INVESTIGATIONS ON NORMAL HUMAN ADULT EPIDERMAL MELANOCYTES

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Publications directly related to the subject of the dissertation

I. I.Szabad G[*], **Kormos B[*]**, Pivarcsi A, Széll M, Kis K, Kenderessy Szabó A, Dobozy A, Kemény L, Bata-Csörgő Zs: Human adult epidermal melanocytes cultured without chemical mitogens express the EGF receptor and respond to EGF. Arch Dermatol Res 299: 191-200, 2007 **IF: 1.596**

*: these two authors contributed equally to this work

II. **Kormos B**, Belső N, Bebes A, Szabad G, Bacsa S, Széll M, Kemény L and Bata-Csörgő Zs: *In vitro* dedifferentiation of melanocytes from adult epidermis. PLoS ONE 6(2): e17197. doi:10.1371/journal.pone.0017197 **IF: 4.351**

Other publications

I. Belső N, Széll M, Pivarcsi A, Kis K, **Kormos B**, Kenderessy Sz A, Dobozy A, Kemény L, Bata-Csörgő Zs: Differential expression of D-type cyclins in HaCaT keratinocytes and psoriasis. J Invest Dermatol 128:634-42, 2008 **IF: 4.829**

II. Belső N, Széll M, Pivarcsi A, Kis K, **Kormos B**, Kenderessy Szabó A, Dobozy A, Kemény L, Bata-Csörgő Zs: A D3 vitamin antipszoriatikus támadáspontjai lehetnek a pikkelysömörben kórosan kifejeződő D típusú ciklinek/D type cyclins have abnormal expression in psoriasis and may be possible targets of the antipsoriatic effect of 1,25-Dihydroxyvitamin D3. Bőrgyógy Vener Szle 84: 3-9, 2008

III. Megyeri K, Orosz L, **Kormos B**, Pásztor K, Seprényi G, Ocsovszki I, Mándi Y, Bata-Csörgo Z, Kemény L: The herpes simplex virus-induced demise of keratinocytes is associated with a dysregulated pattern of p63 expression. Microbes Infect. 2009 Jul-Aug;11(8-9):785-94. **IF: 2.801**

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V. Ungvári K, Pelsőczi KI, **Kormos B**, Oszkó A, Rakonczay Z, Kemény L, Radnai M, Nagy K, Fazekas A, Turzó K: Effects on Titanium Implant Surfaces of Chemical Agents Used for the Treatment of Peri-implantitis. J Biomed Mater Res B Appl Biomater. 2010 Jul; 94(1):222-9. **IF: 2.185**

VI. Kiss M, Dallos A, **Kormos B**, Sántha P, Dobozy A, Husz H, Kemény L: Sortilin is expressed in cultured human keratinocytes and is regulated by cutaneous neuropeptides. J Invest Dermatol. 2010 Nov;130(11):2553-60. **IF: 5.543**

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
BPE	bovine pituitary extract
BSA	bovine serum albumin
cAMP	cyclic adenosine 3', 5' monophosphate
CFSE	carboxyfluorescein diacetate, succinimidyl ester
CNS	central nervous system
CT	cholera toxin
DAPI	4',6-diamidino-2-phenylindole
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ET-1	endothelin-1
ET-3	endothelin-3
FBS	fetal bovine serum
IBMX	3-isobuthyl-1-methylxantine
L-DOPA	L-dihidroxy-phenylalanine
MAPK	mitogen activated protein kinase
MITF	microphthalmia-associated transcription factor
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
SCF	stem cell factor
SCFR	stem cell factor receptor
TBST	Tris buffered saline completed with 0.1% Triton-X
TPA	12-*O*-tetradecanoyl phorbol-13-acetate (TPA=PMA)
TRP-1	tyrosinase-related protein-1
TRP-2	tyrosinase-related protein-2
TSPO	translocator protein
TYR	tyrosinase
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
α-MSH	α-melanocyte stimulating hormone

1. INTRODUCTION

Melanocytes are cells specialized for the production of melanin. Melanocytes are mainly located in the epidermal basal layer and they also exist in the hair matrix, eye, ear and brain (leptomeninges), where they have special functions (hearing, balance, vision, binding of reactive oxygen species etc.) beside melanogenesis. 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" [1]. Synthesis of melanin in melanocytes takes place within highly specialized membrane-bound intracellular organelles called melanosomes. Melanosomes develop through a series of morphologically defined stages from an unpigmented (stage I) to a striated organell enriched in melanin (stage IV). Stage IV melanosomes travel to the dendritic tips in a centrifugal manner and translocate to adjacent keratinocytes where the melanin forms a photoprotective cap over the keratinocyte nuclei [2]. This cap saves the keratinocytes from the UV-induced DNA damage [3].

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 [4]. Additionally, TRP-1 stabilizes the enzymatic activity of tyrosinase [5] and maintains the melanosome structure integrity [6], while TRP-2 has a role in melanocyte survival [7].

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. Unfortunately, the prevalence of melanoma is increasing, especially in young people [8]. Its exact pathomechanism is not known, but some authors stated that melanoma cells arise from undifferentiated melanocytes [9,10]. Another common disorder related to melanocytes is vitiligo, which is characterized by white patches on the skin, because melanocytes are missing. The pathomechanism of vitiligo is also not clear, it has a genetic background, it is considered an autoimmune disease partly because it often associates with other autoimmune diseases, and it can be triggered by environmental effects [11]. One possibility to treat vitiligo

symptoms is autologous melanocyte transplantation. For transplantation, melanocytes should be separated from the patient first, then cultured *in vitro* and finally put back onto the skin. To investigate melanocyte differentiation and melanoma development and for treatment of vitiligo, establishment of appropriate melanocyte culture techniques is important.

1.1 Melanocyte culture techniques

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 [12]. 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 [13]. Because these mitogens alter the physiological responses of the cells, attempts have been made to define culturing techniques free of these substances [14-16]. 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 [17]. In another study melanocyte cultures were established without non-physiological mitogens by co-culturing human epidermal keratinocytes with human epidermal melanocytes [18]. 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 [19]. 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 [20].

1.2 Role of c-Kit and tyrosinase-related proteins in regulation of melanocyte differentiation and pigmentation

In vertebrates, melanocytes arise from the neural crest [21]. Melanoblasts are unpigmented cells containing only immature melanosomes that lack functional tyrosinase, the critical enzyme of melanin synthesis [22]. Fully differentiated melanocytes characterized by

TYR, TRP-1 and TRP-2 activities as well as by numerous mature melanosomes and well-developed dendrites, are located mainly in the epidermis, dermis and hair bulb [23]. Examination of melanoblasts is important to analyze basic mechanisms of cell differentiation, and to study the pathomechanisms of melanoma and genetic disorders of melanocyte development [24].

Melanocyte differentiation is under the control of microphthalmia transcription factor (MITF), a basic helix-loop-helix leucine zipper transcription factor, that activates genes involved in pigment production, such as TYR, TRP-1 and TRP-2 [25] and melanocyte survival, e.g. Bcl-2 [25,26]. Ectopic expression of MITF in embryonic fibroblast induces growth inhibition and morphologic changes consistent with melanocyte differentiation [27]. The transcriptional activity of MITF is modified by mitogen-activated protein kinase (MAPK) phosphorylation [28].

The proto-oncogene c-Kit encodes a membrane receptor protein (Kit/SCFR) with intrinsic tyrosine kinase activity. Stimulation of the c-Kit with its ligand, stem cell factor (SCF) induce dimerization and activation of its intrinsic tyrosine kinase activity, leading to autophosphorylation and activation of the Ras/Raf/MEK/ERK signaling pathway which regulate melanocyte proliferation, differentiation and migration [29] by stimulation of MITF (Fig. 1). In mouse epidermis, Kit^{+} cells differentiate into $Mitf^{+}$ and/or TRP-2^{+} cells first and then into TRP-1^{+} cells after UV exposure [30]. 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-2^{+}TRP-1^{-} cells represent a reserve population of precursor melanocytes [31]. In mouse, towards the completion of hair follicle morphogenesis, several distinct follicular melanocytic cell populations were defined: undifferentiated, non-pigmented c-Kit-negative melanoblasts in the outer root sheath and bulge and highly differentiated melanocytes adjacent to the hair follicle dermal papilla above the Auber's line [32]. Interestingly, autocrine SCF stimulation of Kit receptor seems to be an important step in melanoma genesis in its early phases, but it is down-regulated in later stages [33,34]. Similarly, it was also shown that c-Kit expression was downregulated in melanocytes following malignant transformation and SCF inhibited the growth of melanoma cell lines, indicating that c-Kit plays a more important role in melanogenic differentiaton than in cell proliferation [35,36].

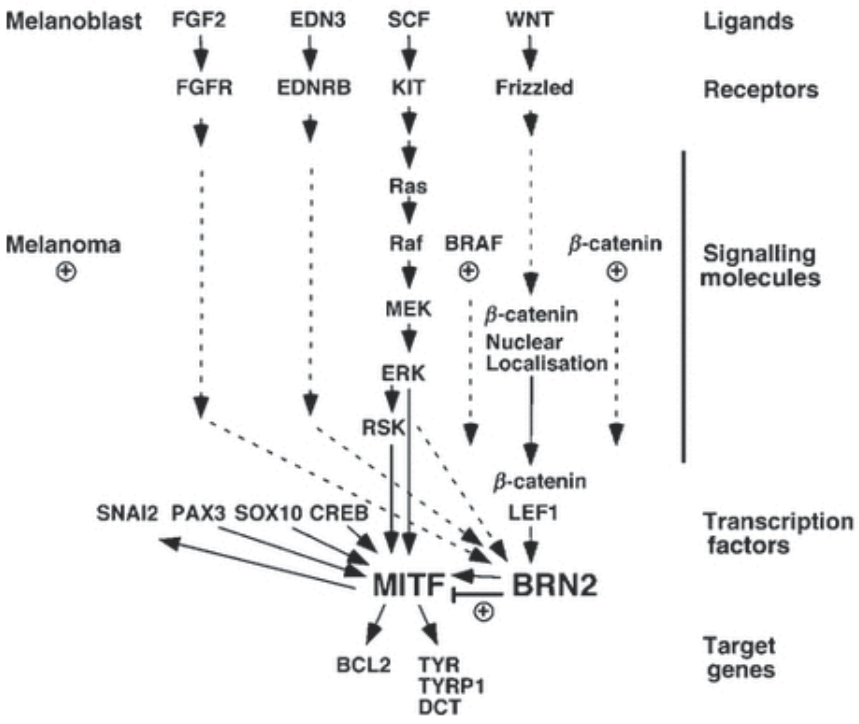

Figure 1. Schematic representation of MITF regulation.

1.3 Epidermal growth factor (EGF) and its receptor, epidermal growth factor receptor (EGFR)

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. The EGFR regulates the intracellular effects of ligands such as EGF and TGFα [37]. EGFR (ErbB1, HER1) is the first discovered member of the EGFR family, which has three other members: ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4) [38]. EGFR has an extracellular portion that contains four domains (two ligand-binding (L1 and L2) and two cysteine-rich domains (CR1 and CR2)), a transmembrane domain, a juxtamembrane domain (JM), a kinase domain and a carboxy-terminal domain (CT) (Fig. 2).

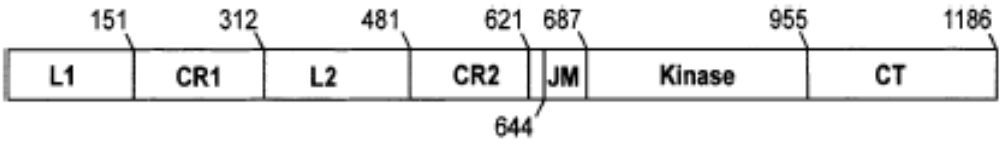

Figure 2. Schematic representation of domains of the EGFR sequence [37].

Upon ligand binding to the EGFR extracellular domains, the receptors dimerize and the enzymatic activity of its intracellular tyrosin kinase domain increases and several different signal transduction pathways can be engaged. The best studied pathways include the Ras/Raf/MEK/ERK and PI3K/PDK1/Akt pathways (Fig. 3). In the modulation of balance between cell proliferation, apoptosis and senescence, the EGFR/PI3K/PDK1/Akt pathway is crucial in promoting sustained proliferation mediated by the EGFR/Ras/Raf/MEK/MAPK pathway [39].

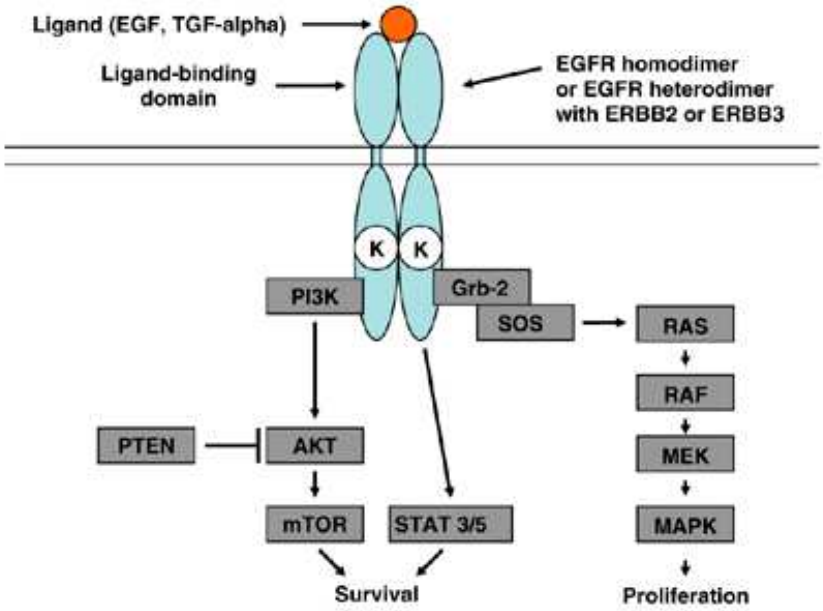

Figure 3. Schematic representation of EGFR signaling [40].

ErbB signaling pathways are found in malignant cells. The loss of growth regulation, by constitutively activated receptors, receptor gene amplification or overexpression of receptors and its ligands, is considered to play a significant role in neoplastic transformation, invasion and metastasis [39]. In melanoma, EGFR amplification may be a supporter of the metastatic potential [41]. In another study, chromosome 7 and EGFR gene copy number changes were detected simultaneously in a large number of primary melanomas and the resulting data was correlated to the clinical outcome of patients [42].

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* [43,44]. 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 [45,46]. 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 [47] a controversy arose around this issue. Later another group also published data supporting the presence of EGFR on normal human melanocytes [48].

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 [20] 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. To prove that the use of dispase prevents fibroblast contamination, we isolated RNA both from the epidermis and the dermis after dispase treatment and performed real-time RT-PCR using primers specific for COL1A2, the gene that encodes for the alpha 2 chain of type I collagen. We could not detect COL1A2 gene

expression in the epidermal samples (Fig. 4). Thus, the use of dispase prevents fibroblast contamination in the epidermal cell cultures.

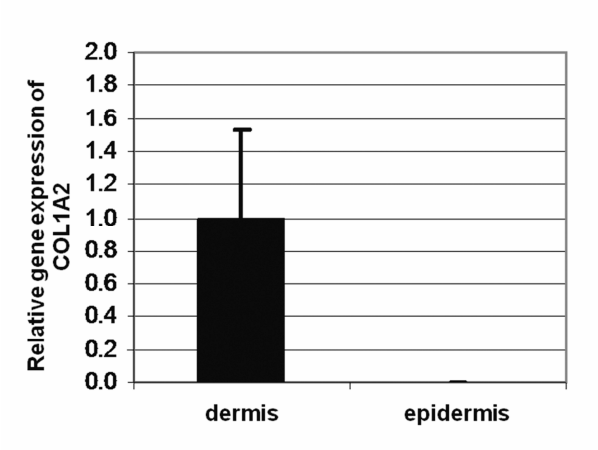

Figure 4. COL1A2 is undetectable in epidermal samples after dispase digestion. Values are shown as relative expressions compared to one of the dermal samples (mean ± SEM). Averages were calculated from three different donors.

Next day the epidermis was peeled of the dermis. The epidermis was put into 0.25% trypsin (Sigma) for 30 minutes at 37 °C. Following 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 centrifuged at 200 *g* for 10 minutes at 4 °C. Epidermal cells were then placed into 75 cm^2 tissue culture dishes (BD Falcon) at a cell density of $2x10^5$ cells/cm^2. In Mel-mix medium at the beginning of the culture melanocytes usually attach in 24 hours, while keratinocytes can attach later between 24-48 hours after plating. We therefore wash the plates between 12-24 hours after plating to remove keratinocytes and supply fresh Mel-mix medium to the cells. If this was not enough to prevent our culture from keratinocyte contamination, at the first passage keratinocytes are completely removed from the cultures by short trypsinization with 0.01% Trypsin/EDTA, Sigma. Due to the different attachment characteristics of keratinocytes and melanocytes to the culture plastic, melanocytes release 2-3 minutes earlier than keratinocytes, thus enabling separation of the two cell populations. Fresh culture media was put on cells three times weekly. Primary melanocyte cultures reached ~90% confluency in 7–9 days. Confluent primary cultures were treated with 0.05% EDTA in PBS and cells were harvested by a short, 2-3 minutes trypsinization. Harvested cells were divided into two equal parts at passages. Cultures were grown at 37 °C in humidified atmosphere containing 5% CO_2 [20].

Keratinocytes prepared from similar epidermal samples were cultured in KSF medium (Life Technologies) as described previously [49].

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

Mel-mix cultured melanocytes in 7^{th} passage were switched into M254 medium or treated with 10 nM cholera toxin, 10 ng/ml PMA and with both 10 nM cholera toxin and 10 ng/ml PMA for one week. Cells were then trypsinized, centrifuged at 200g and supernatants were discarded. Cell numbers were equalized and 5M NaOH was added to the pellets. Cells were then sonicated using ultrasound sonicator for 10 minutes. The solutions were transported into 96 well U-bottom plates and 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

The growth of cultured melanocytes was measured using a rapid colorimetric assay, which determines cell numbers [50] by the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in living cells. 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) in RPMI without phenol-red. After incubation for 3 hours at 37^oC, the medium was gently removed from each well and the chrystallized dye was solubilized with 2% SDS and 0.04 mM HCl in absolute isopropanol. The absorbance of the color reaction was determined by a Multiscan Ex spectrophotometer (Thermo Labsystems, Vantaa, Finland) and Ascent Software (Thermo) at 540 nm.

3.6 RT-PCR

Total RNA was isolated from $1x10^6$ melanocytes by TRIZOL™ reagent (Life Technologies) following the manufacturer's instructions. First strand cDNA was synthesized from total RNA in 20 μl final volume using the First Strand cDNA Synthesis Kit of MBI Fermentas (Vilnius, Lithuania). After reverse transcription, amplification was carried out by PCR using Taq DNA Polymerase and the dNTP set of MBI Fermentas (Vilnius, Lithuania). One quarter of the reverse transcription volume was used as a template. Primers specific for tyrosinase (5'–TTGGCAGATTGTCTGTAGCC–3' and 5'– AGGCATTGTGCATGCTGCTT –3') and for human β-actin (5'–AGAGATGGCCATGGCTGCTT–3' and 5'– ATTTGCGGTGGACGATGGAG–3') were included in the reactions at 0.66 pmol/μl final concentrations. The same PCR conditions were used for both PCR primer pairs: 94 °C 90 sec., 55 °C 90 sec. and 72 °C 120 sec. The numbers of cycles were: 30 cycles for tyrosinase and 25 cycles for β-actin. The concentration of $MgCl_2$ was 1.5 mM in the PCR reactions. 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

Total RNA was isolated from $1x10^6$ cultured melanocytes by TRIZOL™ reagent (Life Technologies) following the instructions of the manufacturer. RNA concentrations were determined by the A_{260} values of the samples. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). RT-PCR was used to quantify the relative abundance of each mRNA (iCycler IQ Real Time PCR, BioRad, Hercules, CA, USA). After reverse transcription, amplification was carried out using FastStart TaqMan Probe Master from Roche (Basel, Switzerland). Primers were as follows: TRP-1 forward: CTTTTCTCACATGGCACAGG, TRP-1 reverse: AAGGCTCTTGCAACATTTCC, probe: #10 from the Roche Universal ProbeLibrary, (cat. no. 04685091001); c-Kit forward: CGTGGAAAAGAGAAAACAGTCA, c-Kit reverse: CACCGTGATGCCAGCTATTA, c-Kit probe: #2 from the Roche Universal ProbeLibrary, (cat. no. 04684982001); nestin forward: TGCGGGCTACTGAAAAGTTC, nestin reverse: TGTAGGCCCTGTTTCTCCTG, nestin probe: #76 (cat. no. 04688996001); 18S RNA forward: CTCAACACGGGAAACCTCAC, 18S RNA reverse: CGCTCCACCAACTAAGAACG, 18S probe: #77 from the Roche Universal ProbeLibrary,

(cat. no. 04689003001). The amplification protocol for TRP-1 and c-Kit expression contained one cycle of initial denaturation at 95 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 15 sec and annealing/extension at 57 °C for 1 min. The same protocol with 25 cycles was applied for the detection of 18S mRNA expression. Primers and probe specific for EGFR (Cat. Number: HS00193306_m1) were purchased from Life Technologies (Applied Biosystems). The amplification protocol contained one cycle of initial denaturation at 95 °C for 6 min followed by 40 times of cycles of denaturation at 95 °C for 15 sec, annealing/extension at 60 °C for 1 min, and one cycle of terminal extension at 72 °C for 10 min.

3.8 Immunocytochemistry

For cytospin preparation cells were harvested by trypsinization. After washing, the cell pellet was resuspended in PBS and the cell density was set at $1x10^6$ cells /ml. 100 µl cell suspension was put into plastic tubes and centrifuged (Cytopro, Wescor, Logan, Utah, USA) onto glass slides, then dried overnight at 25 °C. Air-dried slides were fixed in 2% paraformaldehyde (Sigma) for 20 minutes. Slides were incubated with primary antibodies specific for TRP-1 at a dilution of 1:2000 (Signet Laboratories, Dedham, MA, USA) for c-Kit (BD) at a dilution of 1:250 and for EGFR (Clone: sc-120, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at a dilution of 1:200 in a staining solution containing TBST (Tris buffered saline containing 0.1% Triton-X) (Sigma) and 0.5% BSA (Sigma) for 1 hour. Control slides were incubated with mouse IgG_{2a} (Sigma), the isotype of all primary antibodies. Slides were then incubated with a biotinylated secondary antibody (Vectastain ABC Kit, Vector, Burlingame, USA) for 1 hour at room temperature, followed by the blocking of endogen peroxidase with 1% H_2O_2 (Sigma) in methanol (Spektrum 3D, Debrecen, Hungary) for 20 minutes. After that the slides were incubated with horse radish peroxidase-conjugated streptavidin for 1 hour at room temperature (Vectastain ABC Kit, Vector). The peroxidase activity was visualized using 3-amino 9-ethylcarbazole (AEC, Sigma) as a substrate. Finally, the slides were counterstained with Mayer's hematoxylin (Sigma). The cells were analyzed using a Zeiss Axio Imager microscope and photographed using a PixeLINK digital camera (TissueGnostics, Austria). For quantification of the positive cells, the Cell Counter option of ImageJ software (freely available program from NIH) was used.

3.9 Fluorescent immunocytochemistry

Melanocytes were cultured onto glass coverslips and stained at 3rd passage culture. Cells were washed with PBS then fixed with 2% paraformaldehyde (Sigma) for 20 minutes. After blocking with 0.5%BSA and 1% goat serum in TBST, cells 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 in a staining solution containing TBST overnight at 4°C. Control slides were incubated with mouse IgG_1 (Sigma). After washing in TBST cells were incubated with AlexaFluor 546 conjugated anti-mouse IgG and with AlexaFluor 488 conjugated anti-rabbit IgG (both from Life Technologies) at a dilution of 1:500 in TBST for 3 hours at room temperature. After incubation, cells were washed in TBST and counterstained with DAPI (Sigma) at a dilution of 1:100 for 20 minutes. Cells were washed again in TBST then were mounted with Fluoromount-G mounting medium (SouthernBiotech, Birmingham, AL, USA). 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 Tris-buffered saline (TBS, 150 mM NaCl, 25 mM Tris, pH 7.4) containing 3% non-fat dry milk powder (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°C. 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 in PBS were irradiated with 0, 20.8, 31.2 and 41.6 mJ/cm^2 doses of UVB. After irradiation, prewarmed medium (37 °C) was put on the cells. To examine the indirect effects of UVB, cultured normal human adult epidermal keratinocytes in PBS were irradiated with the same doses of UVB and supernatants of the irradiated keratinocytes were collected 6 and 24 hours after irradiation. These supernatants were mixed with AIM-V medium (v:v) supplemented with 5% FBS (Hyclone, Logan, UT, USA), 1% L-glutamine and 1% Antibiotic Antimycotic Solution and autologous cultured melanocytes in 3rd passage were cultured in them for 24 hours, then total RNA was isolated from the cells. Cell viability was measured during the experiment, these data are included in the Results section.

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. Cells were trypsinized, washed and resuspended in PBS. Stock CFSE was added to the cell suspension at 0.5 µM final concentration. The suspension was incubated for 15 minutes at 37 ºC. After incubation, cells were centrifuged and resuspended in fresh prewarmed medium (37 ºC), then plated and cultured for 96 hours. For control culture (0-hour sample) cells were incubated at 37 ºC in suspension with fresh medium for 30 minutes. CFSE-labeled cells were trypsinized and measured by flow cytometry in every 24 hours between 0 to 96 hours. After a washing procedure, CFSE fluorescence in the FL-1 channel was measured using a dual-laser 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. Briefly, cells were fixed in 2% paraformaldehyde for 15 minutes at room temperature, washed, then incubated with X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) at 1 mg/ml concentration in a special staining solution overnight at 37ºC.

Next day, the cells were mounted with 70% glycerol and were visualized using a Nikon Eclipse microscope and photographed by a Nikon camera. For quantification of the positive cells, the Cell Counter option of ImageJ software (freely available program from NIH) was used.

3.14 Data presentation and statistical analysis

For the evaluation of the MTT assay, the average absorbance of the control wells was regarded as 100% and the percentage of cell growth in each well was calculated (% of control). PCR results were expressed as fold increases over the control values. Data from both MTT assay and real-time PCR analysis were presented as mean ± standard deviation (SD) or standard error of mean (SEM). Data were compared using the one-way analysis of variance (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

4.1.1 The dendritic morphology of melanocytes changes and cells become bipolar during long-term in vitro culturing in Mel-mix medium

Normal human adult epidermal melanocytes cultured in Mel-mix medium show "typical", multi-dendritic morphology in early cultures (Fig. 5A), but during culturing their morphology changes, cells become bipolar (Fig. 5B). This change in cell morphology is indicative for dedifferentiation of the cells.

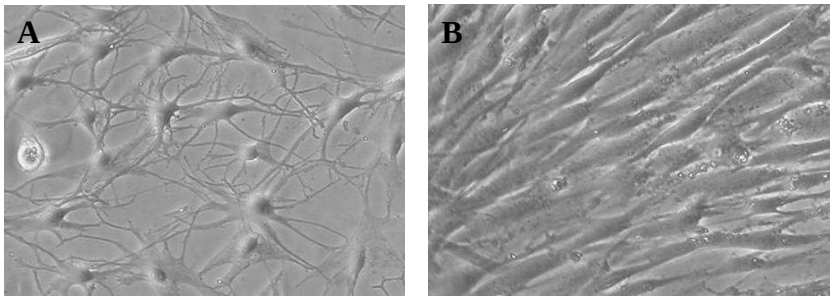

Figure 5. Morphology of Mel-mix cultured melanocytes change during in vitro culture. Melanocytes in 2-week old cultures show multidendritic morphology (A), while in 6-week old cultures they have bipolar shape (B).

4.1.2 Pigmentation of melanocytes decreases in Mel-mix culture

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 (Fig. 6A, L-DOPA stained cultured cell in 2nd passage), while further cultured cells did not (Fig. 6B, L-DOPA stained cell in 8th passage).

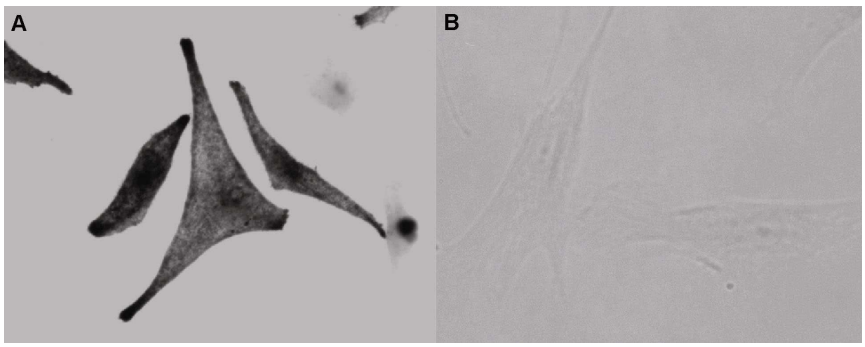

Figure 6. Pigmentation of melanocytes decreases in Mel-mix culture. Cultured cells in 2nd passage (A) show pigmentation, but in 8th passage (B) they do not as determined by L-DOPA staining.

4.1.3 In vitro cultured normal human adult melanocytes express tyrosinase mRNA

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 (Fig. 7).

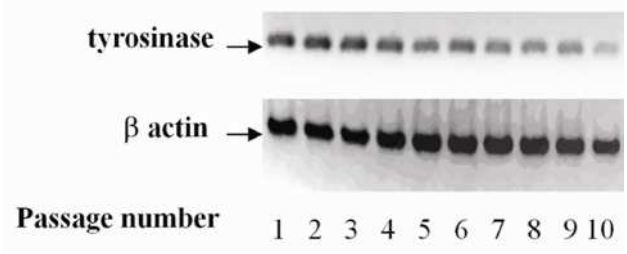

Figure 7. Tyrosinase mRNA is expressed in long term cultured melanocytes. Cells were grown in complete Mel-mix medium and passaged several times. Tyrosinase mRNA expression was determined by RT-PCR in every passage, α-actin was used as an internal control. Tyrosinase mRNA expression was detected in the cultured cells even in 10th passage, although compared to the actin there was a gradual decrease in the intensity of the tyrosinase mRNA bands, indicating a decreasing tendency for the amount of tyrosinase mRNA in older cultures.

4.1.4 TRP-1 and c-Kit expressions decrease in human adult melanocytes cultured in Mel-mix medium

The expression of TRP-1, an also indispensable enzyme of melanin synthesis and c-Kit, a melanocyte differentiation marker were also checked. Cytospins were prepared from melanocyte cultures at an early phase (first passage in culture, approximately 7-10-day-old culture), at 3rd passage in culture (3p, about three weeks after primary plating) and at late phase in the 7th passage cultures (generally five weeks into culture). TRP-1 protein expression was detected using a monoclonal antibody (Mel-5). TRP-1 exhibited strong expression in primary melanocyte cultures (100% of the cells were positive), and its expression decreased (33% in 3rd, 0% in 7th passage culture) during *in vitro* culturing in Mel-mix medium (Figure 8A). c-Kit protein expression was also strong in 30% of the cells in the early cultures (1st and 2nd passages), but melanocytes never stained as uniformly with the anti-c-Kit antibody as with the Mel-5 antibody (Figure 8B). In 3rd passage cultures only 4% of the cells were positive for c-Kit protein (Figure 8B) and all cells were TRP-1 and c-Kit negative in the five-week-old, 7th passage melanocyte cultures (Figure 8A and 8B). 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 (Figure 8C). This is in line with previous results, showing that TRP-1 is regulated at the transcriptional [51] level. The decrease in TRP-1 mRNA levels from the 4th passage to the 7th passage samples were statistically significant compared to the 1st passage samples. c-Kit mRNA values showed decreasing tendency, but changes in mRNA expressions were not statistically significant (Figure 8D). Values are shown as relative expressions compared to the first passage samples. Averages were calculated from three independent experiments.

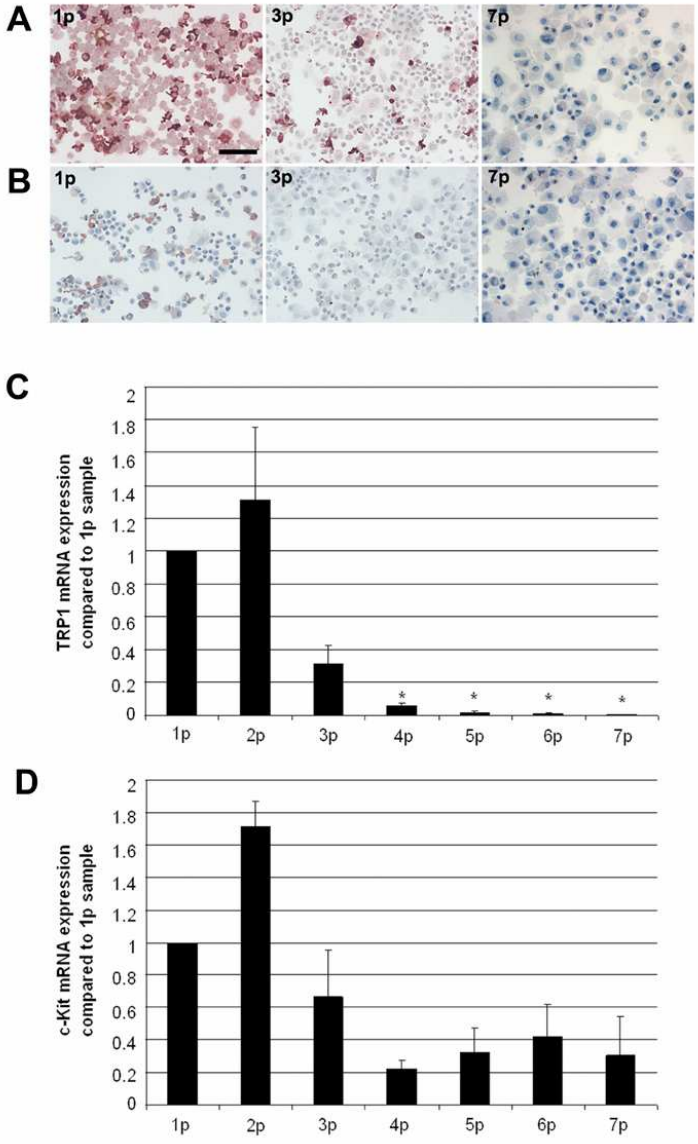

Figure 8. TRP-1 and c-Kit expressions decrease in human melanocytes cultured in Mel-mix medium. Both TRP-1 (A) and c-Kit (B) protein expressions were present in primary cultures, 100% of cells expressed TRP-1 and 30% expressed c-Kit (1p), the expression of both decreased during *in vitro* culturing. Melanocytes in 7th passage (7p) expressed neither TRP-1 nor c-Kit proteins. Bar: 100 µm. TRP-1 and c-Kit mRNA expressions were detected in cultured melanocytes at every passage by real-time RT-PCR analysis. TRP-1 (C) mRNA expression decreased significantly, while c-Kit (D) mRNA expressions showed a decreasing trend during *in vitro*

culturing. Values are shown as relative expressions compared to the first passage (1p) sample (mean±SD from three independent experiments; *significant at p≤0.05, Univariate ANOVA).

4.1.5 Nestin, a neuronal precursor marker, is expressed both at the mRNA and the protein level in Mel-mix cultured melanocytes indicating a dedifferentiation process in the cells

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 have found a correlation between nestin positivity and melanocyte proliferation status (Fig 9A, Isotype control: Fig 9B).

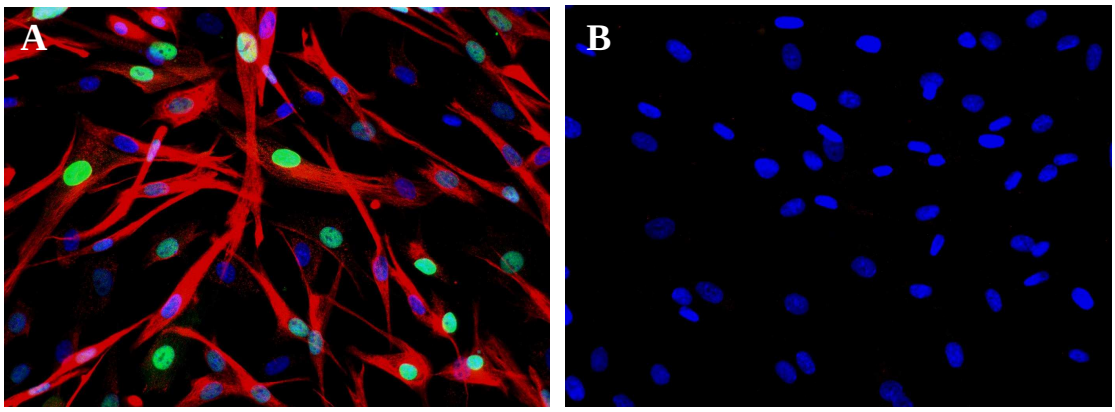

Figure 9. Melanocytes cultured in Mel-mix medium express nestin protein. Most melanocytes in the culture express nestin (red). There are melanocytes which co-express nestin and cyclin D1 (green), there are melanocytes which express either nestin or cyclin D1 and finally there are some cells which express none of these proteins (A). Cell nuclei were counterstained with DAPI (blue). Isotype control (B).

Nestin mRNA expressions were examined and compared in passages from 1-3-7 using real-time RT-PCR. The expression of nestin was significantly higher in 7p cultures compared to 1p or 3p cultures (Fig. 10). Values are shown as relative expressions compared to one of the first passage samples. Averages were calculated from three different donors.

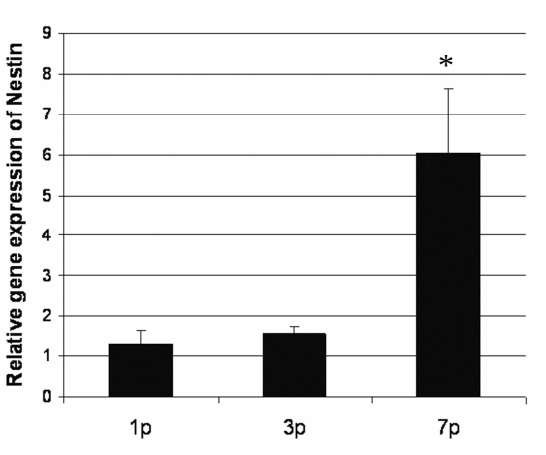

Figure 10. Melanocytes cultured in Mel-mix medium express more nestin mRNA in late passage cultures compared to early cultures. (mean±SEM from three independent donors; *significant at $p \leq 0.05$, Univariate ANOVA).

4.2 Investigation of differentiation potential on dedifferentiated melanocytes

4.2.1 Pigmented, mature melanocytes dedifferentiate in vitro when PMA containing medium is switched to PMA-free Mel-mix

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 (Figure 11A) and expressed TRP-1 (100% of the cells were positive, Figure 11C) and c-Kit (100% of the cells were positive, Figure 11E) proteins and showed a generally week EGFR staining (only 32% of the cells showed weak positivity, Figure 11G), while cells cultured in Mel-mix for two weeks became bipolar (Figure 11B), their TRP-1 (Figure 11D) and c-Kit (Figure 11F) protein expressions drastically decreased (TRP-1^+cells: 16%, c-Kit^+cells: 25%), and their EGFR expression strongly increased ($EGFR^+$ cell: 80%) (Figure 11H). These changes indicated that in Mel-mix, without PMA, melanocyte dedifferentiated *in vitro*.

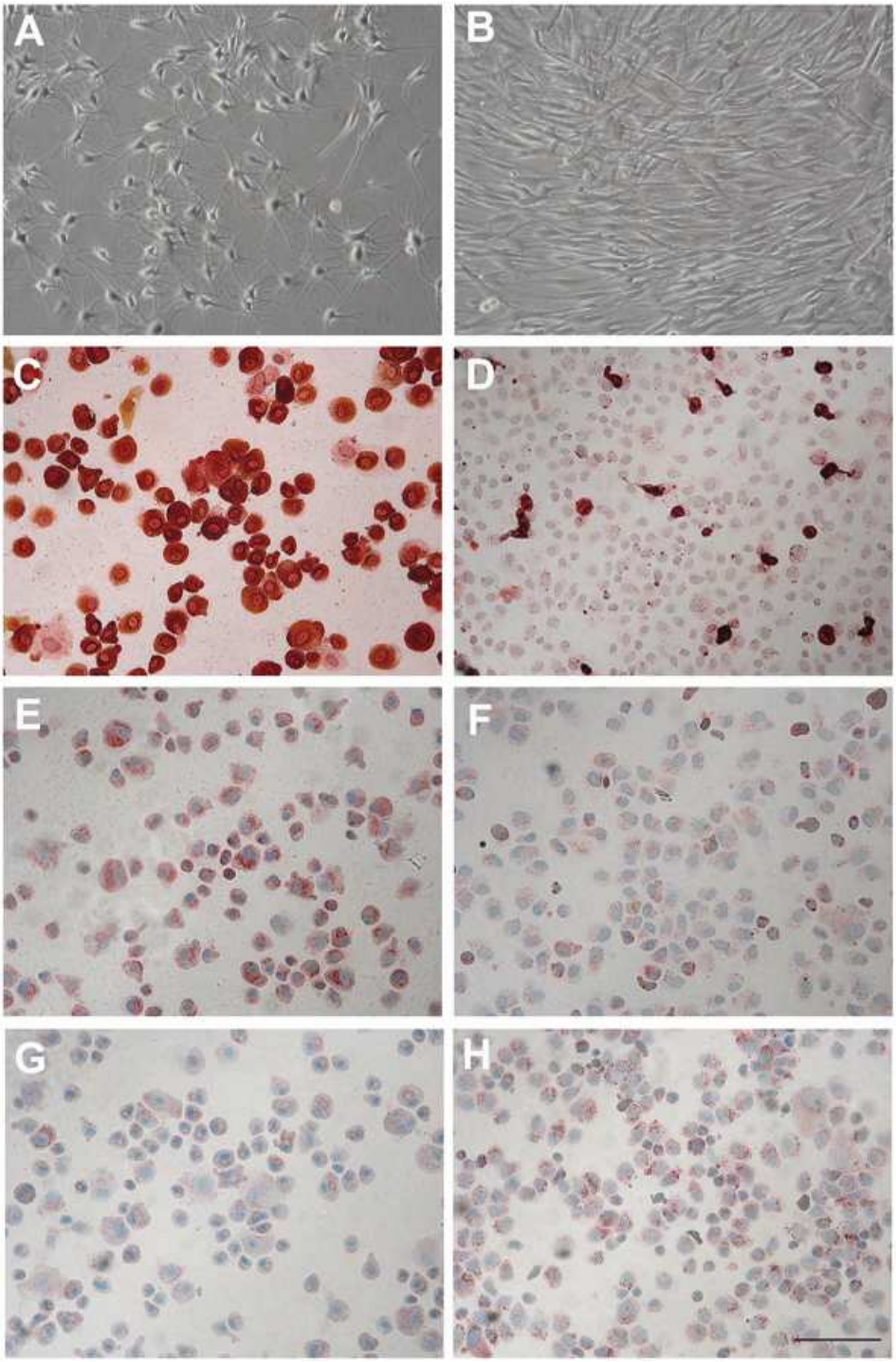

Figure 11. Switching the PMA-containing M254 medium to PMA-free Mel-mix medium results in cell morphology changes and loss of c-Kit and TRP-1 expressions.

When melanocyte cultures were initiated in PMA-containing M254 medium, cells were dendritic, and expressed TRP-1 as well as c-Kit proteins (100 % of the cells expressed TRP-1 and c-Kit). At week 4, when cells were in 4^{th} passage, half of the cultures were switched to PMA-free Mel-mix medium. Cells in Mel-mix medium became bipolar (B)**,** lost TRP-1 (D) and c-Kit (F), and increased EGFR (H) expressions in two weeks, while melanocytes continuously cultured in M254 medium remained dendritic (A) expressed both TRP-1 (C) and c-Kit (E) proteins, and showed only a week EGFR staining (G). Bar: 100 µm.

4.2.2 Cholera toxin and PMA induce dendrite formation, a significant c-Kit, a slight TRP-1 mRNA expressions and the reappearance of c-Kit and TRP-1 proteins in dedifferentiated melanocytes

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 (Figure 12A) for 1 week in this environment. CT+PMA treatment induced dendrite formation in bipolar, dedifferentiated melanocytes (Figure 12B). Immunocytochemical staining demonstrated that TRP-1 protein expression was induced in 7% of the cells (Figure 12D), while c-Kit protein expression was induced in 12% of the dedifferentiated melanocytes (Figure 12F) compared to untreated cultures (TRP-1^{+}cells: 0%, c-Kit^{+}cells: 0%) (Figure 12C and E).

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 (Figure 12G). 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 (Figure 12H). This difference was statistically significant. Values are shown as relative expressions compared to the first passage samples (mean±SD from three independent experiments; *significant at $p \leq 0.05$, Univariate ANOVA).

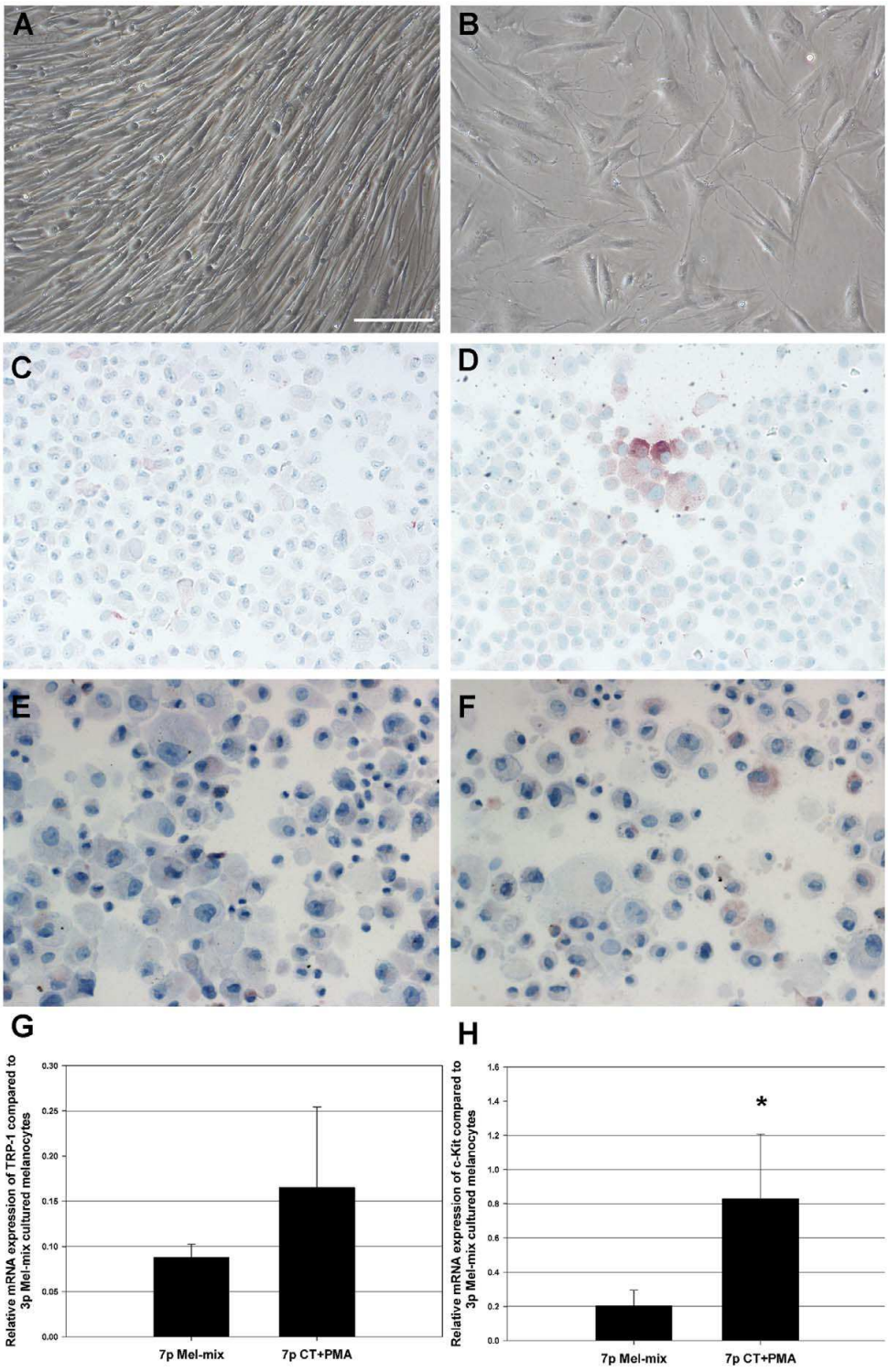

Figure 12. CT and PMA induce dendrite formation, TRP-1 protein and c-Kit mRNA and protein expressions in dedifferentiated melanocytes. Cultured, 7th passage melanocytes showed a uniform, bipolar morphology in Mel-mix medium (A), while treatment of melanocytes with CT and PMA induced dendrite formation (B). CT+PMA induced both TRP-1 (D) and c-Kit (F) protein expressions in melanocytes compared to untreated cultures (C and E) as demonstrated by immunocytochemistry. Bar: 100 µm. At the mRNA level, CT and PMA caused a 2-fold increase in TRP-1 (G) and 4-fold increase in c-Kit (H) expressions compared to untreated controls. Values are shown as relative expressions compared to 3rd passage Mel-mix cultured cells (mean±SD from three independent experiments; *significant at $p \leq 0.05$, Univariate ANOVA).

CT and PMA treatment also increased the melanin-content of the cells. Mel-mix cultured melanocytes in 7th passage were switched into M254 medium or treated with 10 nM cholera toxin, 10 ng/ml PMA and with both 10 nM cholera toxin and 10 ng/ml PMA for one week. An individual melanocyte in 7th passage culture growing in PMA-free Mel-mix medium contained 39 pg of melanin. Switching the PMA-free Mel-mix medium to PMA-containing M254 medium increased the melanin content in the cells to 58 pg melanin. PMA treatment of Mel-mix cultured melanocytes raised pigment content to 68 pg/cell. Cholera toxin caused only a slight increase in melanin-production, melanocytes in this culture contained 47 pg/cell melanin. Simultaneous addition of CT and PMA showed similar result than CT treatment alone (48 pg/cell) (Fig. 13). Results are from one experiment.

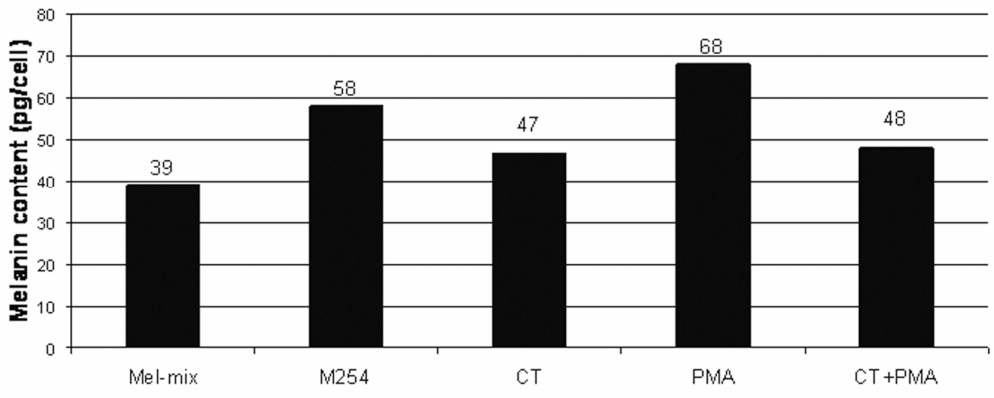

Figure 13. CT and PMA treatment increased the melanin-content of melanocytes.

4.2.3 Direct UVB effect decreases, while indirect, keratinocyte-mediated UVB effect increases the relative mRNA expression of TRP-1 in dedifferentiated melanocytes cultured in Mel-mix

To study the effect of UVB on melanocyte differentiation, we irradiated 3rd passage melanocytes cultured in Mel-mix medium with 20.8, 31.2 and 41.6 mJ/cm^2 doses of UVB. Cell viabilities 24 hours after irradiation were: 98±2% at 20.8 mJ/cm^2, 84±12% at 31.2 mJ/cm^2 and 77±14% at 41.6 mJ/cm^2 compared to the viability of non-irradiated control cultures. 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 (Figure 14A). As melanocyte differentiation is also influenced by its *in vivo* environment, we irradiated keratinocytes with the same UVB doses to detect the keratinocyte driven UVB effect on melanocytes. Keratinocyte supernatants were collected 6 and 24 hours after UVB irradiation and third passage cultured autologous melanocytes were treated with these supernatants for 24 hours. Keratinocytes were more

sensitive to UVB irradiation in comparison with melanocytes. 24 hours after UVB irradiation 61±11% of keratinocytes were alive at 20.8 mJ/cm^2 dose, 54±11% at 31.2 mJ/cm^2 and 34±13% at 41.6 mJ/cm^2 compared to non-irradiated control cells. Keratinocyte supernatants collected 6 hours after irradiation caused a slight increase in TRP-1 mRNA expression (data not shown), 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. The increase was almost three times higher with supernatants collected from 20.8 and 41.6 mJ/cm^2 UVB irradiated keratinocytes and more than five times higher with supernatants from 31.2 mJ/cm^2 UVB irradiated keratinocytes (Figure 14B). Neither indirect nor direct UVB irradiation influenced cell morphology (data not shown). Values are shown as relative expressions compared to non-irradiated samples (mean±SEM from two independent experiments).

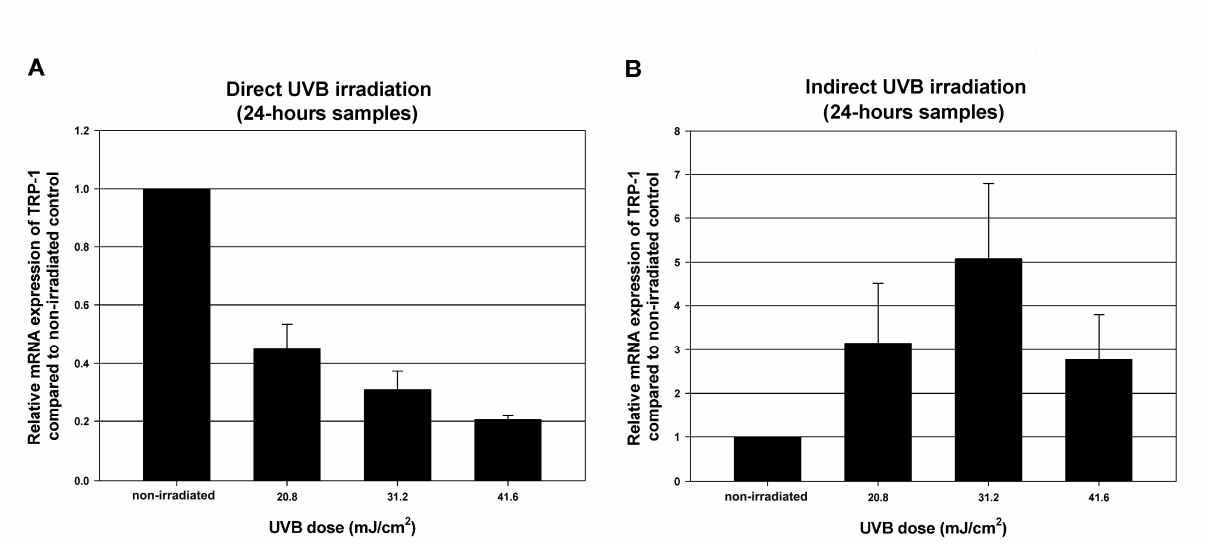

Figure 14. Direct UVB irradiation decreases, while indirect, keratinocyte-mediated UVB effect increases the relative mRNA expressions of TRP-1 in dedifferentiated melanocytes. Dedifferentiated melanocytes cultured in Mel-mix medium were irradiated with 20.8 31.2 and 41.6 mJ/cm^2 doses of UVB. Direct UVB irradiation decreased TRP-1 mRNA expression in melanocytes (A). Keratinocytes were also irradiated with the same UVB doses. Supernatants were collected from irradiated keratinocytes and autologous melanocytes were treated with them. The "indirect" UVB irradiation caused an increase in TRP-1 mRNA expression (B). Values are shown as relative expressions compared to non-irradiated samples (mean±SEM from two independent experiments).

4.2.4 Melanocytes cultured in Mel-mix medium proliferate more rapidly than cells in PMA containing M254 medium

To characterize the proliferative capacity of melanocytes cultured in Mel-mix medium, CFSE analysis was performed. Melanocyte culture was initiated from one donor in Mel-mix medium. At the 1st passage, cells were divided into two parts: one part was continuously cultured in Mel-mix, the other part was cultured in PMA containing M254 medium. Both cultures were in 3rd passage when the CFSE analysis was performed. After labeling, samples were collected in every 24 hours for 120 hours and the fluorescence intensity was measured. 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. Based on CFSE peak fluorescence intensity halving, the calculated number of cell divisions was only 4.5 for melanocytes cultured in M254 medium, while cells cultured in Mel-mix medium completed 8 dividing cycles in the 120-hour experimental period (Figure 15).

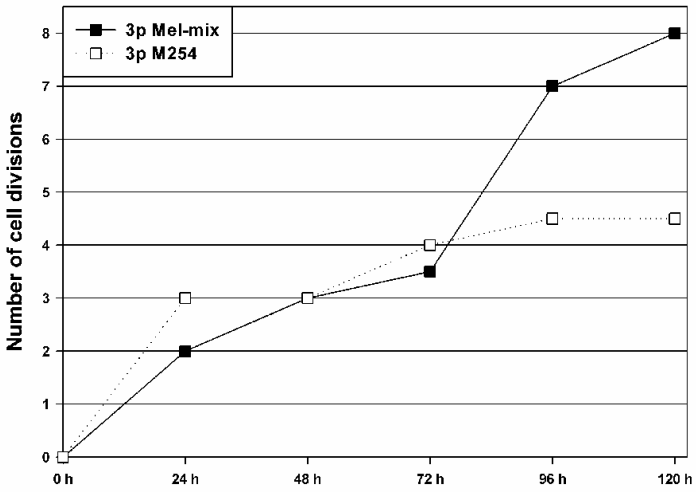

Figure 15. Melanocytes cultured in PMA-free Mel-mix medium proliferate more rapidly than melanocytes cultured in PMA-containing M254 medium. CFSE measurements were performed on melanocytes cultured in Mel-mix and M254 medium. Based on CFSE peak fluorescence intensity halving, cell division numbers for melanocytes cultured in Mel-mix medium (solid line) was 8, while for cells cultured in M254 medium (dashed line) was only 4.5 in the 120-hour experimental period.

4.2.5 Senescent cells are equally present in PMA-free and PMA-containing melanocyte cultures

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. In 3rd passage cultures 50 % of the cells were β-galactosidase+ in Mel-mix and 57% in M254, in 8th passage cultures 57% β-galactosidase+ cells were in Mel-mix and 48% in M254 (Figure 16). Blue cells appeared flat without dendrites in cultures, irrespective of the used media.

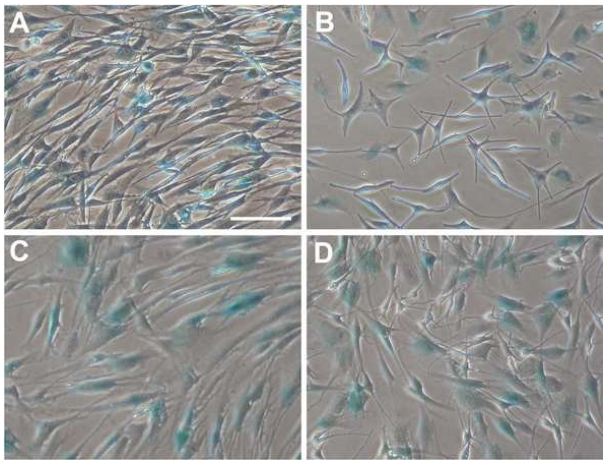

Figure 16. Melanocyte cultures contain about equal number of senescent cells regardless of culture conditions. β-galactosidase assay was performed to detect senescent cells (blue cells) in the cultures. 3rd passage melanocytes cultured in Mel-mix medium (A), 3rd passage melanocytes cultured in M254 medium (B), 8th passage melanocytes cultured in Mel-mix medium (C), 8th passage melanocytes cultured in M254 medium (D). Bar: 100 µm.

4.2.6 Nestin, a neuronal precursor is expressed by dedifferentiated melanocytes

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 (Figure 17A). 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 (Figure 17A). Values are shown as relative expressions compared to the 2nd passage sample. On Western blot, although a faint nestin specific band was visible in all samples (Figure 17B and C), 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 (Figure 17B).

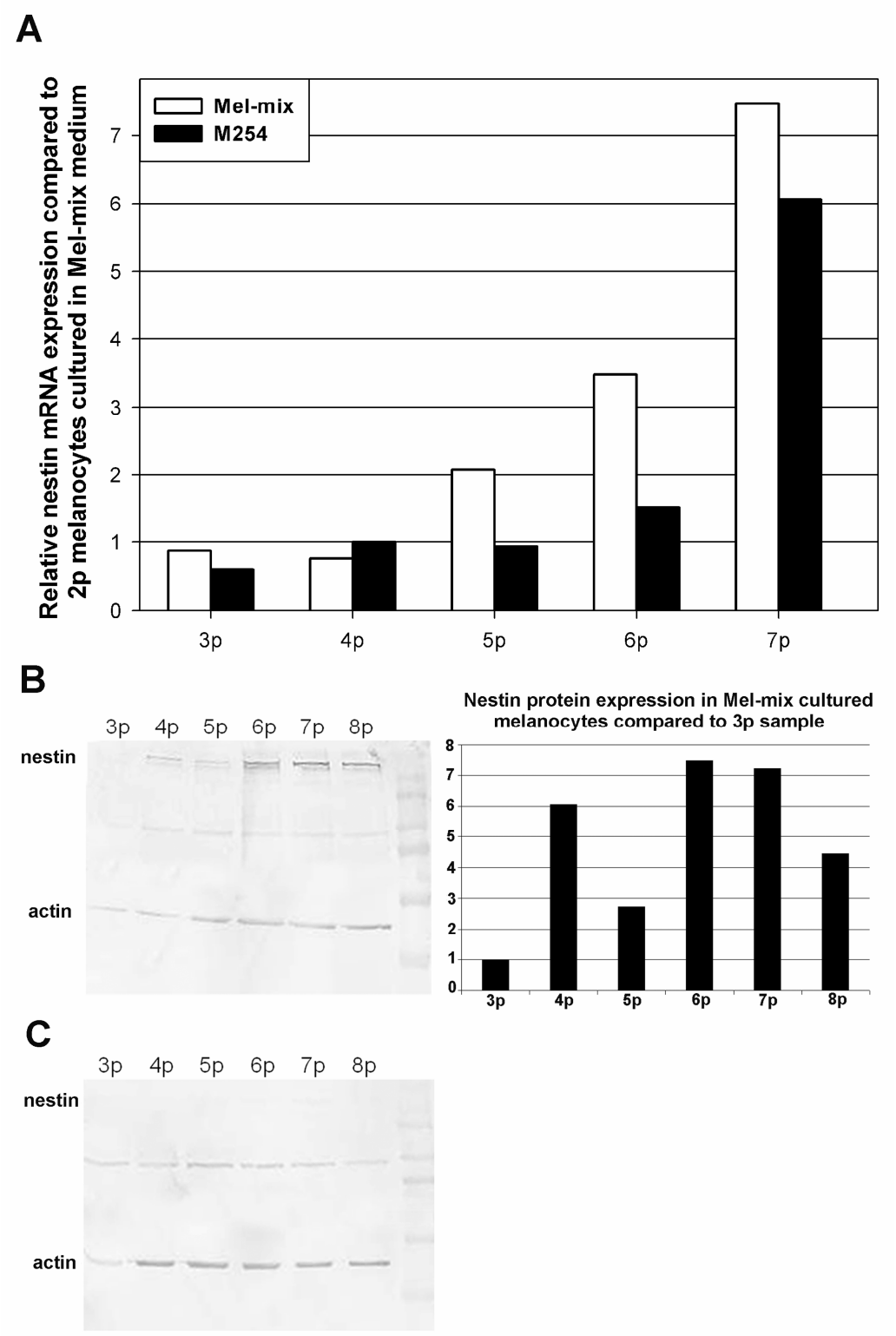

Figure 17. Nestin is strongly expressed in dedifferentiated melanocytes. Although nestin mRNA increased with increasing passage number in all cultured cells this increase was more pronounced in dedifferentiated melanocytes (A). Using a monoclonal antibody, nestin was easily detectable in dedifferentiated cells (B), while in differentiated melanocytes only weak specific bands, not detectable by densitometry, were visible on the blot (C). Densitometry showed increasing expression in dedifferentiated cells during culturing (B).

4.3 The role of EGF and EGFR in melanocytes.

4.3.1 Cultured normal adult epidermal melanocytes express EGFR mRNA and protein

Using real-time RT-PCR, we examined the EGFR mRNA expression both in normal human adult epidermal melanocytes, cultured in the complete chemical-free Mel-mix medium until 3-4th passages and in cultured normal human epidermal keratinocytes in 3rd passage. Cultured melanocytes expressed EGFR mRNA, although on average at a three times lower level than keratinocytes (Fig. 18A). The differences were not statistically significant ($p<0.05$, ANOVA). Cultured melanocytes were from eight and seven different donors (3rd passage n=8 and 4th passage n=7), keratinocytes from seven different donors (3rd passage n=7).

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 (Fig. 18B). As isotype control, mouse IgG_{2a} was used.

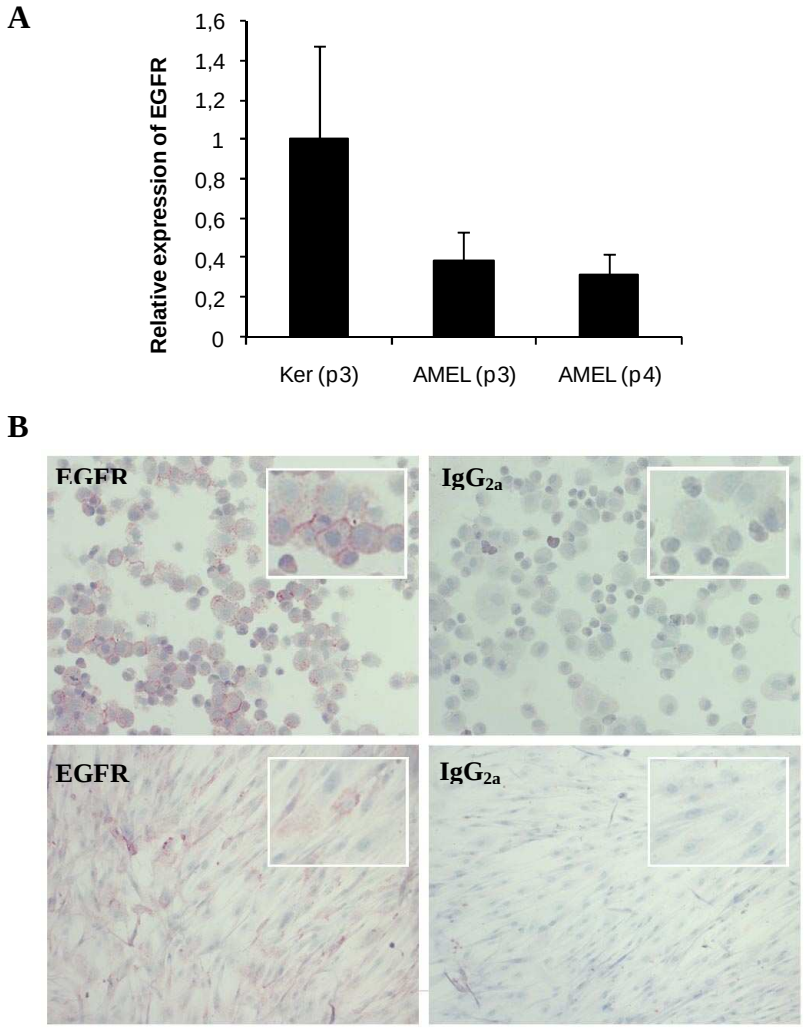

Figure 18. Normal human adult epidermal melanocytes express EGFR mRNA (A) and protein (B). EGFR mRNA was determined in melanocytes in 3rd and 4th passage and in keratinocytes in 3rd passage using real-time

RT-PCR. EGFR mRNA expression was normalized to 18S ribosomal RNA. The EGFR mRNA expression in melanocytes is expressed relative to the EGFR mRNA expression in keratinocytes. Values represent means ± SEM from seven different donors (n=7) for keratinocytes, and for melanocytes eight different donors (n=8) in 3rd passage (p3) and seven different donors (n=7) in 4th passage (p4) (A). Cultured cells in 3rd passage were used to detect the presence of EGFR on cytospin and on slide preparations by immunocytochemical analysis. EGFR were uniformly expressed by all cells in the culture (cytospins: upper figures, cells grown on slide: lower figures). Mouse IgG_{2a} stained cells served as isotype controls. Original magnification: 200x, inserts magnified digitally by 2x (B).

4.3.2 EGF has a dose dependent mitogenic effect on cultured normal human adult epidermal melanocytes

Since we were able to detect the expression of EGFR on the cells, 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 (Fig. 19).

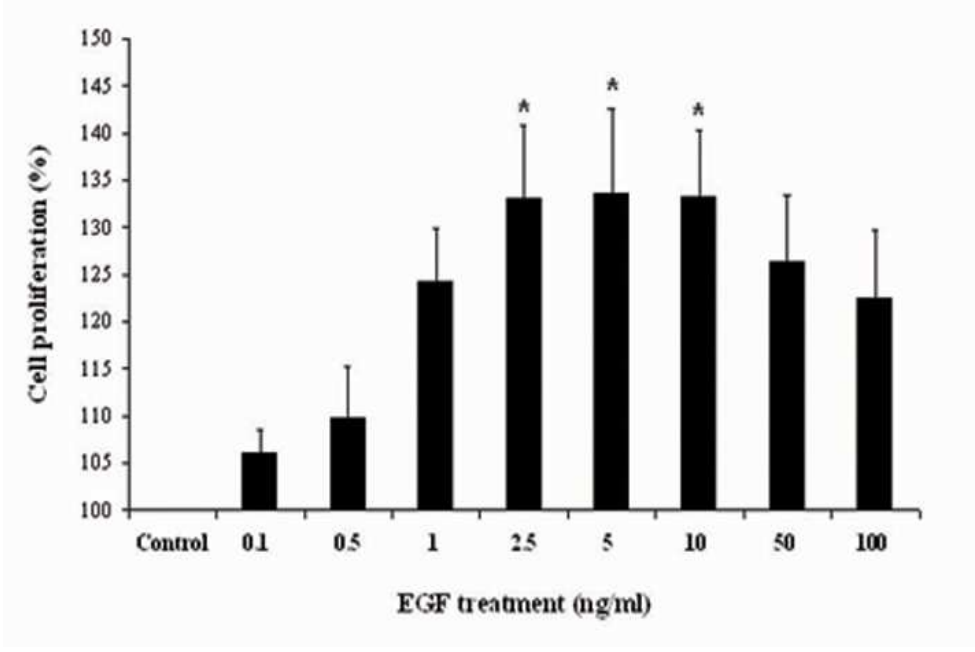

Figure 19. EGF exerts a dose dependent mitogenic effect on melanocyte growth. Normal human melanocytes in 4th passage cultured in Mel-mix medium without FBS and BPE, were exposed to different concentrations of EGF for three days. Cell numbers were determined by MTT assay. Cell numbers increased significantly in cultures at 2.5 ng/ml, 5.0 ng/ml and 10.0 ng/ml EGF concentrations. The graph shows percent changes relative to control (cells cultured without EGF in Mel-mix medium) with means ± SEM of 6 independent experiments, each from 8 samples. *significantly different from the control (ANOVA, $p < 0.05$).

4.3.3 EGF does not influence the relative expression of EGFR mRNA in cultured normal human epidermal melanocytes

There is evidence obtained in human epidermoid carcinoma cells, that EGF receptor gene expression can be modulated by EGF. 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 applied EGF concentrations were chosen based on the optimal mitogenic effect of EGF on the cells. 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 (Fig. 20).

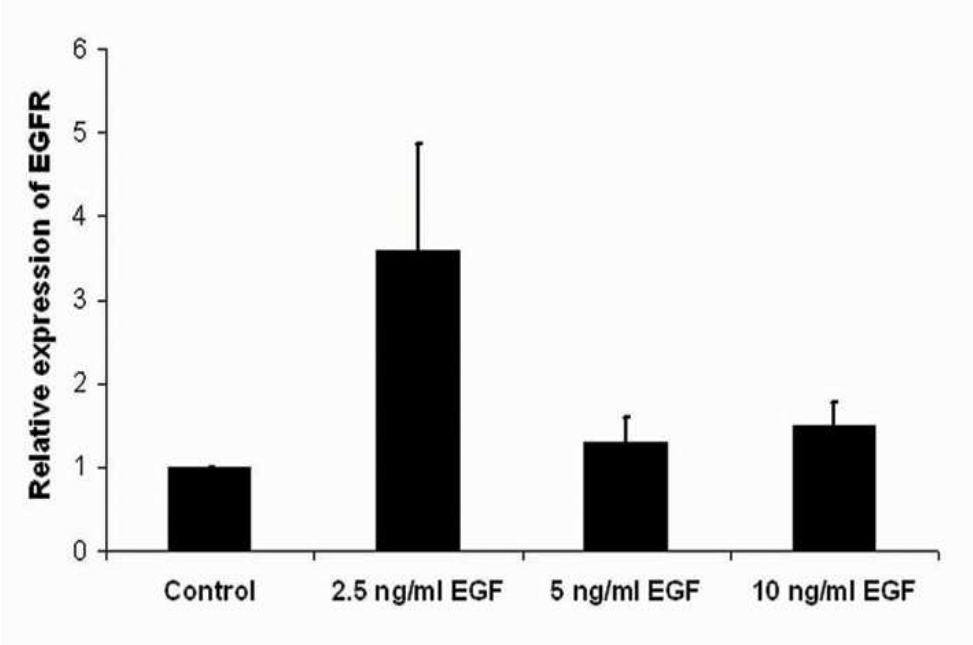

Figure 20. EGF at mitogenic concentrations has no effect on EGFR mRNA expression in normal human melanocytes. Cultured melanocytes in 5th passage were exposed to EGF at mitogenic concentrations (2.5-5-10 ng/ml). The relative expression of EGFR mRNA was determined by real-time RT-PCR, mRNA expressions were normalized to the expression values of 18S ribosomal RNA and compared to the controls. Values represent means ± SEM from three independent experiments.

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 [23]. Melanocytes cultured without PMA, CT or IBMX show pigmentation loss and decreased dendrite formation [19,20,52]. 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 [53]. Activation of c-Kit by stem cell factor results in Mitf phosphorilation [28]. Microphtalmia associated transcription factor (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 [54]. There is also evidence that c-Kit, tyrosinase and TRP-1 gene expression are coordinated in melanocytes [55]. 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. These cells showed characteristic dendritic morphology and pigment production. Among cells that were grown in Mel-mix medium without PMA, c-Kit expression appeared in fewer cells, and when c-Kit positive cells from PMA containing medium were switched into Mel-mix, the expression of c-Kit protein 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. Similarly TRP-1 positive cells grown in PMA containing medium lost their TRP-1 expression after changing their culture environment to PMA-free Mel-mix. In Mel-mix medium, TRP-1 and c-Kit mRNA expression also showed a decreasing trend. The decrease in c-Kit mRNA level was not significant, less dramatic than the disappearance of c-Kit protein expression in the cells. These data indicate that differentiation is a reversible process in epidermal melanocytes. Depending on the culture conditions, cells can revert to a less differentiated form. In human skin, c-Kit positive cells consist of two populations, one expresses TRP-1 and the other does not [31]. It has been

proposed that the c-Kit+ TRP-1- cell population, also characterized by strong BCL-2 expression, represents less differentiated melanocytes. If differentiated melanocytes die, these cells could serve as a source to repopulate the skin. It is also possible that under certain conditions, when the need for melanization subsides, cells could revert to a less differentiated form [31].

In vitro studies have shown that TPA (PMA) induces neural crest cell differentiation into melanocytes and stimulates proliferation and differentiation of normal melanocytes [24,56]. 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 [54].

It has been reported that withdrawal of cAMP inducers (CT+IBMX) from the medium in melanocyte cultures causes cells to become senescent [57]. 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. This high proliferative rate of Mel-mix cultured adult melanocytes typically lasts until the 10^{th} passage, then proliferation slows, and by about the 15^{th} passage, cell growth arrests. 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. Human TRP-1 has tyrosine hydroxylase activity, it has a role in processing and stabilizing the enzymatic activity of tyrosinase, thereby it takes part in maintaining the structural integrity of melanosomes [58,59]. Data indicate that UVB can influence the expression of TRP-1 [60,61]. Repeated UVB irradiation induced TRP-1 expression in melanocyte-keratinocyte co-culture [62]. 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 [63]. 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 [64]. Our results are in line with numerous data indicating that melanocyte differentiation and melanogenesis are influenced by tissue environment, in which keratinocytes are keys (for a review see [23]).

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 [65]. Cell differentiation is not unidirectional; under certain stimuli *in vitro* or during regeneration, differentiated cells may recover properties of immature cells [66]. 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) [67]. 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 [54]. 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 [68-70].

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. Nestin first appears in cells of nervous tissue formed during the embryonic period of ontogenesis [71]. Upon differentiation, nestin is down-regulated, but its re-expression has been demonstrated in a variety of primary central nervous system (CNS) tumors, in injured tissues of CNS, and in melanoma. It has been suggested that nestin was an indicator of cell-dedifferentiation in melanocytes, as nestin protein was found to be abundant in melanoma [72,73]. In a recent study, nestin expression was detected in cultured normal human foreskin melanocytes [74]. 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 [71]. 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. To prove this, more data through functional investigations are

needed. 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 (data not shown). Translocator protein 18 kDa, the peripheral benzodiazepine receptor by its earlier name, is a mitochondrial membrane protein associated with the mitochondrial permeability pore. In the healthy adult brain, TSPO expression is restricted to glial cells. However, in developing or damaged neural regions, TSPO appears in differentiating/regenerating neurons similar to nestin. TSPO mRNA and protein, while missing from mature neurons, are present in neural stem cells and also in postmitotic neuronal precursors [75]. Further studies are needed to clarify how abundant TSPO expression is in our dedifferentiated melanocytes and how it translates to protein expression.

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. Data from malignant melanoma were available for more than 20 years. Evidence suggests that abnormal expression and signaling of EGFR play a central role in melanoma. In one study EGFR was detected immunhistochemically in two of 11 dysplastic nevi, in three of 16 radial-growth-phase primary melanomas and in eight of 10 metastases, mature dermal nevi were found negative [76]. In another study increased EGFR immunoreactivity was found in different types of benign and neoplastic melanocytic lesions [43]. Human melanomas harboring RAS mutations appear to have elevated EGFR immunoreactivities and overexpression of EGFR correlates with melanoma progression [77,78]. In RAS-dependent melanomas of transgenic mice the oncogenic and tumor maintenance program involves activation of EGFR signaling [79]. EGF is expressed by melanoma cells and can stimulate their proliferation [80], high EGF production, linked to functional polymorphism in the EGF gene, might be important in the development of melanoma [81]. Data on the expression of EGFR in normal human melanocytes were contradictory. Reviews on melanocyte biology categorically statd that normal melanocytes did not express EGFR [45], this statement was the title of a letter published in 2004 in the Journal of Investigative Dermatology [46]. In contrast Gordon-Thomson and colleagues (2001), and later Mirmohammadsadegh et al (2005) have clearly demonstrated the presence of EGFR in normal cultured melanocytes [47,48]. 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 [82]. We found the amount of EGFR mRNA enhancing in the cells with passages while cells were becoming less melanized (data not shown). In cultures populated predominantly by melanized cells, Gordon-Thomson et al have also observed reduced EGFR expression [47]. The amount of EGFR mRNA was much lower in melanocytes compared to normal cultured keratinocytes. The regulation of EGFR expression is complex, involving multiple stimulatory as well as inhibitory transcription factors [83]. In a human carcinoma cell line it was shown that EGF was able to enhance mRNA expression of its receptor, EGFR [84]. 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 [85,86]. EGFR specifically affects proliferation and migration of neural progenitor cells in the subventricular zone of the mouse brain [87]. In the developing central nervous system (CNS), activation of the EGFR pathway induces astrocyte differentiation. In the adult CNS, the EGFR pathway is absent from astrocytes but is highly up-regulated and activated following neuronal injury [86]. In our experimental setup, EGFR expression was constitutive in dedifferentiated melanocytes. In addition, melanocyte proliferation was inducible by EGF treatment indicating a functional EGFR signaling.

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 [20]. 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.

7. REFERENCES

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