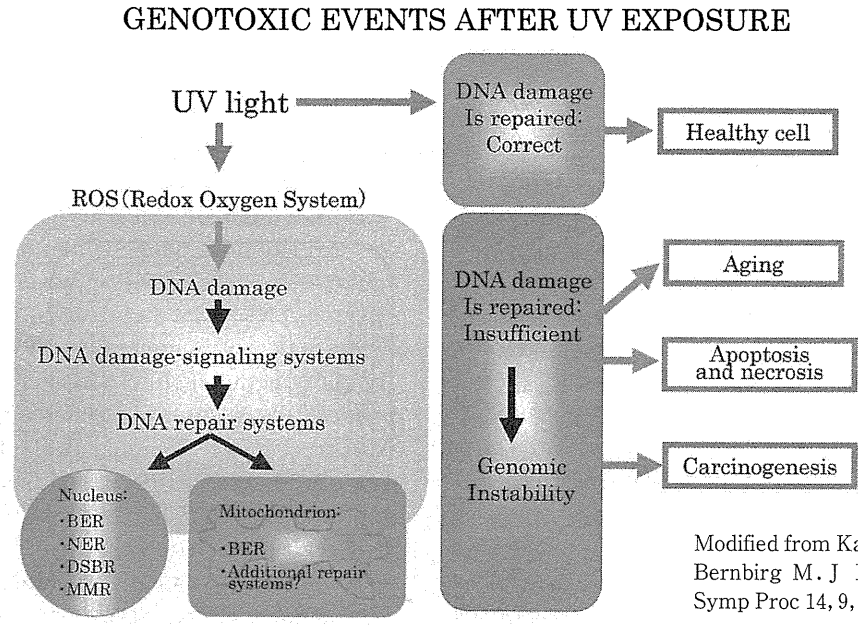

Figure 3: UV-induced genotoxic effects. UVR causes always the DNA damage to some extent. If DNA damage is repaired insufficiently, it will result in either apoptosis or necrosis of cells which will accentuate the aging process. It will also result in genomic instability which may become carcinogenic.

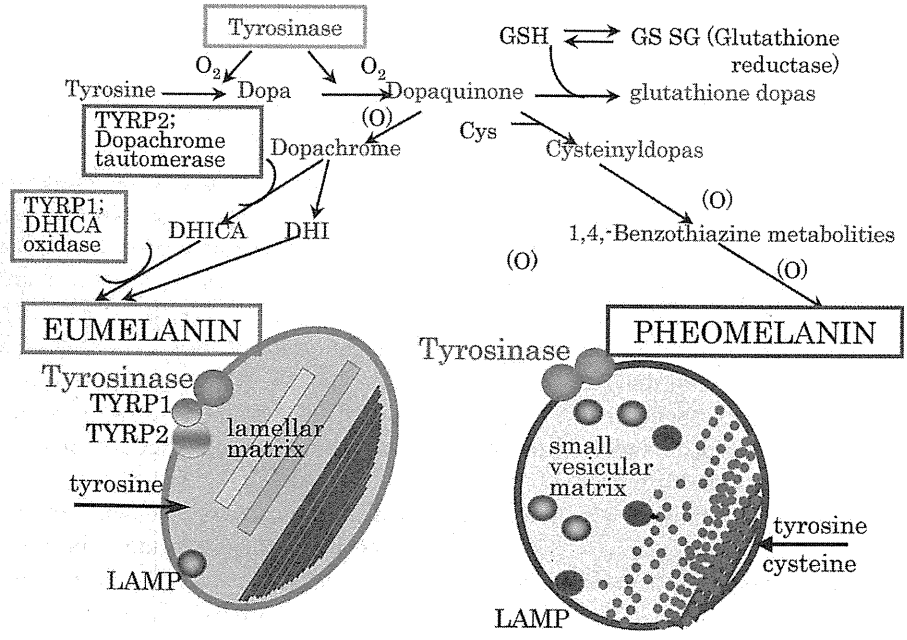

Figure 4: Cascade of eumelanin and pheomelanin biosynthesis. Two forms of melanin pigment are present in mammals. Eumelanin is synthesized by conversion of tyrosine to dopa and dopaquinone in the presence of tyrosinase. Through the interaction of TYRP 1 (DHICA oxidase) and TYRP 2 (dopachrome tautomerase: DCT) or auto-oxidation of dopa quinone, eumelanin will be produced within ellipsoidal granules that contain lamellar matrix inside. In contrast, pheomelanin will be produced after binding of dopaquinone with cysteine to form cysteinyldopa that will be auto-oxidized to form benzothiazine metabolites and result in pheomelanin in oval granules that contain small vesicular matrix.

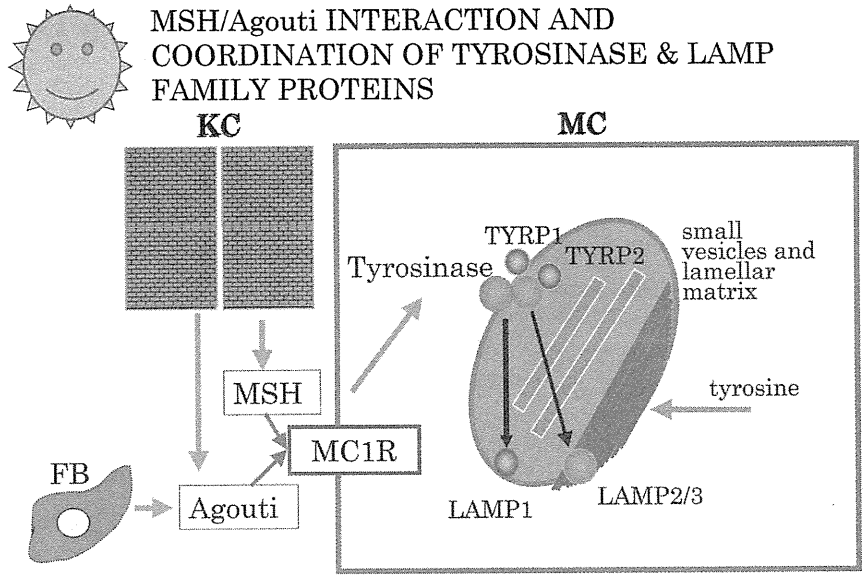

Figure 5: MSH/Agouti signaling protein interaction and coordinated expression of tyrosinase and LAMP family proteins after exposure to UVR.

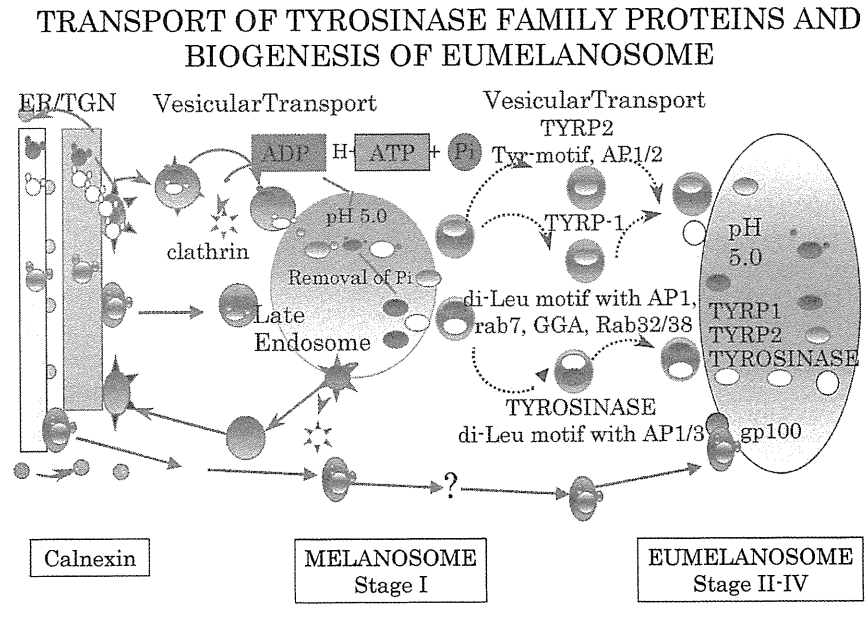

Figure 6: Transport of tyrosinase family proteins and biogenesis of eumelanosomes. Tyrosinase family proteins including TYRP 1 and DCT will transported from Golgi after glycosylation maturation in the presence of molecular chaperone, calnexin, by vesicular transport to late endosomal compartments which are delineated to Stage I melanosomes. Tyrosinase, TYRP 1 and DCT will take the different vesicular transport to Stage II melanosomes in which melanin biosynthesis starts.

can directly stimulate the expression of tyrosinase and LAMP gene families. Importantly, tyrosinase and TYRP-1 coordinate together to up-regulate LAMP-1 molecule which is likely to scavenge some of the toxic species generated during melanin metabolism (hydroxyl radicals) through high content of both N-and O-linked oligosaccharides by continuously coating the inner surface of melanosomal membrane (Fig. 4, 5).[20,21,40]

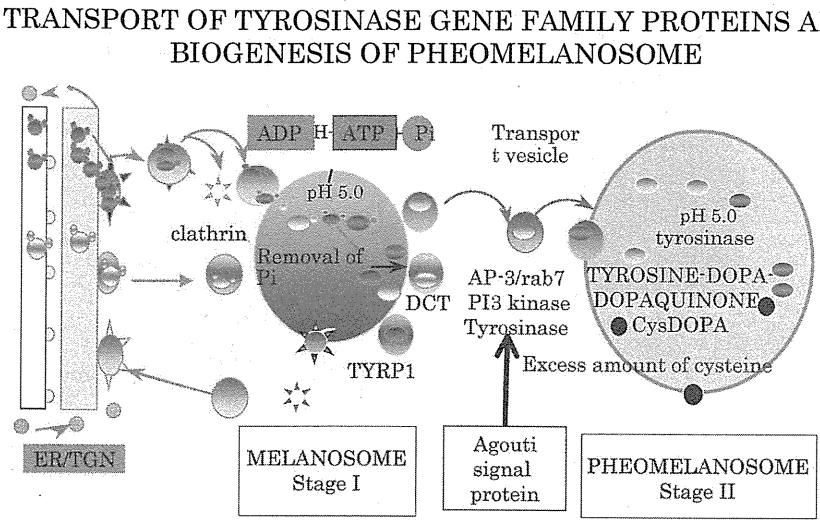

Figure 7: Vesicular transport of tyrosinase and biogenesis of pheomelanosomes.

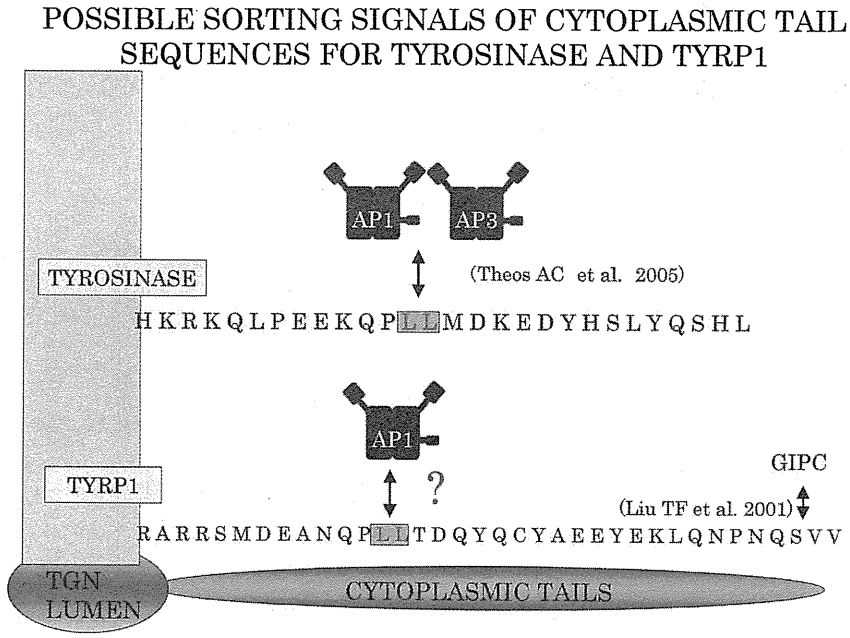

Figure 8: Possible sorting signals of cytoplasmic tail sequences for tyrosinase and TYRP 1. Cytoplasmic tail motifs of tyrosinase and TYRP 1 are referred from the reports of Theos AC et al. and Liu TF et al. respectively.[21,36]

Both tyrosinase and TYRP 1 are transported from the trans-Golgi network (TGN) to melanosomes via endosomal compartments (Fig. 6, 7). Adaptor proteins (APs) generate transport vesicles, sorting proteins and assembling clathrin to the local membrane in endosomes.[42] AP 3, one of the four known mammalian APs, has been reported to bind to a dileucine motif of tyrosinase.[5,14] A mutation of β 3 A subunit in AP 3 results in Hermansky-Pudlak syndrome (HPS)-2 in humans and pearl in mice.[8] Accumulating evidence suggests that both tyrosinase and TYRP 1 interact with APs and travel from endosomes to melanosomes, though the exact transport pathway is still not clear. It has recently been reported that tyrosinase can use both AP 1 and AP 3 for its proper sorting while TYRP 1 can use only AP 1.[53] It is also clear that

both tyrosinase and TYRP 1 are not transported to lysosomes. Tyrosinase may, however, be dedraded in lysosomes if its transport from TGN to melanosomes is blocked.[10]

3. Eumelanin biosynthesis and biological role of tyrosinase-related proteins

Mutations that effect different stages of melanocyte development have been best characterized in experimental mice. Both albino mutation of mouse tyrosinase and brown mutation of tyrosinase-related protein 1 (Tyrp-1) alter cysteine in the EGF motif to serine in albino mutation and different cysteine to tyrosine in brown mutation. Proof of the mutation status of the alteration has, in both cases, been provided by analysis of revertants which restore wild type function. In addition, comparison of the phenotypes with those of deletion of the loci indicates that both mutations have complete loss of function of the gene product. On the other hand, Tyrp-2 containing the slaty mutation, which has arginine to glutamine change at the first copper -binding sites may still have considerable DCT activity (Fig 8).

The function of TYRP/ tyrp-1 is still not fully understood, however, it is believed to be involved in eumelanin synthesis since the brown mutation only affects the eumelanic animals. It has shown that demonstrated gene changes in Tyrp-1 mRNA expression and in Tyrp-1 gene structure in certain mouse brown b-locus mutants. These findings have provided additional evidence of allelism and allowed an explanation of the phenotypes. The human homologue of TYRP-1 of the mouse brown gene maps to the short arm of chromosome 9 and extends the known region of homology with mouse chromosome 4.

Both recessive and dominant mutations of Tyrp-1 gene have been examined. Some recessive mutations involve single amino acid substitutions, changing an arginine residue to the single sequence cysteine, whereas others affect the levels of Tyrp-1 mRNA present in the melanocytes. These different mutations result in either complete loss of function, partial loss of function or temperature sensitive function. Dominant mutations affecting the Tyrp-1 gene have also arisen as a result of a base pair mutation. This type of mutation may destabilize the melanosomal membrane and allow the intermediates of

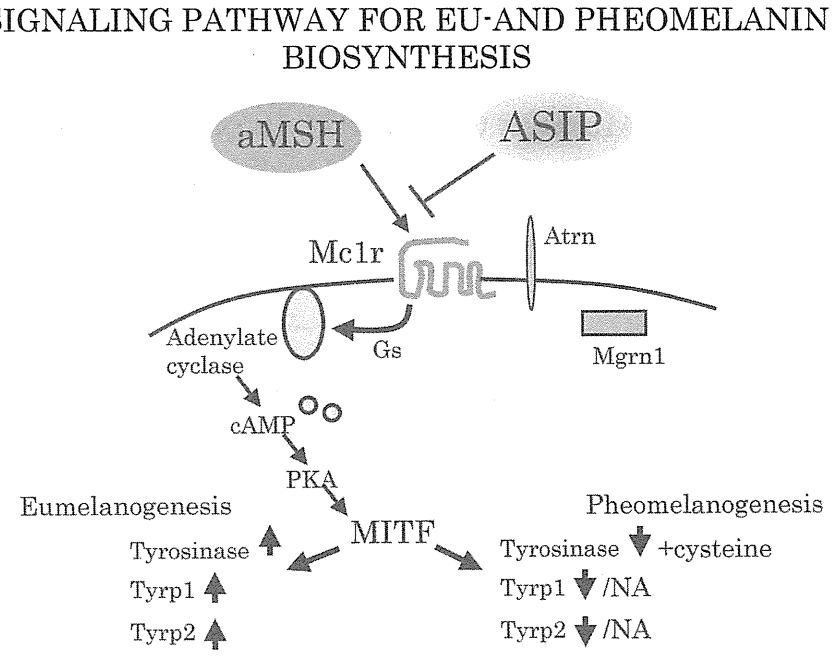

Figure 9: Agouti signaling protein is the key protein molecule which is involved in pheomelanin biosynthesis and which antagonizes to largely unknown MSH binding with Mc 1 r (melanocortin 1 receptor) on the melanocyte cell membrane. Attractin (Atrn) binds N terminal of Agouti/ASIP and supports this binding to Mc 1 r. Biological role of mahogunin (Mgrn) is still unknown.

melanogenesis, which are normally sequestered in the melanosomal compartment, to interfere with normal melanocyte function. Another type of dominant mutation may arise due to the Tyrp-1 gene having undergone some rearrangement, as a result Tyrp-1 is not properly transcribed, leading to melanocyte dysfunction and death. It has been therefore suggested that mutant TYRP-1 protein is involved in toxicity of melanocytes which is associated with inherently toxic melanogenesis process.[20,23]

4. Pheomelanin biosynthesis and its elevation in melanoma and dysplastic nevi

The diverse patterns of mammalian coat color are determined by the quantity and distribution of just two types of organic pigment: eumelanin (black to brown) and pheomelanin (yellow to red). Both are produced by melanocytes in the skin and hair.[20,21] Two major loci are central to pigment-type switching in mouse. One is the agouti locus encoding agouti signal protein (ASIP), with mutants including non-agouti(*a*), giving a eumelanic black mouse in the absence of other mutations) and dominant yellow (*Ay*) ; the other is the melanocortin-1 receptor (*Mc 1 r*) locus, formerly extension (*e*), also with both eumelanic and pheomelanic mutants (e.g. recessive yellow, $Mc1r^{e}$) (Fig. 9). MC 1 R is a cell-surface G-protein-coupled receptor for which the best-known agonist is the soluble peptide αMSH, cleaved from the precursor pro-opiomelanocortin (POMC) in the pituitary and skin. Binding of αMSH to MC 1 R is known to activate adenylate cyclase and cAMP synthesis, promoting eumelanin synthesis through both post-translational and transcriptional pathways via microphthalmia-related transcription factor (MITF).[3] MITF is a master regulator for eumelanogenesis, melanocyte differentiation, proliferation and survival. It promotes transcription of melanocyte-specific gene products including melanosomal enzymes tyrosinase, TYRP 1 and DCT and the matrix protein SILV/PMEL. Synthesis of both eumelanin and pheomelanin start from tyrosine oxidation catalyzed by tyrosinase. The resulting dopaquinone can be a substrate for eumelanin synthesis, promoted by TYRP 1 and DCT, or pheomelanin in the presence of high cysteine concentrations and/or low tyrosinase activity.

ASIP is a soluble protein of 131 amino acids, apparently secreted by dermal papilla cells in hair bulbs. It competitively antagonizes αMSH at the MC 1 R and inhibits the eumelanogenic signal, down regulating melanogenic enzymes

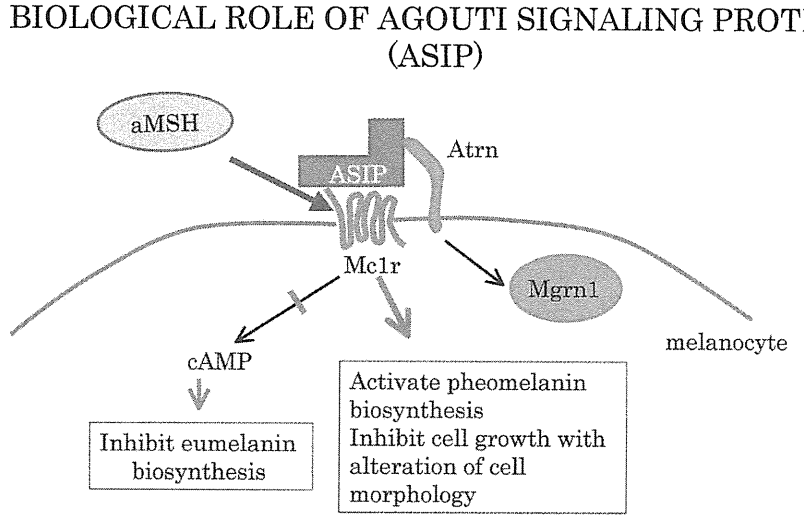

Figure 10: Activation of ASIP will result in not only eumelanin production inhibition and pheomelanin production activation but also the differentiation and proliferation of melanocyte. It is likely that there is an alternating signal transduction cascade beside cAMP cascade in this process.
This un-clarified, new cascade passes through Mc 1 r but is independent to cAMP cascade and does not include the pathway of Attractin (Atrn)/ mahogunin 1 (Mgrn 1).

and leading to pheomelanin synthesis (Fig. 10). For better understanding of ASIP signaling, we have sought to develop culture conditions under which ASIP contributes to overt pheomelanin synthesis by the melan-a immortal murine melanocyte line.[11]It was demonstrated that ASIP signaling can be reduced melanocyte growth and induce morphological dedifferentiation as well as affecting pigmentation (Fig. 10). These biological effects are mimicked in genetically yellow melanocytes, incomplete in Atrn- and *Mgrn1*-null melanocytes, appear independent of cAMP down-regulation, and require the amino terminus of ASIP.

As discussed in our previous report [18], significantly high content of pheomelanin was found in melanoma and dysplastic melanocytic nevi (DMN) lesions by analysis using high-pressure liquid chromatography (HPLC)[2,4,15-17,26,45], indicating that these abnormal melanosomes may be pheomelanic.[44,52] Our previous electron microscopic studies showed that melanocytes of dysplastic melanocytic nevi DMN were rich in abnormal melanosomes which exhibited features consistent with pheomelanosomes. It would be important to examine the type of melanogenesis in abnormal pigmentation in the aged skin such as senile freckle and solar lentigo. [11]

3. UV-photoproducts of pheomelanin and its precursors, and enhancement of skin aging process

Catalase structure and activity was seriously affected by photo-oxidation of its own substrates, hydrogen peroxide, owing to cleavage of its porphyrin active site.[2,54] Recently the over-expression of mitochondrial catalase in the murine model increased the lifespan of the mice by 40%, indicating the importance of this enzyme in the aging process.[47,54] UVA-irradiated pheomelanin altered the structure of catalase and decreased its activity in human skin.[35] Electron-spin resonance spectroscopy experiments by Sealy et al. on black and red melanin, suggested that red melanin (pheomelanin) contained a specific kind of free radical (s) not present in black eumelanin.[46]Chedekel et al. found that pheomelanin was photo-destroyed, in the presence of oxygen, by UVR.[5] Haryanvi et al., using the reversion test of Ames, demonstrated that pheomelanin became mutagenic after exposure to UVR.[11] Koch, Chedekel and Meresca et al. described photo-initiated DNA damage by melanogenic intermediates of 5-S-cysteinyldopa origin.[30,35] The binding of these molecules to DNA was activated by 300 nm UVR and resulted in single-strand breaks. However, while biosynthesis process and biological role of eumelanin after exposure to UVR are well characterized, the nature and photo-biological role of pheomelanin may still be largely unknown.

Summary and Perspective

Melanin pigmentation of the skin has been generally regarded to possess a protective role against exposure to UVR. Melanin pigment, specifically eumelanin, may therefore provide a protective role against development of sunburn, solar degeneration and skin aging as well as cancer.[24,25] Evidence accumulated recently indicates that the photoprotective role of the melanin pigment may be restricted to eumelanin as well as to the visible and UVA ranges and that in the UVB and UVC ranges both eumelanin and pheomelanin components may behave as photosensitizers. UV photolysis studies using catechols, catecholmines, and hydroxylated indole derivative (i.e., the eumelanin precursors) have demonstrated the formation of hydrated electrons, hydrogen atoms, semiquinone/ semiquinone imine radicals, and indoloxyl radicals. The photolysis of pheomelanin precursors leads to the formation of hydrated electrons, hydrogen atoms, and to alanyl and aryl thiyl radicals. Thus, while eumelanin may act as a photoprotector, its precursors may be still phototoxic.

The photoprotection by melanin pigment can be accomplished by scavenging the reactive free radicals, by quenching excited states, and by decreasing oxygen concentration in exposed tissues. The phototoxicity is related to the generation of reactive free radicals by low molecular eumelanic and pheomelanic intermediates. It may, however, be possible to modulate the photosensitivity of the pigmentary system by affecting the redox status of endogenous melanin pigments, or by switching melanin pigmentation from eumelanogensis to pheomelanogenesis, or *vice versa*, or by using sensitizers activated by red light, which is poorly absorbed by melanin.

Further investigation of the biological role in eu- and pheomelanin biosynthesis after UVR is required to provide a new insight for the better understanding the skin aging process.

Acknowledgment

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ORIGINAL ARTICLE

Usefulness of alkaline hydrogen peroxide oxidation to analyze eumelanin and pheomelanin in various tissue samples: application to chemical analysis of human hair melanins

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Summary

Eumelanin and pheomelanin in tissue samples can be specifically measured as the markers pyrrole-2,3,5-tricarboxylic acid (PTCA) and 4-amino-3-hydroxyphenylalanine after acidic permanganate oxidation and hydroiodic acid hydrolysis, respectively. Those degradation methods, although widely applied, are not easily performed in most laboratories. To overcome this difficulty, we developed alkaline H_2O_2 oxidation in 1 M K_2CO_3 that produces, in addition to the eumelanin marker PTCA, thiazole-2,4,5-tricarboxylic acid (TTCA) and thiazole-4,5-dicarboxylic acid (TDCA) as markers for pheomelanin and pyrrole-2,3-dicarboxylic acid (PDCA) as a marker for 5,6-dihydroxyindole-derived eumelanin. Those four degradation products can be easily separated by HPLC and analyzed with ultraviolet detection. The alkaline H_2O_2 oxidation method is simple, reproducible and applicable to all pigmented tissues. Its application to characterize eumelanin and pheomelanin in human hair shows that PTCA and TTCA serve as specific markers for eumelanin and pheomelanin, respectively, although some caution is needed regarding the artificial production of TTCA from eumelanic tissue proteins.

Introduction

Melanocytes in mammals and birds produce two chemically distinct types of melanin: black to brown eumelanin and yellow to reddish-brown pheomelanin (Ito and Wakamatsu, 2003; Simon and Peles, 2010; Simon et al., 2009). Both eumelanin and pheomelanin are derived from the common precursor dopaquinone that is produced from tyrosine by the action of tyrosinase (Cooksey et al., 1997). Dopaquinone is a highly reactive

Significance

The color of hair, skin, and eyes is mainly determined by the quantity and ratio of eumelanin and pheomelanin produced in melanocytes. The alkaline H_2O_2 oxidation method described herein gives relative values for eumelanin as PTCA, pheomelanin as TTCA, and dihydroxyindole (DHI)-derived eumelanin as pyrrole-2,3-dicarboxylic acid (PDCA). Thus, the ratios of TTCA PTCA, PDCA PTCA, and TTCA 4-AHP can be used to estimate relative contents of pheomelanin in melanin, DHI units in eumelanin, and benzothiazole units in pheomelanin, respectively. The measurement of PTCA, TTCA, PDCA, and TDCA in a single chromatographic analysis provides valuable information for characterizing mixed melanogenesis.

intermediate, and in the absence of thiol compounds, it undergoes intramolecular cyclization to give dopachrome (Ito and Wakamatsu, 2008). When not accelerated by any additional factors, dopachrome undergoes a mostly decarboxylative rearrangement to form 5,6-dihydroxyindole (DHI). However, dopachrome tautomerase (also known as tyrosinase-related protein-2) catalyzes the tautomerization of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Tsukamoto et al., 1992). The dihydroxyindoles DHI and DHICA are then further oxidized to produce the eumelanin polymer. On the other hand, in the presence of cysteine, its addition to dopaquinone proceeds quickly, giving 5-*S*-cysteinyldopa (5SCD) and 2-*S*-cysteinyldopa (2SCD) in a ratio of 5.3:1 (Ito and Prota, 1977). The oxidation of cysteinyldopas by dopaquinone gives rise to pheomelanin polymers via benzothiazine intermediates. A gradual conversion of benzothiazine units to benzothiazole takes place during the late stage of pheomelanin production (Wakamatsu et al., 2009).

It is the quantity and the ratio of eumelanin to pheomelanin that mainly determines the color of hair, skin, and eyes (Ito and Wakamatsu, 2003). Furthermore, it is generally accepted that eumelanin is photoprotective, while pheomelanin is phototoxic to tissues (Simon and Peles, 2010; Simon et al., 2009). Thus, the measurement of eumelanin and pheomelanin in tissue samples such as hair and skin and in cultured melanocytes provides valuable information about the 'chemical' phenotype of the sample. Fortunately, a number of specific degradation products from eumelanin and pheomelanin were identified by extensive studies in Naples in the 1960s (Prota, 1992). Based on this knowledge, we developed melanin assay methods in which eumelanin and pheomelanin could be analyzed as specific degradation products, pyrrole-2,3,5-tricarboxylic acid (PTCA) and a mixture of aminohydroxyphenylalanine isomers (AHPs), after acidic $KMnO_4$ oxidation and hydroiodic acid (HI) reductive hydrolysis, respectively (Figure 1, Ito and Fujita, 1985; Wakamatsu and Ito, 2002). The $KMnO_4$ oxidation method was later improved to make the calibration curve more linear (Ito and Wakamatsu, 1994), and the HI hydrolysis method was also improved by analyzing a more specific marker 4-amino-3-hydroxyphenylalanine (4-AHP; Wakamatsu et al., 2002). However, although PTCA is highly specific for DHICA-derived eumelanin, the $KMnO_4$ oxidation has some disadvantages in that the procedure is rather complex and involves ether extraction and the yield of pyrrole-2,3-dicarboxylic acid (PDCA) is too low for use as a specific marker for DHI-derived eumelanin such as dopamine melanin. This shortcoming was mostly overcome by introducing H_2O_2 oxidation in 1 M K_2CO_3 heated at 100°C for 20 min (Ito and Wakamatsu, 1998). That improved method was successfully applied to characterize neuromelanin isolated from the *substantia nigra* of human brain tissue in which pheomelanic units

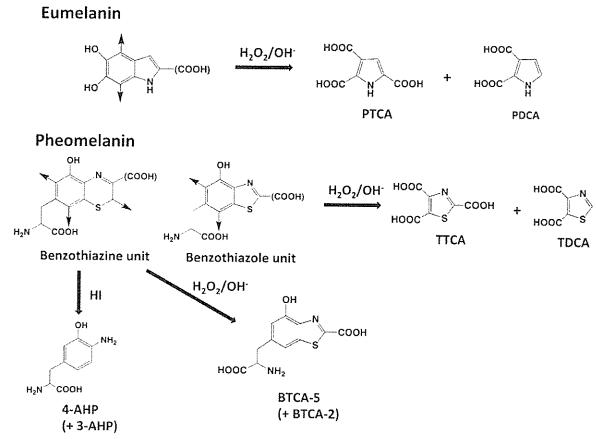

Figure 1. Chemical degradation of eumelanin and pheomelanin. Upon H_2O_2 oxidation, DHICA-derived units in eumelanin give PTCA while DHI-derived units gave PDCA. Upon H_2O_2 oxidation, benzothiazole units in pheomelanin give TTCA and TDCA. Upon H_2O_2 oxidation, benzothiazine units in pheomelanin give BTCA-5 and BTCA-2, while upon HI hydrolysis, they give 4-AHP and 3-AHP.

were analyzed as thiazole-2,4,5-tricarboxylic acid (TTCA) and thiazole-4,5-dicarboxylic acid (TDCA; Wakamatsu et al., 2003). That method was found to work well for isolated melanin samples, but did not give yields higher than those with the $KMnO_4$ oxidation (Ito and Wakamatsu, 1998). Recently, we evaluated H_2O_2 oxidation in 1 M K_2CO_3 at 25°C (alkaline H_2O_2 oxidation) to make it more useful for simultaneously analyzing eumelanin and pheomelanin in tissue samples. We have been using this method for some time (e.g., Wakamatsu et al., 2009) and have found it to be suitable for broader use among researchers compared with the previous $KMnO_4$ oxidation and HI hydrolysis methods. The alkaline H_2O_2 method reported herein is simple, reproducible, and applicable to all pigmented tissues. Its application to characterize eumelanin and pheomelanin in human hair is also described.

Results

Method evaluation

In preliminary experiments, we compared the oxidation of melanin in 1 M K_2CO_3 and in 1 M NaOH, the latter method being used by Prota and his successors for some time (Greco et al., 2009; Napolitano et al., 2000; Panzella et al., 2007). Many interfering peaks were observed in HPLC chromatograms of human and mouse hair samples oxidized with H_2O_2 in 1 M NaOH. Those interfering peaks were not found after oxidation in 1 M K_2CO_3. Therefore, we did not further evaluate the 1 M NaOH conditions, and we then examined the time course of H_2O_2 oxidation in 1 M K_2CO_3 at 25°C. Yields of PTCA and PDCA from human black hair and those of TTCA, TDCA, and PTCA from human red hair became

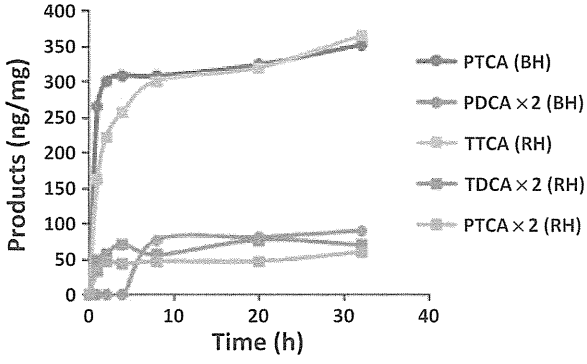

Figure 2. Time course of alkaline H_2O_2 oxidation of human black hair and human red hair. The production of PTCA and PDCA in black hair and of TTCA, TDCA, and PTCA in red hair was followed in duplicate. BH and RH denote black hair and red hair, respectively.

nearly constant at 8 h and continued to increase slightly to 32 h (Figure 2). It is interesting that PDCA appeared only after 8 h while nearly 80% of PTCA appeared as early as 1 h, indicating a complex, sequential process of the oxidation. Based on these results, we chose 20 h as the standard reaction time for the convenience of sample handling.

We then examined the linearity of the method and found that human black hair gave excellent linearity for PTCA and PDCA from 0.1 mg to 2.5 mg (r = 0.999) while human red hair also gave excellent linearity for TTCA over the same range (r = 0.999; Figure S1). The reproducibility of PTCA and PDCA in human black hair and that of TTCA and TDCA in human red hair (n = 5) were excellent to good with coefficients of variance of 2.3, 2.9, 6.6, and 11.0%, respectively (Table S1). Black hair samples cut in a length less than 5 mm gave almost the same values of PTCA with a slightly lower reproducibility (CV = 3.7%). It should be stressed that the PDCA PTCA ratio in black hair gave an excellent reproducibility (CV = 0.9%). The TTCA PTCA ratio in red hair also gave a good reproducibility (CV = 5.0%).

The application of the alkaline H_2O_2 oxidation method to synthetic melanins and tissue samples is summarized in comparison with acidic $KMnO_4$ oxidation and H_2O_2 oxidation in 1 M NaOH in Table 1. The absorbance at 500 nm (A500) after solubilization in Soluene-350 was also analyzed. In the H_2O_2 oxidation, 6-alanyl-2-carboxy-4-hydroxybenzothiazole (BTCA-5) and its isomer 7-alanyl-2-carboxy-4-hydroxybenzothiazole (BTCA-2) were also analyzed as an additional set of pheomelanin markers (Greco et al., 2009). In comparison with acidic $KMnO_4$ oxidation, yields of PTCA were increased 2-fold while those of PDCA were increased more than 10-fold, as previously reported (Ito and Wakamatsu, 1998). Upon alkaline H_2O_2 oxidation of synthetic eumelanins, the yield of PTCA increased with DHICA content while the opposite held for PDCA. This suggests the usefulness

Table 1. Contents of melanin markers in various synthetic melanins and tissue samples[a,b]

		Alkaline H_2O_2 Oxidation (ng)						Acidic $KMnO_4$ Oxidation (ng)				HI Hydrolysis (ng)	
Sample	A500	PTCA	PDCA	TTCA	TDCA	BTCA-5	BTCA-2	PTCA	PDCA	TTCA	TDCA	4-AHP	3-AHP
DHI melanin	13.8	1940	2020	<40	<40	ND[c]	ND	553	<20	<20	<20	ND	ND
DHI+DHICA (1:1) melanin	11.8	26500	1620	<40	<40	ND	ND	6850	<20	<20	<20	ND	ND
DHICA melanin	6.55	92900	<40	<40	<40	ND	ND	21200	<20	<20	<20	ND	ND
Synthetic pheomelanin[d]	4.65	7510 (5780)	2270 (1610)	14200 (15300)	4560 (3830)	51100 (98400)	10800 (17100)	197	<20	8480	2570	248000	47400
Mouse black (*a a*) hair[d]	0.534	2000 (1490)	20 (9.4)	97 (67)	<20 (<20)	<4.0 (<4.0)	<4.0 (<4.0)	1030	<2.0	14	9.1	28	17
Mouse yellow (*e e*) hair[d]	0.072	121 (109)	45 (<4.0)	243 (273)	29 (48)	273 (454)	106 (156)	20	<2.0	59	55	2790	977
Human black hair[d]	0.277	340 (339)	51 (36)	98 (177)	42 (<20)	<4.0 (<4.0)	<4.0 (<4.0)	144	<2.0	31	17	3.7	24
Human blond hair	0.054	39	7.5	38	15	<4.0	<4.0	23	<2.0	<2.0	7.5	2.5	2.3
Human red hair[d]	0.055	62 (82)	5.9 (7.3)	89 (113)	46 (59)	84 (123)	20 (20)	27	<2.0	52	13	363	77
Human melanocytes	0.048	87	28	153	8.9	375	105	26	<0.5	90	50	434	132
Human epidermis	ND	101	<20	53	<20	ND	ND	43	<10	18	<10	16	9.4

[a]Values are absorbance at 500 nm or ng per mg melanin, mg hair, 10^6 cells, or epidermis (19.6 mm^2).
[b]Mouse hair data are averages of 3 samples; other data are averages of duplicate assays.
[c]ND = Not determined.
[d]In parentheses are values obtained with oxidation in 1 M NaOH.

of the PDCA PTCA ratio as a measure to estimate the DHI DHICA ratio. High levels of PTCA were detected in eumelanic mouse and human black hair samples. The pheomelanin markers TTCA and TDCA were detected in pheomelanic tissues such as mouse yellow hair and human red hair. Cultured human melanocytes also gave a high level of TTCA (TTCA PTCA ratio = 1.76) in parallel with a high level of 4-AHP, a specific pheomelanin marker. Human epidermis gave detectable levels of PTCA and TTCA (TTCA PTCA = 0.52). This difference in the TTCA PTCA ratio between melanocytes and epidermis may be surprising; however, it was reported that human melanocytes in culture are more pheomelanic compared with the epidermis from the same individuals, with the AHPs PTCA ratio being approximately 10 times greater in the melanocytes (Hunt et al., 1995). Synthetic pheomelanin gave a high level of BTCA-5, although that level was about one-fourth that of 4-AHP. Some samples were also analyzed with H_2O_2 oxidation in 1 M NaOH (Table 1). Yields of BTCA-5 and BTCA-2 were increased 2-fold compared with the oxidation in 1 M K_2CO_3 while the yields of PTCA and TTCA were similar.

Typical HPLC chromatograms of H_2O_2 oxidation products are shown in Figure 3. PTCA was well separated from other peaks in all samples, and the other markers (PDCA, TTCA, and TDCA) were also detected in most samples. None of those markers was detected in albino hairs from mice or from humans. Among the four markers, the separation of TTCA from PDCA tended to be somewhat troublesome. When they appeared too close to each other, better separation could be achieved by changing the column temperature from 45 to 50°C. We can usually analyze more than 300 oxidized mixtures per column without any deterioration in the separation although the retention times became faster by 25%.

We next evaluated the correlation of PTCA levels obtained with alkaline H_2O_2 oxidation to that with $KMnO_4$ oxidation in 38 human hair samples. Figure 4 shows the excellent correlation (r = 0.947) with a slope of 2.00. This indicates that alkaline H_2O_2 oxidation is as reliable as $KMnO_4$ oxidation to analyze eumelanin as the specific marker PTCA.

Application to human hair samples

The usefulness of alkaline H_2O_2 oxidation was evaluated using a large (n = 228) set of human hair samples taken from students at the University of Arizona (Valenzuela et al., 2010). TTCA is a marker for benzothiazole units in pheomelanin (Figure 1). In fact, the TTCA PTCA ratios in red hair (n = 16) were high with an average of 5.98 ± 2.77 (Figure 5A), indicating the usefulness of this ratio as a measure to estimate the relative proportion of

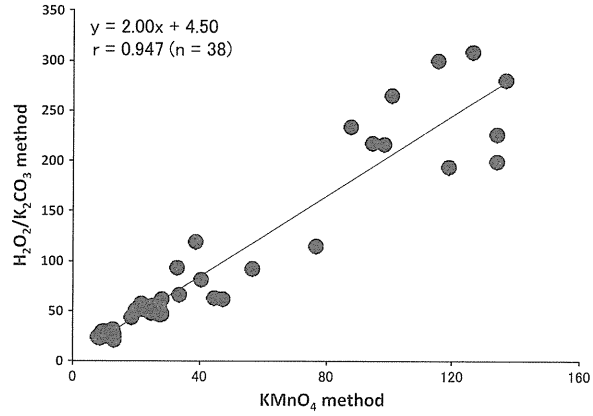

Figure 4. Correlation of PTCA levels between alkaline H_2O_2 oxidation and acidic $KMnO_4$ oxidation in human hair samples. Correlation was significant at P < 0.0001.

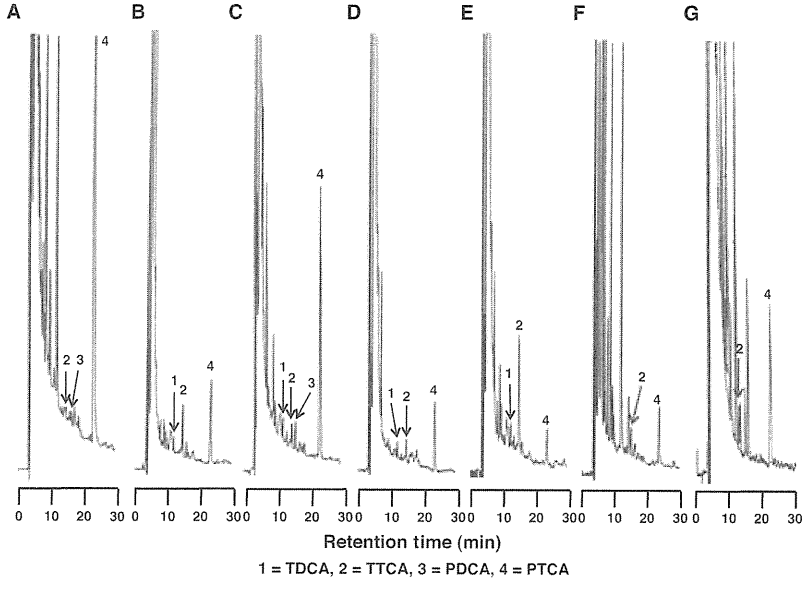

Figure 3. HPLC chromatograms of alkaline H_2O_2 degradation products. (A) mouse black (*a a*) hair, (B) mouse yellow (*e e*) hair, (C) human black hair, (D) human blond hair, (E) human red hair, (F) human melanocytes, (G) human epidermis. Amounts of samples used were 1 mg for A – E, 0.75 10^6 cells for F, and 9.8 mm^2 for G. Sensitivities were 16 mV full scale for A, B, C, and F, 8 mV for D and E, and 4 mV for G.

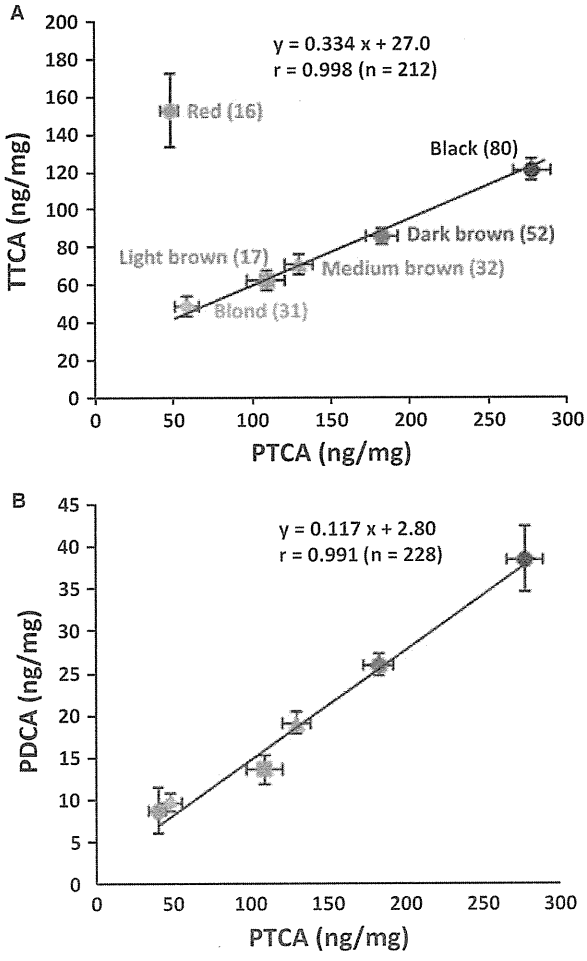

Figure 5. Correlation of PTCA levels with TTCA levels (A) and PDCA levels (B) in alkaline H_2O_2 oxidation of human hair samples. Correlations are significant with $P < 0.0001$ in A (excluding red hairs) and in B. Bars represent SEM.

pheomelanin. However, the TTCA level was also proportional to the PTCA level with a slope of 0.334 in eumelanic, black to blond hair ($r = 0.998$). This result suggested an artificial production of TTCA in eumelanic hairs. To test this possibility, we analyzed protein-bound dopa and 5SCD in eumelanic hair samples (Ito et al., 1984). A good correlation ($r = 0.800$) was found between levels of protein-bound 5SCD and TTCA, with a tendency to correlate with protein-bound dopa (Figure S2A,B).

The PDCA level was proportional to the PTCA level with an average ratio of 0.148 ± 0.013, irrespective of hair color ($r = 0.991$; Figure 5B). That ratio is much higher than that obtained for eumelanin prepared from equimolar DHI and DHICA (0.061 in Table 1) and that for mouse black hair (0.010), indicating a higher proportion of DHI in human hair.

Next, the correlation of TTCA level to 4-AHP level was examined in red hair ($n = 16$), and there was a

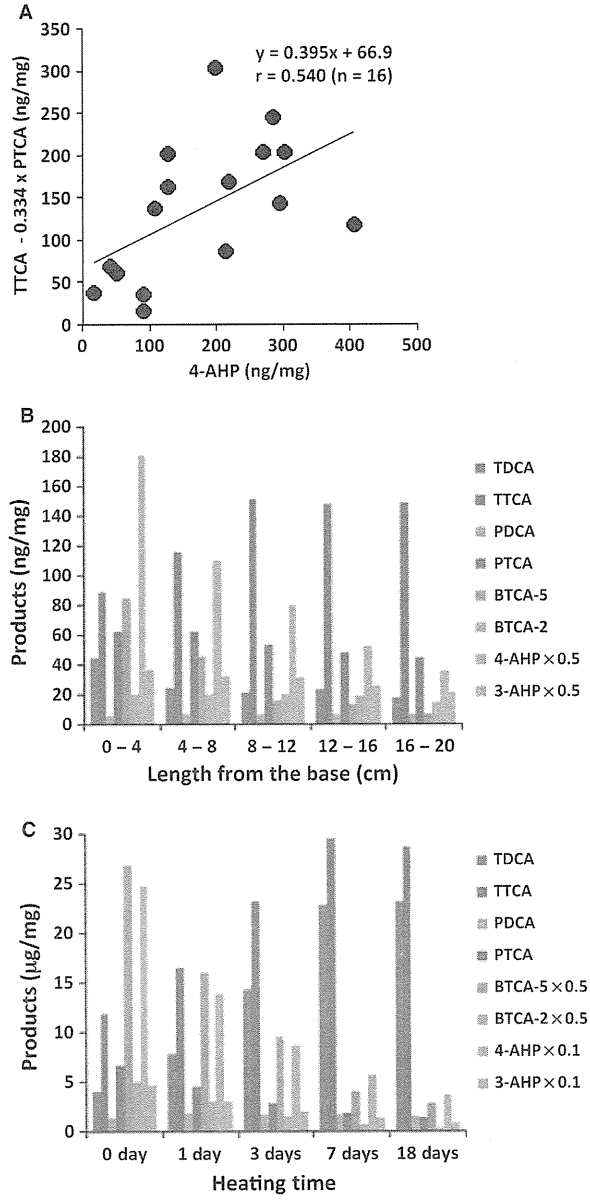

Figure 6. Evaluation of pheomelanin markers, TTCA, BTCA-5, and 4-AHP. (A) Correlation of TTCA levels with 4-AHP levels in human red hair. Correlation is significant at $P = 0.0313$. (B) (Photo)degradation of pheomelanin in vivo. Red hair sample was cut to 4 cm length. Similar trend of results were obtained with two other red hair samples. (C) Thermal degradation of synthetic pheomelanin. Melanin powder was heated at 100°C for the indicated time in the dark.

weak correlation ($P = 0.0313$, Figure 6A). In other words, the TTCA 4-AHP ratio varied considerably from one red hair sample to another, indicating variation in the benzothiazole benzothiazine ratio, as suggested by Greco et al. (2009). This possibility was reexamined by analyzing red hair samples that were cut at a 4 cm length for TTCA, AHPs, and BTCAs. The TTCA level

increased 1.7-fold from the base (0–4 cm) to the middle (8–12 cm) and then remained constant, while 4-AHP level decreased 5-fold from the base to the tip (16–20 cm) (Figure 6B). As a result, the TTCA 4-AHP ratio increased 8-fold, from 0.25 at the base to 2.0 at the tip. To mimic the degradation of pheomelanin in vivo, synthetic pheomelanin was heated at 100°C for up to 18 days (Figure 6C). After heating for 7 days, the TTCA level showed a nearly 3-fold increase to the maximum while the 4-AHP level decreased 4-fold. As a result, the TTCA 4-AHP ratio increased 10-fold, from 0.048 in the fresh pheomelanin to 0.50 in the pheomelanin heated for 7 days.

Finally, the 'chemical' phenotype (eumelanin and pheomelanin content) was evaluated against the 'visual' phenotype in human hair. Eumelanin contents were calculated by multiplying the PTCA contents by a factor of 80. This factor was based on the value of 160 in acidic $KMnO_4$ oxidation (Ozeki et al., 1996) and the slope of 2.0 (Figure 4). Pheomelanin contents were calculated by multiplying the TTCA contents (minus 0.334 PTCA) by a factor of 34. That factor is based on the 2.96% yield of TTCA after heating for 7 days at 100°C (Figure 6C). When pheomelanin contents were calculated by multiplying the 4-AHP contents by a factor of 26, the pheomelanin content in red hairs became identical to the value obtained with TTCA. This conversion factor of 26 is in close agreement with the 3.66% yield of 4-AHP after heating for 18 days at 100°C (Figure 6C).

As shown in Figure 7A, black to blond hairs contained small amounts of pheomelanin (based on the TTCA content) at nearly constant levels of 0.85–0.99 μg mg while eumelanin contents varied greatly depending on the intensity of color from black, dark brown, medium brown, light brown to blond, with levels of 22.2, 14.6, 10.4, 8.7, and 4.7 μg mg, respectively. Only red hairs contained comparable levels of eumelanin and pheomelanin at 3.8 and 4.7 μg mg, respectively. These results indicate an excellent correlation between the 'chemical' and the 'visual' phenotypes. Figure 7B shows that the level of combined amounts of eumelanin and pheomelanin correlated well with the total melanin level measured spectrophotometrically ($r = 0.992$). The slope (0.89) was close to 1, indicating the reliability of both methods.

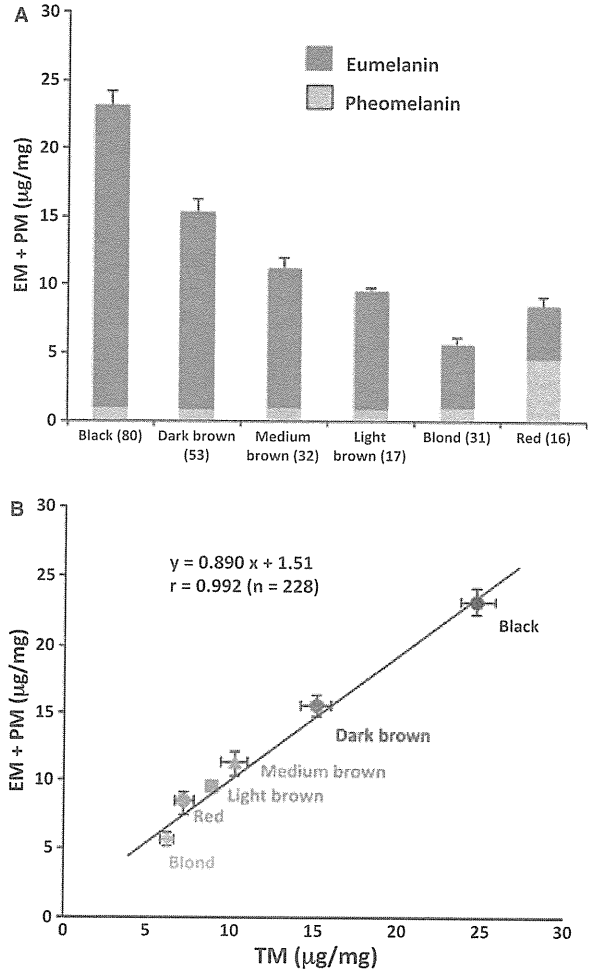

Figure 7. Chemical analysis of human hair melanin. (A) Contents of eumelanin and pheomelanin (chemical phenotypes) in human hair samples of various colors (visual phenotypes). For the conversion factors, see Results. (B) Correlation of combined amounts of eumelanin and pheomelanin with total melanin (TM) contents in human hair samples. Correlation is significant at $P < 0.0001$. Bars represent SEM.

Discussion

The alkaline H_2O_2 oxidation method described is a simple procedure because it requires only the mixing of a tissue homogenate with H_2O_2 in 1 M K_2CO_3 for 20 h at room temperature followed by acidification and direct injection into an HPLC. The extraction with ether used in $KMnO_4$ oxidation is not necessary. The method is highly reproducible because of its simplicity and has a high linearity because of the mild conditions. These are advantages over the previously used acidic $KMnO_4$ oxidation (Ito and Wakamatsu, 1994). However, because of the mild conditions, the artificial production of PTCA from pheomelanin is unavoidable, as seen in the much higher PTCA value in synthetic pheomelanin (7510 ng mg in H_2O_2 oxidation versus 197 ng mg in $KMnO_4$ oxidation) and in pheomelanic mouse yellow hair (121 ng mg versus 20 ng mg). The artificial production of TTCA in eumelanic tissues is also unavoidable, but it can be compensated for by subtracting 0.334 PTCA from the TTCA value (Figure 5A).

The measurement of PTCA is very reproducible because of the high yield and excellent separation in HPLC. Likewise, the measurement of TTCA in pheomelanic tissues is also reproducible. However, some caution is necessary to make sure of the identification of

PDCA and or TTCA present at trace to low levels. This can be achieved by co-injecting samples with standards or by comparing the ultraviolet spectrum with standards on a photodiode array detector.

PTCA can be used as a specific marker for eumelanin. More specifically, PTCA is a marker for DHICA-derived units in eumelanin. Another advantage of the alkaline H_2O_2 method is that PDCA can now be used as a marker for DHI-derived units in eumelanin (Ito and Wakamatsu, 1998). The PTCA A500 ratio has been used to estimate DHICA content in eumelanin (Lamoreux et al., 2001; Ozeki et al., 1995). In addition to this, the PDCA PTCA ratio can now be used to estimate DHI content in eumelanin. The high reproducibility is an advantage of the PDCA PTCA ratio (Table S1). In fact, the PDCA PTCA ratio remains constantly high among eumelanic hairs that vary from black to blond in color (Figure 5B). This suggests that DCT activity is rather low or negligible in those tissues. In this regard, Commo et al. (2004) showed that in individuals older than 45 yr, DCT protein in hair bulbs is not detectable in brown hair or in black hair, of African, Asian, or Caucasian origin. In the present study, we used hair samples from younger subjects. Therefore, the effect of aging on the DHI DHICA ratio should be explored in a more carefully controlled study.

We have been using the 4-AHP content as a measure to estimate pheomelanin content because it is highly specific for pheomelanin and is highly sensitive because of the use of electrochemical detection. On the other hand, TTCA is not as specific as 4-AHP as a marker for pheomelanin and is much less sensitive because of the use of UV detection. Nevertheless, it is a great advantage of TTCA that it can be analyzed simultaneously with PTCA (and PDCA). In fact, the TTCA PDCA ratio was successfully used to estimate cysteinyldopamine-derived units in isolated neuromelanin (Wakamatsu et al., 2003). On the other hand, the measurement of 4-AHP requires the evaporation of HI, which is rather tedious to perform in biological laboratories. Previously, the 4-AHP PTCA ratio has been used to compare the relative pheomelanin content in mixed melanogenesis (Ito and Wakamatsu, 2003), and the TTCA PTCA ratio can be used for the same purpose. It should be remembered however that 4-AHP is produced from benzothiazine units in pheomelanin in an early stage of pheomelanin production while TTCA is from benzothiazole units in its late stage (Wakamatsu et al., 2009). It is thus expected that the TTCA 4-AHP ratio may be used to follow the (photo)aging process of pheomelanin (Figure 6B,C)(Greco et al., 2009). To obtain more constant ratios of TTCA 4-AHP in human red hair samples, we need to cut hair samples from the base (0–4 cm) and keep them refrigerated.

Alkaline H_2O_2 oxidation affords another interesting pheomelanin marker, BTCA-5 (and BTCA-2). Napolitano et al. (2000) introduced BTCA-5 that is produced by H_2O_2 oxidation in 1 M NaOH to estimate pheomelanin content. Nezirević Dernroth et al. (2010) have recently identified BTCA-5 and BTCA-2 in the urine of patients with diffuse melanosis of melanoma. How BTCAs are produced from pheomelanin by H_2O_2 oxidation is not clearly understood at present. However, the BTCA-5 levels in red hair and aging experiments of synthetic pheomelanin (Figure 6B,C) correlate very well with the 4-AHP levels ($r = 0.984$ and 0.996, respectively). That suggests that during alkaline H_2O_2 oxidation, BTCAs arise from benzothiazine units in pheomelanin through base-catalyzed ring contraction to benzothiazole units (McCapra and Razavi, 1975) followed by oxidative fission to form the carboxyl group. The usefulness of BTCA-5 as a pheomelanin marker was not thoroughly examined in this study. However, it is apparent that the advantage of this marker is the high 5.1% yield (9.8% in 1 M NaOH) from synthetic pheomelanin. But, the yields of BTCA-5 (even with 1 M NaOH) were not high enough in pheomelanic hairs compared with those of 4-AHP (Table 1). Furthermore, HPLC analysis of BTCAs requires a higher concentration of methanol, and under these conditions, only PTCA can be adequately analyzed while PDCA, TTCA, and TDCA emerge too early in HPLC. Therefore, we prefer TTCA to BTCA-5 as a pheomelanin marker for broader applications. Nevertheless, it is fair to mention that the artificial production of TTCA from eumelanic hairs is a disadvantage of TTCA. It is likely that this TTCA is produced from protein-bound forms of dopa, 5SCD, and or related metabolites present in eumelanic hairs (Ito et al., 1984).

In the present study, the alkaline H_2O_2 oxidation method was evaluated as an approach to simultaneously measure eumelanin and pheomelanin. Application to human hair samples shows that the PTCA and TTCA values ('chemical' phenotype) obtained with this method accurately reflect the 'visual' phenotype. Pheomelanic red hairs are quite distinctive from eumelanic hairs with a high TTCA level (153 ng mg) and a high TTCA PTCA ratio (5.99). On the other hand, blond hairs have a lower TTCA level (49 ng mg) and a lower TTCA PTCA ratio (0.95) while both have similar PTCA levels (48 ng mg in red hairs versus 59 ng mg in blond hairs). The constant yet low level of pheomelanin in eumelanic hairs (from black to blond) is consistent with the casing model of mixed melanogenesis in which pheomelanin is always produced first, and then, eumelanin is deposited on the preformed pheomelanin (Ito and Wakamatsu, 2008; Simon and Peles, 2010; Simon et al., 2009). This process of mixed melanogenesis is exemplified in cultured epidermal and uveal melanocytes (Wakamatsu et al., 2006, 2008). In this connection, the time course of H_2O_2 oxidation (Figure 2) is worthwhile to comment. From red hair sample, the production of TTCA reached a maximal at 8 h, while that of PTCA required only 2 h. This result is consistent with the casing model of mixed melanogenesis. Although this oxida-

tion condition was not intended to study the casing model, milder conditions of H_2O_2 oxidation may serve to prove the model by following the time course of appearance of PTCA and TTCA in samples containing both eumelanin and pheomelanin.

In this study, we used a conversion factor of 34 for TTCA (minus 0.334 PTCA) to calculate pheomelanin content. The same value of pheomelanin level in red hairs can be obtained with a conversion factor of 26 for 4-AHP instead of nine (Wakamatsu et al., 2002). Interesting, pheomelanin levels in eumelanic hairs (black to blond) were several-fold greater with TTCA than with 4-AHP. Which marker, i.e. TTCA or 4-AHP, is more accurate in estimating trace levels of pheomelanin in eumelanic hairs (and other pigmented tissues) needs to be examined in future studies.

In conclusion, the alkaline H_2O_2 oxidation method can be applied to characterize eumelanin and pheomelanin in all pigmented tissues and should be suitable for routine and comparative purposes on large numbers of samples.

Methods

Melanin markers, melanins, and tissue samples

The melanin markers PTCA and PDCA were prepared as described in Ito and Wakamatsu (1998) and TTCA and TDCA were prepared as described in Wakamatsu et al. (2003) with minor modifications to improve the yields. As a working standard, a solution containing 1 μg each of PTCA, PDCA, TTCA, and TDCA in 1 ml of 0.1 mol l potassium phosphate buffer (pH 2.1) is routinely used (and will be provided upon request). The preparation of 4-AHP was as described in Wakamatsu et al. (2009). 3-AHP was purchased from Sigma-Aldrich (St. Louis, MO, USA). BTCA-5 and BTCA-2 were prepared according to the methods reported in Napolitano et al. (1996) and Greco et al. (2009), respectively.

DHI melanin, DHI+DHICA (1:1) melanin, and DHICA melanin were prepared under the drastic conditions described for pheomelanin (Wakamatsu et al., 2009; manuscript in preparation). Synthetic pheomelanin was prepared according to the mild conditions described in Wakamatsu et al. (2009).

Mouse black (*a a*), recessive yellow (*e e*), and albino (*c c*) hair samples were obtained from Dr. Tomohisa Hirobe of the National Institute of Radiological Sciences (Chiba, Japan). Data from $KMnO_4$ oxidation and HI hydrolysis were taken from those published in Hirobe et al. (2011).

Pigmented hair was collected from 228 students at the University of Arizona, aged 18-40 years (Valenzuela et al., 2010). Based on visual examination by trained personnel, hair samples were divided into black (n = 80), dark brown (52), medium brown (32), light brown (17), blond (blond brown and blond combined, n = 31), and red (blond red and red combined, n = 16). Hair samples were cut 1 cm from the base. For experiments to evaluate the alkaline H_2O_2 oxidation method, we used black hair from a Japanese male (66 years old) and red hair from a German female (35 year old). Human white hair from a tyrosinase-negative albino patient was obtained from Dr. Yasushi Tomita (formerly of Nagoya University).

Human melanocytes were cultured at Beiersdorf (Hamburg, Germany). Human suction blister epidermis (5 mm in diameter, 19.6 mm^2) was obtained from an individual with phototype III-IV at Beiersdorf.

Chemical analyses

Alkaline H_2O_2 oxidation to measure eumelanin (as PTCA and PDCA) and pheomelanin (as TTCA and TDCA) was performed as described in Wakamatsu et al. (2009). In brief, 100 μl of water suspensions of samples (0.1 mg synthetic melanin, 1.0 mg hair, 0.75 10^6 cells, or 9.8 mm^2 skin) was placed in 10-ml screw-capped conical test tubes, to which 375 μl 1 mol l K_2CO_3 and 25 μl 30% H_2O_2 (final concentration: 1.5%) were added. The tubes were mixed vigorously at 25 ± 1°C for 20 h on a test-tube mixer. The residual H_2O_2 was decomposed by adding 50 μl 10% Na_2SO_3 and the mixture was then acidified with 140 μl 6 mol l HCl. Each reaction mixture was centrifuged at 4000 *g* for 1 min, and an aliquot (80 μl) of each supernatant was directly injected into the HPLC system. H_2O_2 oxidation products were analyzed with an HPLC system consisting of a JASCO 880-PU liquid chromatograph (JASCO Co., Tokyo, Japan), a Shiseido C18 column (Capcell Pak MG, 4.6 250 mm, 5 μm particle size; Shiseido, Tokyo, Japan), and a JASCO UV detector at 269 nm (JASCO Co., Tokyo, Japan). The mobile phase was 0.1 mol l potassium phosphate buffer (pH 2.1) methanol, 99:1 (v v). Analyses were performed at 45 or 50°C at a flow rate of 0.7 ml min. BTCA-5 and BTCA-2 were analyzed with a mobile phase of 0.1 mol l potassium phosphate buffer (pH 2.1) methanol, 94:6 (v v) at 50°C and 289 nm.

Acidic $KMnO_4$ oxidation to measure eumelanin (as PTCA) was performed as described by Ito and Wakamatsu (1994). HI reductive hydrolysis to measure pheomelanin (as 4-AHP and 3-AHP) was performed as described in Wakamatsu et al. (2002).

The method for the measurement of protein-bound dopa and 5SCD is described in Wolber et al. (2008). Data presented are the amounts of free and protein-bound forms combined, as the amounts of free forms of dopa and 5SCD were less than one-tenth in mouse hairs (Ito et al., 1984).

Soluene-350 solubilization to measure total melanin was performed as described in Ozeki et al. (1996) with a minor modification. In brief, 100 μl water suspension containing 1 mg of each hair sample was taken in a 10-ml screw-capped conical test tube, to which 900 μl Soluene-350 (Perkin-Elmer, Waltham, MA, USA) was added. The tube was vortex-mixed and heated at 100°C (boiling water bath) for 15 min. The tube was vortex-mixed again and was heated at 100°C (boiling water bath) for an additional 15 min. The mixture was centrifuged at 4000 *g* for 3 min, and the supernatant was analyzed for absorbance at 500 nm (A500). Data were corrected against the absorbance of 0.020 for the background (Ozeki et al., 1996). To convert to the absolute amount of total melanin (TM), the A500 values are multiplied by a factor of 101 μg (Ozeki et al., 1996).

Statistical analyses

Students' *t* test and multivariate statistics were employed with JMP 7.01 software (SAS Institute Inc., Cary, NC, USA).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Linearity of alkaline H_2O_2 oxidation of human black hair and human red hair. The production of PTCA and PDCA in black hair and of TTCA in red hair were examined.

Figure S2. Correlation of TTCA level with levels of (A) protein-bound dopa and (B) protein-bound 5SCD. (A) Correlation with protein-bound dopa; there was a tendency of correlation with $P = 0.0783$. (B) Correlation with protein-bound 5SCD; the correlation was significant at $P < 0.0001$.

Table S1. Reproducibility of alkaline H_2O_2 oxidation.

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ORIGINAL ARTICLE

Rab7 is a critical mediator in vesicular transport of tyrosinase-related protein 1 in melanocytes

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ABSTRACT

How melanosomal proteins such as enzymic proteins (tyrosinase and tyrosinase-related proteins, Tyrps) and structural protein (gp100) are transported from Golgi to melanosomal compartments is not yet fully understood. A number of small GTPases have been found to be associated with melanosomes and we have identified one of them, Rab7, a regulator of vesicular transport, organelle motility, phospholipid signaling and cytosolic degradative machinery, as being involved in the transport of Tyrp1 from Golgi to stage I melanosomes. This study further characterizes the role of Rab7 as a regulator of differential sorting of melanosomal proteins in this process. Murine melanocytes were transiently transfected with a plasmid encoding either wild-type (Rab7WT), constitutively active (Rab7Q67L) or dominant-negative (Rab7N125I and Rab7T22N) Rab7. Through immunocytostaining and confocal laser scanning microscopy, we quantitatively compared the bio-distribution of melanosomal proteins between Rab7WT-expressing cells and mutant Rab7-expressing cells. We also characterized their differential elimination from melanosomal compartments by Rab7 by utilizing a proteasome inhibitor, MG132. Our findings indicate that Rab7 plays an important role in differential sorting of tyrosinase, Tyrp1 and gp100 in early melanogenesis cascade, and that it is more specifically involved with Tyrp1 than tyrosinase and gp100 in the trafficking from Golgi to melanosomes and the specific exit from the degradative process.

Key words: gp100, melanogenesis, melanosome, tyrosinase, tyrosinase-related protein, Tyrp1.

INTRODUCTION

Melanosomes are melanocyte-specific organelles that produce and store melanin pigments inside. They are lysosome-related organelles, sharing several important properties common to lysosomes, such as the presence of luminal and membrane lysosomal proteins, an acidic luminal environment, accessibility to endocytic tracers and the ability to fuse with phagosomes.[1] They undergo four maturation stages with distinctive morphological changes.[2,3] Once they fully mature, they are stored in the peripheral area or in the cell processes and are transferred to adjacent keratinocytes.

Tyrosinase and its related proteins (tyrosinase-related proteins 1 and 2, Tyrp1 and 2) are the membrane glycoproteins and major melanosomal enzymes. Tyrosinase is a key enzyme that catalyzes the oxidation of tyrosine to dopaquinone which is a source for both black/brown eumelanin and red/yellow pheomelanin.[4,5] Tyrp1 has dihydroxyindole carboxylic acid oxidase activity while Tyrp2 has

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dopachrome tautomerase activity, and both are related to the eumelanin synthesis.[4,6] Tyrp1 is also associated with tyrosinase in melanosomes and functions as a tyrosinase-stabilizer.[7] The loss or dysfunction of tyrosinase and Tyrp1 results in oculocutaneous albinism types 1 and 3, respectively.[8,9] gp100 (also known as Pmel17) is another unique melanosomal glycoprotein that is synthesized as a type 1 integral membrane protein and is cleaved off enzymatically in post-Golgi compartments.[10] The cleaved fragments accumulate in the lumina of stage I/II melanosomes and form unique striated structures on which newly-synthesized melanin is deposited.[3] gp100-associated albinism has not been reported in humans, but dysfunction of gp100 in mice causes premature death of follicular melanocytes, resulting in a unique hair color pattern (*silver* phenotype). Not only the dysfunction of these melanosomal proteins but also their transport failure, results in depigmentation disorders such as Hermansky–Pudlak syndrome.[11]

Both tyrosinase and Tyrp1 are transported from the *trans*-Golgi network to melanosomes through endosomal compartments. Adaptor proteins (AP) generate transport vesicles, sort proteins and assemble clathrin in the post-Golgi network.[12] AP-3 binds to a dileucine motif of tyrosinase.[13–15] Tyrosinase can use both AP-1 and AP-3 for its proper sorting while Tyrp1 can use only AP-1.[2,14,16] This suggests that they use different transport pathways even though they have similar protein structures and functional domains, including the dileucine motif.

Rab7 is a member of the Rab small glutamyl transpeptidase (GTP)-binding protein family and, by affecting its effector molecules, is essential for the regulation of vesicular transport, organelle motility, phospholipid signaling, phagocytosis and autophagocytosis.[17–29] Our previous study of charactering the fractionated melanosome-associated proteins found that Rab7 was associated with the melanosomal membrane of B16 murine melanoma cells and was involved in the transport of Tyrp1.[30] We also reported that exogenously-expressed Tyrp1 was localized to early endosomes in human amelanotic melanoma cells when Rab7 was inhibited.[31] These findings suggest that the transport of Tyrp1 requires functional Rab7 and that Tyrp1 passes through endosomal compartments. However, it is still not clarified whether Rab7 is involved in the transport of other major melanosomal proteins such as tyrosinase and gp100, and if it is, to what extent. It also remains uncertain whether Rab7 functions in the trafficking of melanosomal proteins in non-tumorigenic melanocytes. Here, we transfected immortal melanocytes with plasmids carrying cDNA of wild-type and mutant Rab7, and analyzed their differential effects on the intracellular trafficking of endogenous tyrosinase, Tyrp1 and gp100 by single-cell observation with immunofluorescent staining and confocal laser scanning microscopy. We found that the inhibition of Rab7 led to preferential Tyrp1 elimination from melanocytes. Our results indicate that the transport pathway of Tyrp1 to melanosomes is different from that of tyrosinase and gp100 and that Rab7 is a crucial regulator for Tyrp1 sorting in the endosomal compartment, promoting its exit from the degradative process.

METHODS

Vector construction

Rab7WT, a cDNA of wild-type Rab7, was amplified from pGEM-canine Rab7[32] by using the N-terminal flag sequence-containing primer with the *Hin*dIII site and C-terminal primer with the same restriction site. Oligonucleotides used for the amplification were 5′-CCC AAG CTT ACC ATG *GAC TAC AAG GAT GAC GAT GAC AAG* ACC TCT AGG AAG AAA GTG TTG-3′ and 5′-CCC AAG CTT TCA GCA ACT GCA GCT TTC CGC-3′ (underlines indicate the *Hin*dIII site, and italics the flag sequence). A cDNA of Rab7N125I was constructed through polymerase chain reaction-mediated mutagenesis[33] by which a partial sequence of the flag-tagged *Rab7WT* oligonucleotide was modified as A374T for *Rab7N125I*. The fragment of flag-tagged *Rab7N125I* was inserted into the *Hin*dIII site of pcDNA3.1/Hygro (+) (Invitrogen, Carlsbad, CA, USA). The inserted cDNA was verified by nucleotide sequencing. p3xFLAG-CMV-7.1 fused with cDNA of *Rab7T22N* or *Rab7Q67L* was described previously.[34]

Cell culture and transient transfection

Melan-A cells, kindly provided by Dr Dorothy C. Bennett, UK, were cultured on coverslips in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum, 200 nmol/L phorbol myristate acetate, 100 U/mL penicillin and