen I coating as melanocyte niche have already been shown through disintegrating melanocytic spheroids and reconstituting morphological and physiological features of melanocytes [49]. This additional feature of collagen I is particularly worthy since melanocytes tend to be the limiting factor in co-cultured epidermal and dermal grafts and they are the first cell type to suffer loss [50]. Due to melanocyte sensitivity in grafts, providing a proper biological carrier niche is essential for grafting them. Moreover, collagen I has already been used as dressing and coating in melanocyte grafting applications, including Vitiligo treatment [51].

Enabling retainment of melanocyte dendritic morphology, melanotic function and migratory capabilities, along with biodegradability and resorption features, are precisely the demands for a good melanocyte graft. Bovine collagen I decellularized membrane scaffold fulfilled all of these requirements and provided a friendly niche for melanocytes derived from the ORS of hair follicles.

As one of the most putative skin protein constituents, collagen I represented a reliable adhesion surface for melanocyte grafts. Decellularized collagen scaffolds retained the geometry of the original extracellular matrix, providing 'cell-slots' that could be populated by epidermal cells. Such ready-to-wear skin architecture offered a niche for primary cell grafting that was very close to physiological terms [23]. Furthermore, proven benevolent effects of collagen I on melanocytes strengthened the features of CCC membrane as melanocyte carrier.

In this study, we have already shown that the hair follicle ORS-derived melanocytes successfully nested in the decellularized bovine collagen type I scaffold, protruded dendrites and interfaced with scaffold architecture, expressed melanocyte markers and produced melanin. The HM cells were able to populate the CCC membrane scaffold, they interfaced its inner surfaces, penetrated the depth of 100 µm (Figure 7) and their melanocyte features even exceeded those of the HM cells cultivated on adherent polystyrene surfaces. The HM proliferation rate on CCC membranes exceeded that of the HM in polystyrene-adherent culture by 28% (Figure 9); furthermore, the HM cultivated on CCC membranes produced 17% more melanin than polystyrene-adherent cells (Figure 10) which showed the advantages of three-dimensional cultivation and physiologically compatible surface.

The enhanced melanotic function in the scaffolds in comparison to polystyrene adherent cultures speaks even more in favour of CCC membranes as a good support for melanocytes.

HM melanocytes retained their migratory capabilities on collagen I membranes, giving a fair chance of migration and re-distribution capability within the niche of native skin, after picking up the queue of their new in vivo post-graft environment.

Biodegradability and resorption capability of collagen I should enable optimal blending of such melanocyte graft into either epidermal or dermal tissue upon transplantation, without disturbing the organic architecture of the local matrix.

Both the promising HM cultivation improvements and the collagen I as graft constituent remain to be further explored in future. The cultivation procedure leaves room for acceleration in terms of differentiation of ORS stem cell and progenitor pool into melanocytes. Use of collagen I for melanocyte grafting offers prospects for a variety of upgrades in the direction of using human-derived scaffolds and introducing chemical modifications.

All of the above makes collagen I by all means a very decent candidate for the main component of melanocyte graft carriers.

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