INVESTIGATIONS ON NORMAL HUMAN ADULT EPIDERMAL MELANOCYTES

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1. INTRODUCTION

Melanocytes are cells specialized for the production of melanin. In the skin, melanocytes project their dendrites into the epidermis where they transfer melanosomes to keratinocytes. These dendritic contacts produce interfaces with multiple keratinocytes, thought to number approximately 36 per melanocyte, giving rise to the "epidermal melanin unit".

The rate limiting catalytic activity in the production of melanin is the oxidation of tyrosine by tyrosinase (TYR). Tyrosinase is a melanocyte-specific copper-binding enzyme with homology to polyphenol oxidases and some hemocyanins. It also shows high homology with tyrosinase-related protein-1 (TRP-1) and -2 (TRP-2). The latter is also known as dopachrome tautomerase (DCT). They catalyze different biochemical reactions in eumelanogenesis. Additionally, TRP-1 stabilizes the enzymatic activity of tyrosinase and maintains the melanosome structure integrity, while TRP-2 has a role in melanocyte survival.

Many skin disorders are associated with pigmentation and/or pigment cell dys-or malfunction. Uncontrolled hyperproliferation of melanocytes can lead to melanoma initiation, which is the most aggressive skin cancer, mainly due to its high metastatic potential. Another common disorder related to melanocytes is vitiligo, which is characterized by white patches on the skin, because melanocytes are missing. One possibility to treat vitiligo symptoms is autologous melanocyte transplantation. To investigate melanocyte differentiation and melanoma development and for treatment of vitiligo, establishment of appropriate melanocyte culture techniques is important.

For a long time, the study of melanocyte proliferation *in vitro* was restricted to the use of melanoma cells, because attempts to establish normal melanocytes in culture using the standard, serum-supplemented medium, failed. The first reproducible melanocyte culture technique was published in 1982. Generally, melanocyte culturing *in vitro* is accomplished by using specific mitogens to enhance melanocyte and to suppress keratinocyte and fibroblast growth. Usually the melanocyte culture media is supplemented with the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA or PMA) and the intracellular cyclic adenosine 3', 5' monophosphate (cAMP) enhancer, cholera toxin (CT) and in some cases 3-isobutyl-1-methylxanthine (IBMX) is used. Because these mitogens alter the physiological responses of the cells, attempts have been made to define culturing techniques free of these substances. It was reported that *in vitro* growth of melanocytes could be sustained without chemical mitogens in fibroblast conditioned medium, containing 15% horse serum and polyamines, but these cultures were contaminated by keratinocytes. In another study melanocyte cultures were established without non-physiological mitogens by co-culturing human epidermal keratinocytes with human epidermal melanocytes. Basic fibroblast growth factor (bFGF), endothelin-1 (ET-1) and α-melanocyte stimulating hormone (α-MSH) together could substitute for TPA and BPE in *in vitro* cultures of human melanocytes, however at the critical initial phase of the cultures TPA was used to establish the selective *in vitro* melanocyte growth. In most cultures of human melanocytes, cells were obtained from newborn prepucium. Data concerning adult epidermal melanocytes therefore were scares. We have shown that normal human adult epidermal melanocytes can grow in a medium (referred to as Mel-mix) that lacked the above listed mitogens.

In vertebrates, melanocytes arise from the neural crest. Melanoblasts are unpigmented cells containing only immature melanosomes that lack functional tyrosinase, the critical enzyme of melanin synthesis. Fully differentiated melanocytes characterized by TYR, TRP-1 and TRP-2 activities as well as by numerous mature melanosomes and well-developed dendrites. Melanocyte differentiation is under the control of microphthalmia transcription factor (MITF), a basic helix-loop-helix leucine zipper transcription factor, which activates

genes involved in pigment production, such as TYR, TRP-1 and TRP-2 and melanocyte survival, e.g. Bcl-2. The proto-oncogene c-Kit encodes a membrane receptor protein (Kit/SCFR) which regulates melanocyte proliferation, differentiation and migration by stimulation of MITF. In mouse epidermis, Kit^+ cells differentiate into $Mitf^+$ and/or $TRP\text{-}2^+$ cells first and then into $TRP\text{-}1^+$ cells after UV exposure. In human skin, the presence of Kit reactive cells is consistently demonstrated in the basal layer of the epidermis, follicular infundibula and eccrine coils and ducts. In the follicular infundibula, $Kit^+Bcl\text{-}2^+TRP\text{-}1^-$ cells represent a reserve population of precursor melanocytes. Interestingly, c-Kit expression is down-regulated in melanocytes following malignant transformation and SCF inhibits the growth of melanoma cell lines, indicating that c-Kit plays a more important role in melanogenic differentiation than in cell proliferation.

One of the major growth promoting soluble factors in the epidermis is the EGF. Its role has been extensively studied in epidermal keratinocyte growth regulation, but data on melanocytes concerning the role of EGF and its receptor were controversial. It has been reported that melanocytic lesions in the skin express epidermal growth factor receptor (EGFR) and EGFR mRNA could be detected in melanocytes and melanoma cells *in vivo*. Moreover, in previous studies both the mitogenic effect of epidermal growth factor (EGF) on normal cultured melanocytes, as well as the expression of EGFR on cultured cells, were reported to be missing by different authors. Because Gordon-Thomson et al. reported on not only the presence of EGFR on normal human cultured melanocytes, but also on its tyrosine kinase-mediated signaling in response to EGF a controversy arose around this issue. Later another group also published data supporting the presence of EGFR on normal human melanocytes.

2. AIMS

The aims of our study were:

- to characterize the proliferation and differentiation of normal human adult epidermal melanocytes cultured *in vitro* in a newly defined more physiological medium that lacks the conventionally used mitogenes, such as phorbol-esters, IBMX and cholera toxin
- to investigate the differentiation potential of dedifferentiated normal human adult epidermal melanocytes
- to examine the role of EGF and EGFR in normal human adult epidermal melanocytes

3. MATERIALS AND METHODS

3.1 Culture media

Mel-mix medium contains AIM-V serum free lymphocyte medium and Keratinocyte Serum Free Medium (early name: Keratinocyte Basal Medium) both from Life Technologies, Carlsbad, CA, USA), v:v, supplemented with 2.5% fetal bovine serum (FBS, Life Technologies), 2.5 ng/ml epidermal growth factor (EGF, Life Technologies), 25 μg/ml bovine pituitary extract (BPE, Life Technologies), L-glutamine and Antibiotic Antimycotic Solution containing 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Sigma Laboratories, St. Louis, MO, USA). We also used a commercially available melanocyte medium, M254 Medium (Life Technologies). This medium is supplemented with Human Melanocyte Growth Supplement (HMGS, Life Technologies) containing bovine pituitary extract (BPE), fetal bovine serum, bovine insulin, bovine transferrin, basic fibroblast growth factor, hydrocortisone, heparin and phorbol 12-myristate 13-acetate (PMA). To induce melanocyte differentiation, the Mel-mix medium was supplemented with cholera toxin (CT, Sigma) at 10 nM and phorbol 12-myristate 13-acetate (PMA, Sigma) at 10 ng/ml

concentration, respectively.

3.2 Cell culture

Adult epidermal melanocytes were isolated and cultured as previously described from breast or trunk skin specimens of healthy Caucasian donors undergoing plastic surgery. The study was approved by the Human Investigation Review Board of the University of Szeged: it complied with the ethical standards of research, in accordance with the Helsinki Declaration. Written informed consent was obtained from all donors involved in the study.

Skin specimens were first washed in Salsol A solution (Human Rt, Godollo, Hungary) supplemented with 2% Antibiotic Antimycotic Solution (Sigma). The subcutis and part of the dermis was removed and the tissue was cut into small strips. Overnight incubation in Dispase solution (Grade II, Roche Diagnostics, Mannheim, Germany) was carried out at 4 °C to separate the dermis from the epidermis. Next day the epidermis was peeled of the dermis and after a short trypsinization, the epidermis was mechanically torn apart and vigorously washed to release epidermal cells. The epidermal cell suspension was filtered through a 100 μm nylon mesh (BD Falcon, San Jose, CA, USA) and after centrifugation they were placed into 75 cm^2 tissue culture dishes (BD Falcon) at a cell density of $2x10^5$ cells/cm^2. Cultures were grown at 37 °C in humidified atmosphere containing 5% CO_2.

Keratinocytes prepared from similar epidermal samples were cultured in KSF medium (Life Technologies) as described previously.

3.3 L-DOPA staining

The melanocytes were washed in PBS then fixed in 5% formalin for 30 minutes at 4°C. After fixation the cells were washed in distilled water and incubated with 0.1 % L-DOPA (Sigma) solution for 3 hours at 37 °C. The cells were visualized using a Nicon Eclipse TS100 microscope and photographed by a Nicon Coolpix 4500 digital camera.

3.4 Direct melanin measurement

Cells were trypsinized, and pellets with equalized cell numbers were solved in 5M NaOH. Absorbance was measured by a Multiscan EX (Thermo Scientific) plate reader at 495 nm. Standard curve was prepared using synthetic melanin (Sigma) solved in NaOH.

3.5 Measurement of cell growth by MTT assay

Melanocytes were seeded into 96-well culture plates at a density of $5x10^3$ cells/well and were exposed to different concentrations of EGF (0.1 to 100 ng/ml) for 72 hours. The supernatant was then replaced with 0.5 mg/ml MTT solution (Sigma. After incubation for 3 hours at 37^{o}C, the chrystallized dye was solubilized and the absorbance was determined by a Multiscan Ex spectrophotometer (Thermo Labsystems, Vantaa, Finland) and Ascent Software (Thermo) at 540 nm.

3.6 RT-PCR

The following specific primers were used:

Tyrosinase	forward	TTGGCAGATTGTCTGTAGCC
	reverse	GGCATTGTGCATGCTGCTT
β-actin	forward	AGAGATGGCCATGGCTGCTT
	reverse	ATTTGCGGTGGACGATGGAG

The yielding 198 bp (tyrosinase) and 406 bp (β-actin) products were run on 2% agarose gel, stained with ethidium bromide, photographed and evaluated by Kodak EDAS

290 densitometer (Kodak, Rochester, NY, USA) and Kodak 1D Digital Science software (Kodak).

3.7 Real-Time RT-PCR

The following specific primers were used:

TRP-1	forward	CTTTTCTCACATGGCACAGG
	reverse	AAGGCTCTTGCAACATTTCC
c-Kit	forward	CGTGGAAAAGAGAAAACAGTCA
	reverse	CACCGTGATGCCAGCTATTA
Nestin	forward	TGCGGGCTACTGAAAAGTTC
	reverse	TGTAGGCCCTGTTTCTCCTG
18S	forward	CTCAACACGGGAAACCTCAC
	reverse	CGCTCCACCAACTAAGAACG

Primers and probe specific for EGFR (Cat. Number: HS00193306_m1) were purchased from Life Technologies (Applied Biosystems). To quantify the relative abundance of each mRNA, iCycler IQ Real Time PCR (BioRad, Hercules, CA, USA) was used.

3.8 Immunocytochemistry

For TRP-1, c-Kit and EGFR protein detection, cytospins were stained with the following antibodies: anti-TRP-1 at a dilution of 1:2000 (Signet Laboratories, Dedham, MA, USA), anti-c-Kit (BD) at a dilution of 1:250 and anti-EGFR (Clone: sc-120, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at a dilution of 1:200. Control slides were incubated with mouse IgG_{2a} (Sigma), the isotype of all primary antibodies. For visualization Vectastain ABC Kit (Vector, Burlingame, USA) was used according to the manufacturer's instruction. The cells were analyzed using a Zeiss Axio Imager microscope and photographed using a PixeLINK digital camera (TissueGnostics, Austria).

3.9 Fluorescent immunocytochemistry

Melanocytes grown on glass coverslips were incubated with primary antibodies specific for nestin (Abcam, Cambridge, UK) at a dilution of 1: 1000 and cyclin D1 (NeoMarkers, Fremont, CA, USA) at a dilution of 1:200. Control slides were incubated with mouse IgG_1 (Sigma). AlexaFluor 546 and 488 conjugated anti-mouse IgG (both from Life Technologies) at a dilution of 1:500 was used for visualization. Nuclei were counterstained with DAPI (Sigma) at a dilution of 1:100. The cells were analyzed using a Zeiss Axio Imager microscope and photographed using a PCO Pixelfly digital camera (TissueGnostics, Austria).

3.10 Western blot

Cells were trypsinized and harvested by centrifugation, and the pellet was then gently resuspended in protein lysis buffer (20 mM HEPES, 150 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, 0.5% Triton X-100, 0.1% Igepal® CA-630) containing 0.5% protease inhibitor cocktail (all components from Sigma). Protein concentrations were determined with the BCA detection kit (Thermo Scientific, Waltham, MA, USA). SDS-PAGE was carried out with 40 µg protein samples, blotted to a nitrocellulose membrane (Bio-Rad). Membranes were blocked in 3% non-fat dry milk (Bio-Rad). Mouse anti-human nestin (Abcam) was used at 1µg/ml concentration and rabbit anti-human α-actin (Sigma) was diluted at 1:400 and incubated the nitrocellulose membrane with them overnight at 4^oC. Anti-rabbit and anti-mouse IgG alkaline phosphatase-conjugated secondary antibodies (Sigma) were applied and the bands were visualized using SigmaFAST BCIP/NBT (Sigma).

3.11 Direct and indirect UVB irradiation

For UVB irradiation, a VL-6LM light source (Vilber Lourmat, Marne-la-Vallée, France) was used. The light emitted from this lamp was within the UVB range (280-320 nm) and the peak emission was at 312 nm. Light intensity was measured by a UVX radiometer (UVP, Upland, CA, USA) before the experiments. To study the direct effect of UVB, 3rd passage, Mel-mix-cultured normal human adult epidermal melanocytes and keratinocytes in PBS were irradiated with 0, 20.8, 31.2 and 41.6 mJ/cm^2 doses of UVB. To examine the indirect effects of UVB, the supernatants of the irradiated keratinocytes were collected 6 and 24 hours after irradiation. These supernatants were mixed with complete AIM-V medium (v:v) and autologous cultured melanocytes were cultured in them for 24 hours.

3.12 Carboxyfluorescein diacetate, succinimidyl ester (CFSE) analysis

For CFSE analysis, CellTrace CFSE Cell Proliferation Kit (Life Technologies) was used according to the manufacturer's instructions. CFSE fluorescence was measured using a FACS-Calibur flow cytometer (Beckton Dickinson, San Jose, CA) and analyzed with CellQuest Software. The number of cell divisions was calculated based on the assumption that the dye of the mother cells would be equally divided into both daughter cells, resulting in halving of fluorescence intensity.

3.13 Senescence-associated β-galactosidase assay

To determine the senescent stage of the cells, 3rd and 8th passage melanocytes cultured in Mel-mix or in M254 medium were stained using Senescence-associated β-galactosidase Staining Kit (Cell Signaling Technology, Danvers, USA) according to the manufacturer's instructions.

3.14 Data presentation and statistical analysis

Data are presented as mean ± standard deviation (SD) or standard error of mean (SEM). Data were compared using the one-way analysis of variance (Univariate ANOVA) followed by Tukey's and Dunnett's *post hoc* test to determine statistical differences after multiple comparisons (SPSS, SPSS, Chicago, Illinois). A probability value of less than 0.05 was considered significant.

4. RESULTS

4.1 Differentiation characteristics of normal human adult epidermal melanocytes cultured in Mel-mix medium

Normal human adult epidermal melanocytes cultured in Mel-mix medium show "typical", multi-dendritic morphology in early cultures, but during culturing their morphology changes, cells become bipolar. This change in cell morphology is indicative for dedifferentiation of the cells. During long-term Mel-mix cultures, the color of the cell pellet at consecutive passaging becomes lighter and lighter. Loss of visible pigmentation was verified by L-DOPA staining in the cultured cells. Cells up to 6th passage contained pigment, while further cultured cells did not. To examine the reason of pigment loss we followed the rate-limiting enzyme of melanin synthesis, tyrosinase mRNA in the cultured cells. The presence of tyrosinase mRNA could be verified in all cultures up to the examined 10th passage, although the amount seemingly decreased with passage numbers. The expression of TRP-1, an also indispensable enzyme of melanin synthesis and c-Kit, a melanocyte differentiation marker

were also checked. TRP-1 exhibited strong expression in primary melanocyte cultures, and its expression decreased during *in vitro* culturing in Mel-mix medium. c-Kit protein expression was also strong in the early cultures, but melanocytes never stained as uniformly with the anti-c-Kit antibody as with the Mel-5 antibody. All cells were TRP-1 and c-Kit negative in the five-week-old, 7^{th} passage melanocyte cultures. TRP-1 and c-Kit mRNA expressions were determined at every passage by real-time RT-PCR analysis. TRP-1 mRNA values decreased during culturing, exhibiting similar tendency as the protein expression. The decrease in TRP-1 mRNA levels from the 4^{th} passage to the 7^{th} passage samples were statistically significant compared to the 1^{st} passage samples. c-Kit mRNA values showed decreasing tendency, but changes in mRNA expressions were not statistically significant.

Nestin protein was detectable in 3p melanocyte cultures by fluorescent immunocytochemistry. Melanocytes were not uniformly stained with anti-nestin antibody. To check that nestin positivity correlates with a more proliferative status of the melanocytes we double-stained the cells with nestin and cyclin D1. We could not found a correlation between nestin positivity and melanocyte proliferation status. Nestin mRNA expressions were examined and compared in passages from 1-3-7 using real-time RT-PCR. The expression of nestin was higher in 7p cultures compared to 1p or 3p cultures.

4.2 Investigation of differentiation potential on dedifferentiated melanocytes

In order to study the possible dedifferentiation of melanocytes, normal human adult epidermal melanocytes were cultured in commercially available PMA containing M254 medium for four weeks. These cells showed dendrite-rich morphology, expressed TRP-1 and c-Kit proteins uniformly and EGFR weakly. After four weeks, cultures were split into two; one part was continuously cultured in M254 medium; the other part was switched to Mel-mix medium for the following two weeks. Melanocytes cultured in the PMA containing M254 medium remained dendritic and expressed TRP-1 and c-Kit and showed a generally week EGFR staining, while cells cultured in Mel-mix for two weeks became bipolar, their TRP-1 and c-Kit protein expressions drastically decreased, and their EGFR expression strongly increased. These changes indicated that in Mel-mix, without PMA, melanocyte dedifferentiated *in vitro*.

To check the reversibility of the observed dedifferentiation, we added cholera toxin (CT) and phorbol esther (PMA) to the Mel-mix medium, and cultured 7^{th} passage dedifferentiated melanocytes for 1 week in this environment. CT+PMA treatment induced dendrite formation in bipolar, dedifferentiated melanocytes. Immunocytochemical staining demonstrated that TRP-1 protein expression was induced in 7% of the cells, while c-Kit protein expression was induced in 12% of the dedifferentiated melanocytes compared to untreated cultures ($TRP\text{-}1^{+}$cells: 0%, $c\text{-}Kit^{+}$cells: 0%). Real-time RT-PCR was performed to determine the relative expression of TRP-1 and c-Kit mRNA in CT+PMA containing and Mel-mix control cultures. The relative mRNA expression of TRP-1 was on average only 2 times higher in cultures treated with CT+PMA compared to untreated melanocytes grown in Mel-mix medium. This difference was not statistically significant. The relative mRNA expression of c-Kit was on average 4 times higher in the CT+PMA-treated cultures. This difference was statistically significant. CT and PMA treatment also increased the melanin-content of the cells.

To study the effect of UVB on melanocyte differentiation, melanocytes cultured in Mel-mix medium were irradiated with 20.8, 31.2 and 41.6 mJ/cm^2 doses of UVB. TRP-1 mRNA expression was used as a marker for melanocyte differentiation. Interestingly, direct UVB irradiation of melanocytes resulted in decreased relative mRNA expression of TRP-1 24 hours after irradiation. To study the influence of keratinocytes in UVB-induced melanocyte

differentiation, keratinocytes were irradiated with the same UVB doses. Keratinocyte supernatants were collected 6 and 24 hours after UVB irradiation and autologous melanocytes were treated with these supernatants for 24 hours. Keratinocyte supernatants collected 6 hours after irradiation caused a slight increase in TRP-1 mRNA expression, while supernatants collected 24 hours after similar UVB irradiation caused a more pronounced increase in TRP-1 mRNA expression in melanocytes compared to cells that were treated with non-irradiated keratinocyte supernatants.

To see the proliferative capacity of differentiated and dedifferentiated melanocytes, we compared melanocyte proliferation in cultures of Mel-mix and M254 medium using CFSE analysis. Cells in both cultures proliferated at a similar rate in the first 72 hours, then melanocytes cultured in Mel-mix medium showed an enhanced rate of division compared to melanocytes cultured in M254 medium.

Senescence-associated β-galactosidase assay was performed on both types of cultures when cells were in 3rd and 8th passages. Blue colored senescent cells were present in comparable numbers in both types of cultures regardless of the applied media. Blue cells appeared flat without dendrites in cultures, irrespective of the used media.

To further characterize the dedifferentiation state of cultured melanocytes we cultured cells in Mel-mix up to 2nd passage then transferred half the cells into PMA containing M254 medium and left the other half in Mel-mix. We then measured the expression of nestin, a neuronal precursor marker in the different cultures. We were able to detect nestin mRNA in all cultured melanocytes irrespective of culture conditions. An increase in nestin mRNA expression was observed in cells as they reached higher passages. The increase of nestin mRNA was more pronounced in dedifferentiated melanocytes cultured in the PMA-free Mel-mix medium. On Western blot, although a faint nestin specific band was visible in all samples, densitometry could not detect bands from samples of PMA containing M254 cultured melanocytes. A gradual increase in nestin protein expression was detected only in Mel-mix cultured, *in vitro* dedifferentiated cells.

4.3 The role of EGF and EGFR in melanocytes.

Using real-time RT-PCR, we showed that EGFR mRNA was expressed in Mel-mix cultured melanocytes, although on average at a three times lower level than in keratinocytes. To detect the expression of EGFR protein in the cells, we used a monoclonal antibody to EGFR for immunocytochemical detection. Cultured cells in 3rd passage uniformly expressed the EGFR protein.

Next we examined the effect of EGF at various concentrations on cell growth. Cells from different donors that were cultured in the chemical-free Mel-mix medium until the 4th passage were exposed to different concentrations of EGF (from 0.1 to 100 ng/ml) for three days. The medium at that time did not contain FBS and BPE. Cells responded to EGF with a dose-dependent increase in proliferation, significant growth induction occurred in the 2.5 to 10 ng/ml concentration range.

To examine the effect of different EGF concentrations on the relative expression of EGFR mRNA in cultured normal human adult epidermal melanocytes, cells in 5th passage were exposed to different concentrations of EGF (2.5-5-10 ng/ml) for three days. The relative expression of EGFR mRNA was determined by real-time RT-PCR. EGF had no significant effect on the EGFR mRNA expression in the cells.

5. DISCUSSION

The traditionally used media for melanocyte culturing contain specific mitogens, such as PMA, CT and IBMX. Melanocytes cultured in media that contains PMA, CT or IBMX show the phenotype of fully differentiated melanocytes. Differentiated melanocytes are characterized by melanin production due to the activities of tyrosinase, TRP-1 and TRP-2; by numerous mature melanosomes and well-developed dendrites. Melanocytes cultured without PMA, CT or IBMX show pigmentation loss and decreased dendrite formation. Pigmentation loss in long-term Mel-mix cultured melanocytes is triggered by decreased amount of tyrosinase mRNA and decreased enzyme activity of tyrosinase protein. Expression of two melanocyte differentiation markers, c-Kit and TRP-1 is also decreased.

In melanocyte development, c-Kit plays a critical role in a number of cellular activities, including differentiation. The expression of c-Kit is considered a key step in pigment cell development, Kit+ nonmelanotic cells in the skin are putative melanocyte precursors. Activation of c-Kit by stem cell factor results in Mitf phosphorilation. Mitf determines the melanocyte fate of multipotent neural crest cells partially by its transcriptional and lineage-specific regulation of three major pigment enzymes, tyrosinase, TRP-1 and TRP-2. There is also evidence that c-Kit, tyrosinase and TRP-1 gene expression are coordinated in melanocytes. The melanocytes that we harvested from the adult human skin uniformly stained for c-Kit and TRP-1, if they were cultured in PMA containing M254 medium. After switching the medium into Mel-mix, the expression of these proteins in the cells decreased dramatically. In the Mel-mix culture, without PMA, cells showed a uniform positivity for TRP-1 in early cultures, whereas in older cultures, we could not detect TRP-1 protein expression in the cells. In Mel-mix medium, TRP-1 and c-Kit mRNA expressions also showed a decreasing trend.

In vitro studies have shown that TPA (PMA) induces neural crest cell differentiation into melanocytes and stimulates proliferation and differentiation of normal melanocytes. Similarly, in our culture system, the addition of cholera toxin and PMA to the Mel-mix medium resulted in dendrite formation and induction of c-Kit mRNA and protein expression in the *in vitro* dedifferentiated melanocytes. The amount of TRP-1 mRNA also showed increasing tendency and in a few cells, TRP-1 protein expression reappeared. The fact that c-Kit expression precedes TRP-1 expression is expected, because c-Kit signaling is essential for the transcription of TRP-1.

It has been reported that withdrawal of cAMP inducers (CT+IBMX) from the medium in melanocyte cultures causes cells to become senescent. Although, our culture medium lacks these mitogens, melanocytes proliferate rapidly in this medium. In fact, melanocyte proliferation was higher in Mel-mix medium than in PMA-containing melanocyte growth medium. We found no difference in senescent cell numbers between cultures of Mel-mix and PMA-containing M254 medium.

We used our culture system to study the direct and indirect effects of UVB on melanocyte differentiation/pigmentation. TRP-1 mRNA expression was used as a marker to determine the effect of UVB on melanocyte differentiation/pigmentation. Data indicate that UVB can influence the expression of TRP-1. In our experiments, direct UVB exposure caused a decrease in TRP-1 mRNA expression in melanocytes. Similar results have already been reported: in the absence of cAMP inducers, UVB radiation inhibited, rather than stimulated, melanogenesis. On the other hand, the indirect effect of UVB irradiation on melanocytes, exerted through keratinocyte soluble factors, resulted in TRP-1 mRNA induction in the cells. The most likely keratinocyte-derived factors which may be responsible for this TRP-1 mRNA induction are α-melanocyte stimulating hormone (α-MSH) and endothelin-1 (ET-1). It is well documented that UVB induces the production of both factors. Our results are in line with

numerous data indicating that melanocyte differentiation and melanogenesis are influenced by tissue environment, in which keratinocytes are keys.

Melanocytes arise from the neural crest, a pluripotent structure of the vertebrate embryo. In addition to melanocytes and many other cell types, neural crest is also the source of neurons and glia of the peripherial nervous system. During the segregation of cell lineages derived from the neural crest, multipotent neural-melanocytic progenitors and bipotent glial-melanocytic precursors are generated. From the bipotent glial-melanocytic precursors, melanoblasts and melanocytes originate. Cell differentiation is not unidirectional; under certain stimuli *in vitro* or during regeneration differentiated cells may recover properties of immature cells. It has been shown that neural crest-derived pigment cells from quail embryo could dedifferentiate/transdifferentiate into glia through a glial-melanocytic progenitor, if treated with endothelin-3 (ET-3). A recent study in mice identified growing nerves projecting throughout the body as progenitor niche containing Schwann cell precursors from which large numbers of melanocytes originate. It is known that cutaneous melanocytes share many signaling molecules with neurons, and *in vitro* melanocyte cultures have already been proposed to be used as model system to study Alzheimer's disease.

To characterize the stage of dedifferentiation of adult melanocytes cultured in Mel-mix medium, we examined the expression of nestin, an intermediate filament, which is a "neural stem/progenitor cell" marker in the cells. We found strong nestin expression both at the mRNA and protein levels in dedifferentiated melanocytes. Differentiated cells expressed less nestin mRNA and almost undetectable nestin protein. Nestin has been suggested to take part in stabilizing cell structure and coordinating changes in intracellular dynamics, which may be needed by dividing and migrating cells. Nestin protein did not uniformly co-expressed with cyclin D1, a known proliferation marker in Mel-mix cultured melanocytes, indicating that nestin is not essential in the proliferation of dedifferentiated melanocytes. We have preliminary results showing that another neural precursor marker, the translocator protein (TSPO) is also expressed in both types of cultured melanocytes at the mRNA level.

EGF is a common mitogen for keratinocytes and it is present in the *in vivo* environment of melanocytes. To find its receptor, EGFR on normal human melanocytes and examine its mitogenic potency on them was important to many researchers concerned with melanocyte biology. EGF is expressed by melanoma cells and can stimulate their proliferation, high EGF production, linked to functional polymorphism in the EGF gene, might be important in the development of melanoma. Data on the expression of EGFR in normal human melanocytes were contradictory. Reviews on melanocyte biology categorically stated that normal melanocytes did not express EGFR; this statement was the title of a letter published in 2004 in the Journal of Investigative Dermatology. In contrast Gordon-Thomson and colleagues (2001), and later Mirmohammadsadegh et al (2005) have clearly demonstrated the presence of EGFR in normal cultured melanocytes. Evidence for receptor activation and signaling was also presented in these papers. It is well known that *in vitro* culturing alters cell characteristics, also, to a large degree cultured cell characteristics depend on the culture environment. In previous works, similar to our culture system for adult melanocytes, neonatal melanocytes were not exposed to chemical mitogens when EGFR expression and signaling could be detected. The conflicting results in the literature on the expression of EGFR in normal human melanocytes may have stemed from the use of chemical mitogens in the culture environment, which induce melanocyte differentiation. On normal adult melanocytes cultured in our chemical mitogen-free medium we found that EGFR was expressed both at the mRNA and at the protein levels. Moreover, we demonstrated that treatment of melanocytes with 2.5-5-10 ng/ml EGF significantly increased their proliferation; this result was in contrast with results of previous studies. The regulation of EGFR expression is complex, involving multiple stimulatory as well as inhibitory transcription factors. In a human carcinoma cell line

it was shown that EGF was able to enhance mRNA expression of its receptor, EGFR. In our cultured melanocytes, EGF at mitogenic concentrations (2.5-5-10 ng/ml) did not show a significant effect on the EGFR mRNA expression. It is also known that EGF and its receptor, EGFR have an important role in neuronal differentiation. In our experimental setup, EGFR expression was constitutive in dedifferentiated melanocytes.

Melanocyte differentiation is usually studied on quail embryo skin and on mouse neural crest cell lines. Until now, there has been no suitable human model system for studying melanocyte differentiation. Our human melanocyte culture can serve as a model system to study melanocyte proliferation/differentiation, and melanoma development. Besides that, melanocytes cultured in chemical mitogen-free medium are applicable in the therapy of pigmentation-associated disorders, like vitiligo. Cells expanded *in vitro* in Mel-mix medium supplemented with autologous human serum instead of FBS and BPE allow for autologous transplantation of cultured melanocytes in vitiligo patients in early passages when their pigmentation is not lost. Further work is needed to examine the ability of neuronal transdifferentiaton of these *in vitro* cultured melanocytes. If these cells were able to transdifferentiate into neuronal precursors, they could also be considered as potential therapeutic tools for different neurodegenerative diseases.

6. SUMMARY

For obtaining pure melanocyte cultures from human adult epidermis a novel culture technique using a cholera toxin and PMA-free medium (Mel-mix) was described by our group. In Mel-mix medium the cultured melanocytes become bipolar, unpigmented and highly proliferative. Further characterization of the cultured melanocytes revealed the disappearance of c-Kit and TRP-1 and induction of nestin expression, indicating that melanocytes dedifferentiated in this *in vitro* culture. Cholera toxin and PMA were able to induce c-Kit and TRP-1 protein expressions in the cells, reversing dedifferentiation. TRP-1 mRNA expression was induced in dedifferentiated melanocytes by UV-B irradiated keratinocyte supernatants, however direct UV-B irradiation of the cells resulted in further decrease of TRP-1 mRNA expression. Expressions of EGFR mRNA and protein and mitogenic effect of EGF on Mel-mix cultured melanocytes were proved. EGFR protein expression was more intensive in dedifferentiated melanocytes. These dedifferentiated, easily accessible cultured melanocytes provide a good model for studying melanocyte differentiation and possibly transdifferentiation. Because melanocytes in Mel-mix medium can be cultured with human serum as the only supplement, this culture system is also suitable for autologous cell transplantation.

OUR NOVEL FINDINGS:

1. We proved that pigment-loss in melanocytes cultured in Mel-mix medium that does not contain non-physiological mitogens, parallels the decrease and loss of tyrosinase, TRP-1 and c-Kit expressions, and dendrite-reduction in the cells. These changes are indicative of a dedifferentiation process in the cultured melanocytes.
2. We showed that melanocytes derived from adult epidermis express the known neuronal stem cell marker, nestin both at the mRNA and protein levels, and we also showed that nestin expression was higher in the dedifferentiated melanocytes compared to the differentiated ones.
3. We provided evidence that the *in vitro* dedifferentiated melanocytes could redifferentiate if treated with CT+PMA or with UVB-irradiated keratinocyte supernatants.
4. We showed that cultured normal human adult epidermal melanocytes express EGFR both at the mRNA and protein levels, and we also showed that treatment of melanocytes with EGF increases cell proliferation, but it does not influence the mRNA expression of EGFR.
5. We observed that EGFR protein expression is stronger in dedifferentiated melanocytes cultured in PMA-free Mel-mix medium compared to differentiated cells cultured in PMA-containing medium.
6. We described a new *in vitro* human melanocyte model system in which melanocyte proliferation, differentiation and neuronal transdifferentiation can be studied.

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