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Class III β-tubulin, a novel biomarker in the human melanocyte lineage

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Abstract

It is generally thought that class III β-tubulin expression is limited to cells of the neural lineage and is therefore often used to identify neurons amongst other cell types, both *in vivo* and *in vitro*. Melanocytes are derived from the neural crest and share both morphological features and functional characteristics with peripheral neurons. Here, we show that these similarities extend to class III β-tubulin (TUBB3) expression, and that human melanocytes express this protein both *in vivo* and *in vitro*. In addition, we studied the expression of class III β-tubulin in two murine melanogenic cell lines and show that expression of this protein starts as melanoblasts mature into melanocytes. Melanin bleaching experiments revealed close proximity between melanin and TUBB3 proteins. *In vitro* stimulation of primary human melanocytes by α-MSH indicated separate regulatory mechanisms for melanogenesis and to TUBB3 expression. Together, these observations imply that human melanocytes express TUBB3 and that this protein should be recognized as a wider marker for multiple neural crest-derived cells.

Introduction

Over the last several years, there has been a notable increase in interest in the culture of skin and skin stem cells, in part for the generation of skin substitutes, in part with view to understanding cutaneous pathologies. It is of great importance to be able to identify the different skin cell subtypes accurately in culture in order to fully understand their biology. A widely used biomarker in this context is class III β-tubulin (TUBB3), which is generally thought to be expressed only in cells of the neural lineage.

Microtubules are one of the main classes of cytoskeletal filaments involved in cellular morphology, motility, division and intracellular transport. They are composed of assemblies of heterodimers one α- and one β-tubulin subunit [1, 2]. There are multiple β-tubulin isotypes and their expression is tissue-specific and depends on the developmental stage [3]. Isotype TUBB3 was first described in avian and mammalian species [4]. Antibodies directed against TUBB3 epitopes distinguished between neurons and glial cells [5, 6], so that it became a standard biomarker for neuronal identification. TUBB3 expression is often used to identify neuronal cells in skin-derived and (epidermal) stem cell cultures. *In vivo* it is used to trace the dendritic processes of neurons in the dermis and epidermis, of which the cell bodies are located elsewhere.

TUBB3 is not exclusively neuron-specific. It is, albeit weakly, expressed in testicular and a few other tissue types [3, 7]. Recently, it was shown to be present in cultures of mesenchymal and epithelial cells [8]. A variety of solid tumors such as ovarian tumors, lung tumors and malignant melanomas also express TUBB3 and it has been suggested that this tubulin isotype is associated with cellular drug resistance [9]. Tubb3 has also been found expressed in developing melanoblasts in mouse and chicken embryo's [10, 11]. Interestingly, in a recent study positive immunoreactivity for the TUBB3 protein was reported in cells in the basal layer of normal human adult epidermis [12]. Although it is suggestive that these cells could be melanocytes, no confirmation has been provided. This is of importance as an identical epidermal immunostaining pattern can be found in a study on human epidermal innervation [13]. A potential TUBB3 expression by melanocytes could therefore mislead neuronal cell identification when using antibodies directed against TUBB3.

In the present study, we have resolved this issue by showing that TUBB3 is expressed in human epidermal and hair follicle melanocytes, both *in vivo* and *in vitro*, and

therefore have identified TUBB3 as a novel marker of melanocytes. In addition, we have explored its relation to melanocyte differentiation and melanogenesis. Because melanocytes do express TUBB3, this has major implications in interpreting results of cultured skin and skin stem cells, as well as in studies on skin innervation.

Materials and Methods

Tissue samples

Human adult retroauricular tissue was obtained during surgery on a 70-year-old female patient. Fetal scalp skin was collected from tissue of 20 weeks gestational age obtained by elective abortion. Both cases were approved by the Medical Ethical Committee of the Leiden University Medical Center (fetal tissue: P08.087) and informed consent was obtained in accordance with the Declaration of Helsinki guidelines. Skin specimens were fixed in 4% formaldehyde in PBS overnight at 4 ^{0}C followed by ethanol dehydration and embedding in paraffin wax using standard procedures.

Cell culture

Fetal scalp skin was mechanically dissociated and cultured for seven days in RPMI 1640 (Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) GOLD (PAA Laboratories GmbH, Pasching, Austria), 200 nM 12-O-tetradecanoylphorbol 13-acetate (TPA, Sigma-Aldrich, Saint Louis, MO, USA), 200 pM cholera toxin (CT, Sigma-Aldrich), 10 ng/ml stem cell factor (SCF, R&D Systems, Inc., MN, USA), GlutaMAX (Life Technologies) and antibiotic antimycotic solution (Sigma-Aldrich). Cultures were maintained in a humidified incubator with 5% CO_2 at 37 ^{0}C. Medium was changed every third day.

Melb-a cells were cultured in RPMI with 10% FBS, 20 ng/ml SCF, 10 ng/ml bFGF (R&D Systems, Inc.), GlutaMAX and antibiotic antimycotic solution. Melan-Ink4a2 cells were cultured in RPMI containing 10% FBS, 200 nM TPA, 200 pM CT, 10 ng/ml SCF, GlutaMAX and antibiotic antimycotic solution. Both cell lines were maintained in a humidified incubator with 10% CO2 at 37 ^{0}C. Medium was changed every other day, and cells were passaged using TrypLE Express (Life Technologies) upon reaching 90% confluency.

Primary normal human melanocytes were cultured in Ham's F10 supplemented with 100 U/ml penicillin, 100 U/ml streptomycin and 292 µg/ml L-glutamine (Invitrogen, Breda, the Netherlands), 1% Ultroser G (Biosepra, Fremont, CA, USA), 5 ng/ml endothelin-1 (Sigma-Aldrich), 5 ng/ml basic-FGF (Sigma-Aldrich), 30 µg/ml CT, 33 µM IBMX (Sigma-Aldrich) and 8 nM TPA. Melanocytes were cultured in a humidified incubator with 7.5% CO_2 at 37 ^{0}C. Medium was changed every

other day, and cells were passaged using 0.01% Trypsin (Life Technologies) upon reaching 90% confluency. Melanocytes were stimulated with 109 U alpha-MSH (Sigma-Aldrich) for 6, 24 and 48 hours.

Immunochemistry and image processing

Paraffin sections (5 μm) of skin specimen were deparaffinized in xylene, rehydrated in a descending ethanol series (100%, 90%, 80%, 70%), and rinsed in distilled water. Antigen unmasking was performed in 0.01M sodium citrate buffer (pH 6.0) for 12 minutes at 97 ^{0}C using a microwave oven. Sections were blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS containing 0.05% Tween-20 (Promega, Madison, WI) for 30 minutes, and incubated with primary antibodies (appropriately diluted in BSA/Tween-20/PBS) overnight at room temperature in a humidified chamber. Secondary antibodies were diluted in 1% normal goat serum (NGS, DAKO, Glostrup, Denmark) in Tween-20/PBS, and sections were incubated for 1 hour at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and sections were mounted in ProLong Gold (Molecular Probes, Life Technologies). Cultures were fixed in 1% formaldehyde in PBS overnight at 4 ^{0}C and, when necessary, stored in PBS. Bleaching was performed in 2% potassium permanganate solution for 30 minutes followed by 0.5% oxalic acid solution for 10 minutes. Cells were permeabilized with ice-cold methanol for 10 minutes, blocked in 5% NGS and 0.05% Tween-20 in PBS, and incubated with primary antibodies (appropriately diluted in NGS/Tween-20/PBS) overnight at 4 ^{0}C. Next, the cells were incubated with fluorochrome-conjugated secondary antibodies (diluted in NGS/Tween-20/PBS) for 1 hour at room temperature and followed by nuclear staining with DAPI added to Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Primary and secondary antibodies, and their dilutions, are listed in Table S1. In all double stainings with melanocytic markers, monoclonal anti-TUBB3 2G10 was used. Due to technical limitations, DCT was used to identify the pigmented melanocytes in deparaffinized sections whereas TYRP1 was used to identify these cells in culture. These enzymes catalyze subsequent steps in melanin biosysthesis. Human dermal fibroblast cultures (HDF-a, ScienCell, Carlsbad, CA, USA) were used as a negative tissue control whereas in specificity controls the primary antibody was omitted. Brightfield, phase contrast and fluorescent images were taken with either a DM5500 microscope and camera (Leica Microsystems, Rijswijk, the Netherlands) or an Olympus IX70 microscope (Olympus, Zoeterwoude, the Netherlands) equipped with a Leica DFC340 FX camera (Leica) using LAS

AF software (Leica). Brightness and contrast adjustments and cell counting was performed using ImageJ 1.45s (National Institutes of Health, http://imagej.nih.gov/ij).

Western blot analysis

For Western blotting, p34 melb-a and p19 melan-Ink4a2 cells were lysed in ice cold lysis buffer (50 mM Tris-HCl (pH 7.5), 100mM NaCl, 0.2% Tween-20, supplemented with 1 mini protease inhibitor cocktail tablet (Roche, Basel, Switzerland) per 10 ml). Human melanocytes were harvested for Western analysis in lysis buffer M-per (Thermo Scientific, Rockford, IL), supplemented with 1 tablet PhosSTOP and 1 tablet protease inhibitor (Roche) per 10 ml. Protein concentration was determined using a Pierce BCA Protein assay kit (Thermo Scientific). Equal amounts of protein were loaded on a 10% SDS-PAGE gel, separated for 90 minutes at 100 V and blotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) for 90 minutes at 300 mA. Membranes were blocked with 3% non-fat dry milk (Elk skimmed instant milk powder; FrieslandCampina, the Netherlands) in TBS containing 0.01% Tween-20 (TBST, pH 7.5) for 1 hour at room temperature followed by incubation with the primary antibodies TUJ1 (1:2000, Covance, Princeton, NJ), 2G10 (1:400, Abcam, Cambridge, UK) and DCT (1:1000, Abcam) overnight at 4 ^{0}C. GAPDH (1:2000, Santa Cruz Biotechnology Inc, Santa Cruz, CA) and β-actin (1:5000, Sigma-Aldrich, St. Louis, MO) were used as loading controls. Subsequently, the membranes were inubated with horseradish peroxidase-conjugated secondary antibodies (Promega) for 1 hour at room temperature in TBST. Protein was detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) using a Gel Doc XR System (Bio-Rad, Hercules, CA). Quantity One 1-D Analysis software (Bio-Rad) was used to determine band intensity for analysis of protein expression levels in the human melanocytes.

qRT-PCR Analysis

RNA from p34 melb-a and p19 melan-Ink4a2 cell cultures was isolated using RNA-Bee (Tel-Test Inc., Friendswood, TX) according to the manufacturer's instructions. Primers against *Mitf*, *Dct* and *Tubb3* were designed with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast). A complete list of the primers used can be found in Table S2. cDNA was generated by incubating 1 μg RNA with 500 ng Random Hexamers (Promega), supplemented with H2O to 10 μl, for 10 minutes

at 70 ^{0}C. Per sample, 100 units M-MLV reverse transcriptase, 1.25 µl 10 mM dNTP mix, 20 units RNAsin ribonuclease inhibitor and 1x buffer (all from Promega) were mixed, incubated at room temperature for 10 minutes, and heated for 50 minutes at 45 ^{0}C and 10 minutes at 70 ^{0}C. Real-time PCR was performed using the IQ5 Real Time PCR Detection System (Bio-Rad) with an initial denaturation step of 15 minutes at 95 ^{0}C followed by 40 cycles of 15 seconds at 95 ^{0}C, 30 seconds at 60 ^{0}C and 30 seconds at 72 ^{0}C. Cycle threshold values were attained and relative expression levels of mRNA were normalized to the housekeeping genes *Hprt* and *Gapdh* and calculated by the ΔΔCT method. qRT-PCRs were performed on two independent samples of each cell line and three technical replicas were performed for each sample.

Statistical analysis

Results are expressed as the mean ± standard deviation. Two-tailed, paired Student's *t*-tests were used to determine statistical significance. A value of $p < 0.05$ was considered to be statistically significant. Analysis was performed using Microsoft Excel 2010 (Microsoft, Redmond, WA).

Primary Antibodies

Antibody	*Host*	*Clone*	*Dilution*	*Company*	*Catalog number*
Melan-A	Rabbit	A19-P	1:200	Novus Biologicals	NBP1-30151
DCT	Rabbit	-	1:200	Abcam	Ab74073
TYRP1	Rabbit	-	1:50	Santa Cruz Biotechnology	sc-25543
TUBB3	Rabbit	TUJ1	1:1000	Covance	MRB-435P
TUBB3	Mouse	2G10	1:200	Abcam	Ab79078

Secondary antibodies

Antibody	*Dilution*	*Company*
Alexa Fluor 488 goat anti-rabbit IgG (H+L)	1:200	Life Technologies
Alexa Fluor 555 goat anti-mouse IgG (H+L)	1:200	Life Technologies
Alexa Fluor 647 goat anti-mouse IgG (H+L)	1:200	Life Technologies

Table S1. List of primary and secondary antibodies for immunochemistry.

GENE	*FORWARD PRIMER*	*REVERSE PRIMER*
Mitf	5'-GGGGCTGCCTGAAACCTTGCT-3'	5'-TACCTGGTGCCTCTGAGCTTGC-3'
Dct	5'-TGACCGTGAGCAATGGCCGA-3'	5'-GGCCGGTCCAGCCGAACTTG-3'
Tubb3	5'-CCGCGTGAAGTCAGCATGAGGG-3'	5'-CCGCTGGGGTCTATGCCGTG-3'
Hprt	5'-TTGCTCGAGATGTCATGAAGGAGA-3'	5'-AGCAGGTCAGCAAAGAACTTATAG-3'
Gapdh	5'-TGCACCACCAACTGCTTAGC-3'	5'-GGCATGGACTGTGGTCATGAG-3'

TABLE S2. LIST OF PRIMERS.

Results

TUBB3 is expressed in human epidermal melanocytes *in vivo*

To confirm the TUBB3 expression reported by Akasaka et al. in the basal layer of human epidermis and to investigate if this was indeed localized to melanocytes, we analyzed human epidermal skin sections by immunohistochemistry for both TUBB3 and two melanocytic markers, melanoma-specific antigen 1 (melan-A) and dopachrome tautomerase (DCT). Melan-A (also known as MART-1) is a protein present both in non-pigmened and in pigmented melanocytes. DCT (also known as tyrosinase-related protein 2, TYRP2) is an enzyme involved in melanogenesis and is a direct target of microphthalmia-associated transcription factor (MITF) and therefore specifically marks the early and terminally differentiated pigment producing melancoytes. In adult human epidermis, most melan-A+ and DCT+ cells in the basal layer of the epidermis immunostained for TUBB3 (Figures 1A and 1B). TUBB3 expression was seen in 83.7% $\pm$ 5.4 (n = 49) of the melan-A+ cells and in all of the DCT+ cells (n = 21). There were also TUBB3+ cells in the basal layer that did not immunostain for melan-A (6.8% $\pm$ 5.0, n = 44) nor for DCT (43.2% $\pm$ 19.3, n = 37). Fetal scalp skin at 20 weeks of gestational age showed a similar staining pattern. Already, melan-A+/TUBB3+ and DCT+/TUBB3+ melanocytes were found in the basal layer (Figures 1C and 1D) and the majority of melan-A+ and DCT+ cells expressed TUBB3 (98.0% $\pm$ 3.0 (n = 50) and 100.0% (n = 27), respectively). Of the TUBB3+ cells, 3.9% $\pm$ 3.4 (n = 51) did not immunostain for melan-A, whereas 42.6% $\pm$ 5.5 (n = 47) were negative for DCT. Taken together, these data show that normal human epidermal non-pigmented and terminally differentiated pigmented melanocytes express TUBB3 *in vivo*.

We also observed TUBB3+ dendritic-like structures in the epidermis (Figure 1C, arrow) which we assumed to be epidermal nerve endings and therefore be of neuronal origin. Interestingly, many of these structures were also melan-A+ (Figure 1C, arrow). Since the dermal nerve fibers did not stain for melan-A (Figure 1C, asterisk), we conclude that these intraepidermal melan-A+/TUBB3+ structures are melanocytic dendrites rather than free nerve endings.

Human hair follicle melanocytes express TUBB3 *in vivo*

Our observations in epidermal melanocytes raised the question of whether hair

follicle melanocytes also expressed TUBB3. In the hair follicle, non-pigmented melanocytes reside within the bulge/sub-bulge area and the outer root sheet of the infundibulum [14 – 16]. They are thought to be the source of the mature melanocytes located around the dermal papilla and lower portion of the medulla, which provide pigment to the hair shaft keratinocytes. In fetal scalp hair follicles, melan-A+ and DCT+ melanocytes were seen in the hair matrix surrounding the basal area of the medulla (Figure 1E-F). As epidermal melanocytes, these hair follicle melanocytes immunostained for TUBB3 (Figures 1E-1F). In addition, TUBB3 expression was observed in cells of the outer root sheath (Figures 1E and 1F, arrowheads). These TUBB3+ cells, however, were negative for melan-A and DCT and it could be surmised that these cells are non-pigmented melanocytes which are known to reside in this area. TUBB3 immunostaining was also seen in the hair shaft medulla keratinocytes (Figure 1E-F, *M*). In summary, in the human hair follicle melan-A+/TUBB3+ and DCT+/TUBB3+ melanocytes were observed in the hair matrix, whereas melan-A-/TUBB3+ and DCT-/TUBB3+ cells were found in the outer root sheath of the hair follicle bulb.

Fetal epidermal and hair follicle melanocytes express TUBB3 *in vitro*

Based on the *in vivo* expression of TUBB3 in both epidermal and hair follicle melanocytes, we anticipated that TUBB3 would also be expressed *ex vivo/ in vitro* in these cells. For this purpose, human fetal scalp skin which contained an abundance of pigmented hair follicles was mechanically dissociated and cultured for seven days in standard culture medium containing additional factors (SCF, TPA and CT, see *Materials and Methods*) to support the whole range of differentiated melanocytes, including the non-pigmented, early-pigmented and the terminally differentiated pigmented melanocytes. This resulted in heterogeneous cultures containing keratinocytes, fibroblasts and melanocytes. The melanocytic cells were identified by means of different types of light microscopy. Using phase-contrast microscopy, melanocytes could be recognized by their bipolar or multipolar dendrites and the halo surrounding their cell body (Figure 2). Bright-field microscopy was used to detect pigment inclusions (Figure 2). Using both approaches, epidermal and hair follicle melanocytes could be distinguished on basis of the criteria formulated by Tobin et al. [17] who showed that cultured epidermal melanocytes have 3-6 dendrites, whereas cultured hair follicle melanocytes are mostly bipolar. Definite confirmation was provided by immunofluorescent staining for tyrosine-related protein 1 (TYRP1) and melan-A.

As expected, we were able to identify TYRP1+/TUBB3+ pigmented epidermal melanocytes in the dissociated skin cultures (Figure 2A). Interestingly, TYRP1-/TUBB3+ cells were also observed (Figure 2A, asterisks). Morphologically, these non- or early-pigmented melanocytes were similar to the TYRP1+/TUBB3+ cells, but they were less dark under bright-field microscopy. Bipolar hair follicle melanocytes were present in large numbers and were TYRP1+/TUBB3+ (Figure 2B). In close proximity to these cells, some bipolar TYRP1-/TUBB3+ were also found (Figure 2B, asterisks). Melan-A staining showed similar results; bipolar melan-A+/TUBB3+ cells were clearly growing out of the hair follicle bulb (Figure 2C). There were no melan-A-/TUBB3+ cells with similar morphology, but some TUBB3 expression was seen in cells with a morphology reminiscent of keratinocytes (Figure 2C, arrowheads). This data show that, as *in vivo*, both human epidermal and hair follicle melanocytes express TUBB3 *in vitro* and that expression starts before initiation of melanogenesis.

Expression of TUBB3 starts upon differentiation of melanoblasts into mature melanocytes

In neurons of the central nervous system TUBB3 expression decreases as the cells mature and can therefore be used to identify young developing neurons. In the peripheral nervous system, however, the opposite is found: TUBB3 levels increase with neuronal cell maturation in dorsal root ganglia[18]. Our experiments with human melanocytes show that TUBB3 is expressed in the end stages of the melanocytic lineage (non-pigmented and pigmented melanocytes). However, expression in the undifferentiated, melanoblast stage remains inconclusive. To further examine this relationship of TUBB3 expression within the melanocytic lineage, we analyzed two widely-used immortalized mouse melanocytic cell lines derived from the C57BL/6J strain. Melb-a is a clonal line of melanoblasts, which do not produce melanin [19]. Using bright-field microscopy, cells could not be seen due to the obvious lack of pigment inclusions (data not shown), confirming their immature state. Although most cells were negative for TUBB3 (Figure 3A), rare TUBB3+ cells could be observed. Interestingly, these TUBB3+ cells demonstrated a more differentiated cell morphology by the presence of dendrites and a halo surrounding their cell bodies. TUBB3- cells were generally flat with few and short dendrites, or none at all (Figure 3A).

To analyze TUBB3 expression in mature murine melanocytes, we used melan-Ink4a2

cells[20]. Cells of this line produce different amounts of melanin, so both non-pigmented and pigmented melanocytes are present which can be visualized under bright field microscopy (Figure 3B). TUBB3 immunostaining showed a gradient of strong to almost no fluorescence (Figure 3B). Our data suggests that there is an inverse relationship between TUBB3 fluorescence and melanin production; cells that showed the brightest TUBB3 fluorescence contained only little or no melanin at all, as evident from the immunofluorescence and bright-field images (Figure 3B, white arrows), whereas the darkest cells demonstrated little or no TUBB3 fluorescence at all (Figure 3B, black arrows). When we analyzed line plots over areas containing large amounts of melanin, we indeed found a relation between fluorescent intensity (Figure 3B, graph, red line) and bright field intensity (graph, black line).

We questioned whether this observed inversed relation of TUBB3 immunostaining and pigment inclusion would be caused by either a down-regulation of TUBB3 upon terminal differentiation, as is the case in neurons from the central nervous system, or by the pigment itself blocking emission of TUBB3 fluorescence, in which case TUBB3 expression would actually be sustained upon terminal differentiation. When we analyzed line plots within cells over areas containing large amounts of melanin, we found a relation between fluorescent intensity (Figure 3B, graph, red line) and bright field intensity (graph, black line), suggestive that the pigment itself blocked fluorescence. Similar line plot profiles were seen with secondary antibodies of different wavelengths (data now show). To further test this, we treated formaldehyde-fixed melan-Ink4a2 cells with potassium permanganate and oxalic acid to bleach the melanin (Figure 3B, bright field). After subsequent immunostaining, TUBB3 fluorescence was significantly increased (Figure 3C). Bright TUBB3 immunostaining was present in nearly all cells (94.2% $\pm$ 3.2, n = 142), whereas in the untreated samples only about a quarter of the cells (25.9% $\pm$ 12.3, n = 192, $p < 0.001$ between groups) were clearly visible (Figure 3B-C). These data indicate that TUBB3 is not only upregulated upon differentiation of melanoblasts into melanocytes, but that this tubulin isotype remains expressed during terminal differentiation into mature pigment-producing melanocytes.

To verify the immunocytochemistry findings, we performed both Western blot and qRT-PCR experiments on the melb-a and melan-Ink4a2 cell lines. Western blotting analysis using two different, commonly used, monoclonal antibodies against TUBB3 (TUJ1 and 2G10) showed corresponding bands at the predicted molecular weight of approximately 50 kDa (Figure 4A). In the case of TUJ1, no band was found in the melb-a cells, whereas a clear band was present in the melan-Ink4a2 cells. Antibody

2G10 showed bands in both cell types with a much higher protein expression in the melan-Ink4a2 cells. Using qRT-PCR we determined *Mitf*, *Dct* and *Tubb3* RNA levels (Figure 4B). *Mitf* and *Dct*, both key molecules in melanogenesis, were 1.67 ± 0.04 and 6.58 ± 0.89 fold increased ($p < 0.03$ and $p < 0.002$) in melan-Ink4a2 cells compared to melb-a cells, again confirming their more differentiated state. Tubb3 was also found in both cell lines, with a 4.12 ± 0.32-fold higher expression level in the melan-Ink4a2 cells ($p < 0.02$). In summary, immunocytochemistry, Western blot and qRT-PCR data indicated that TUBB3 expression starts when melanoblasts commit to the melanocyte lineage and differentiate into pigment producing melanocytes.

α-MSH stimulation does not increase TUBB3 expression in cultured human melanocytes

As TUBB3 expression in the melanocytic lineage coincides with the induction of melanin, we questioned if the melanogenesis pathway could also regulate TUBB3 expression. To assess this, we cultured primary human melanocytes and stimulated the cells with alpha-melanocyte-stimulating hormone (α-MSH) for 6 hours, 24 hours or 48 hours. After exposure, we performed quantitative western blot analysis for DCT and TUBB3 expression on the cell lysates. As α-MSH binds to the melanocortin 1 receptor (MC1R), which in turn promotes transcription of the MITF transcription factor, an observed increase in expression of the MITF target gene DCT would indicate a successful stimulation of the melanogenesis pathway. DCT expression was found to be increased (232%, 175% and 166% after 6 hours, 24 hours and 48 hours, respectively) confirming successful stimulation (Figure 5). In contrast, TUBB3 showed no concomitant increase in expression (108%, 105% and 91%, respectively (Figure 5)). Combined, no association between TUBB3 expression and induction of melanogenesis could be found.

Discussion

In this study, we show that TUBB3 is not only expressed within peripheral neurons but also in the melanocytic lineage. In melanocytes, TUBB3 expression was found both in human epidermal and hair follicle melanocytes. Using skin sections, we showed that nearly all melan-A+ and DCT+ melanocytes express TUBB3. Conversely, TUBB3+ cells in the basal layer of the epidermis were almost always melan-A+, although many did not express DCT. Similar results were found in cultured human fetal epidermal and hair follicle melanocytes. In the melanocytic lineage, melan-A is expressed both by non-pigmented and pigmented melanocytes. However, DCT and TYRP1 are not expressed before terminal diff erentiation into pigment producing melanocytes. This indicates that TUBB3 is widely expressed within the melanocyte lineage, with expression starting before pigment production. Indeed, when we investigated TUBB3 expression in mouse melanoblasts and melanocytes, most melanoblasts did not (as yet) express TUBB3 whereas both non-pigmented and pigmented melanocytes did express TUBB3. This implicates that expression of TUBB3 starts around the point of time where melanoblasts commit to the melanocyte lineage (Figure 5).

Like peripheral neurons, melanocytes are derived from the neural crest. There are also other similarities, both in morphology and functional characteristics. Both cell types have small, round cell bodies and multiple dendritic processes. Both can express similar receptors (e.g. $p75^{NTR}$ and c-Kit) and therefore respond to identical cytokines and neurotrophins [21]. Furthermore, both melanins (produced by melanocytes) and catecholamines (e.g. dopamine, produced by neurons) are derived from tyrosine, and both melanosomes and synaptic vesicle precursors are transported over microtubules by kinesins. In turn, melanosomes and neurotransmitters are both released from their dendrites or axons. Presently, there is an emerging concept that melanocytes could potentially be used to represent neuronal cells in disease model systems. In this light, the expression of neuron-specific TUBB3 in melanocytes might not be so surprising. In the skin, TUBB3 expression is considered to be of neuronal origin, whether it be intraepidermal free nerve endings or nerves projecting to Merkel cells, Langerhans cells or even melanocytes [22 – 24]. As we show that at least a part of these endings belong to melanocytes, it is of great interest to further investigate the extent of TUBB3 expression by skin melanocytes, examine their relationship with surrounding cells and investigate expression of other neuronal biomarkers.

TUBB3 has now been found expressed in chicken, mouse and human melanocytes. This evolutionary conservation is suggestive of an important role of TUBB3 in these cells. We observed initiation of TUBB3 expression in the melanocytic lineage around the time of diff erentiation into pigment producing melanocytes. Melanocyte development as well as the UV-induced pigmentation response are regulated by the MITF transcription factor that modulates multiple diff erentiation and melanin biosynthesis genes. We questioned whether TUBB3 expression could be regulated by the same pathway and stimulated cultured human melanocytes with α-MSH, known to induce MITF expression. Whereas expression of the MITF target gene DCT was induced by α-MSH stimulation, there was no associated increase in TUBB3 expression. Therefore, TUBB3 does not seem to be regulated by the MITF-pathway which regulates melanocyte development and pigmentation response. This is in line with results of studies examining MITF target genes, which consistently found that TUBB3 was not among the targets of this transcription [25 – 27].

Using a well-established bleaching protocol, we show that fluorescent signals emitted by TUBB3 stainings are blocked by melanin. This was in contrast to control experiments with melan-A immunostaining which did not show this clear relationship (Figure S1), indicating that the melanin specifically interfered with the TUBB3 fluorescence. Therefore, we conclude that the diminished TUBB3 fluorescence was caused by specific co-localization of melanin. This could indicate a novel role of TUBB3 in melanosomal transport. It is known that mature melanosomes are transported by kinesin and dynein motor proteins (Marks & Seabra, 2001). These proteins use microtubule tracks to travel in a bi-directional way (Wu et al., 1998). Also, by specifically disrupting microtubules, it has been shown that melanosome transport is microtubule dependent [28]. Potentially, TUBB3 could be the corresponding β-unit isotype (Figure S2). However, its role in cellular processes remains to be investigated.

A recent study by Dalziel et al. (2011) showed that TUBB3 is physically located immediately downstream of MC1R (Figure S3). This receptor binds to melanocortins and plays an important role in melanogenesis through its ability to influence MITF transcription. Aside from showing that TUBB3 is located immediately downstream of MC1R, in itself suggesting a role within melanocytes, Dalziel and colleagues found that chimeric proteins are transcribed by intergenic splicing between these two genes. The monoclonal TUBB3 antibodies used in our study, TUJ1 and 2G10, both recognize the C-terminus of the TUBB3 protein (epitopes EAQGPK [30] and MYEDDEEESEAQGPK [31]) and immunostain the same structures (Figure S4).

In chick and mice, *Tubb3* has only one protein product. In humans, aside from the chimeric proteins, there are four additional TUBB3 isoforms (Figure S3). Both antibodies will recognize at least isoforms 1 and 2 and the chimeric transcript RP11-566K11.2-001. Currently, these antibodies react both with neuronal and melanocytic cells. However, it could be that both cell types express different isoforms of the TUBB3 protein. It will be interesting to investigate the exact expression of these isoforms in normal human neurons and melanocytes to find out whether different and/or identical isoforms are present in these cell types. Together, our results and these findings suggest a dynamic role for TUBB3 in multiple aspects of melanocytic function.

Finally, in addition to TUBB3 expression in melanocytes, we have also observed its presence in hair follicle medulla keratinocytes *in vivo*. Expression of TUBB3 in the mitotic spindle of cultured keratinocytes and fibroblasts has been previously reported [8], something we have observed as well in our cultured melanocytes (Figure S5). This indicates that this biomarker, especially in the skin, cannot be reliably used to differentiate between neuronal cells and other neural crest and ectodermal cell types. Primary skin cultures and differentiation studies on skin stem cells or even (induced) pluripotent stem cells might be prone to this identification issue. Aside from identifying neurons, TUBB3 can be used as a much wider marker of neural crest derived and epithelial cells.

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