(Jiménez-Cervantes et al., 1994; Kobayashi et al., 1994b). The *brown* mutation encodes Tyrp1 that is not properly translocated to melanosomes, resulting in no functional Tyrp1 activity and decreased tyrosinase function. Homozygosity for the mutant *brown* allele significantly suppresses the production of eumelanin (total melanin and PTCA values), as evidenced by comparing wild-type *black* with *brown* and of *black chinchilla* with *brown chinchilla*. The *brown* mutation does not significantly alter the proportion of DHICA in the eumelanin synthesized, but rather, *brown* eumelanin appears to have a smaller molecular size compared to wild-type *black* eumelanin (Ozeki et al., 1997).

In humans, TYRP1 does not appear to function as a DHICA oxidase (Boissy et al., 1998). Although the exact enzymatic function of TYRP1 is not known, it appears to stabilize tyrosinase (and DCT) in mice and in humans (Kobayashi et al., 1998). It is not known whether polymorphisms in the *TYRP1* gene affect normal human pigmentation. However, one mutation at this gene, called OCA3, has been reported to alter the black hair color in Africans to brown (Boissy et al., 1996). A similar hypopigmentation effect caused by OCA3 was also observed in one Caucasian (Rooryck et al., 2006).

Oca2 (p)/OCA2 (P), Slc24a5/SLC24A5 (NCKX5), Slc45a2/SLC45A2 (MATP), and *Si/SILV (PMEL17)* loci

The *pink-eyed dilution* (*p*) gene (now called *Oca2* gene) in mice encodes an integral melanosomal membrane protein with a 12-membrane-spanning structure, and mutations at this locus suppress eumelanin production (Rosemblat et al., 1994), reducing it more than 10-fold (Ozeki et al., 1995). Interestingly, *p*-mutant melanocytes secrete most of the melanin they produce to the culture medium (Hirobe et al., 2002). A similar observation was reported with melan-p1 melanocytes by Potterf et al. (1998). Also, *pink-eyed dilution* mice secrete high levels of melanin-related metabolites, such as 5-*S*-cysteinyldopa, into the blood compared to wild-type mice (Wakamatsu et al., 2007). Thus, it seems likely that the *p* mutation causes a defect in retaining melanin and melanin precursors in melanosomes. Alternatively, the *p* mutation disrupts tyrosinase processing and intracellular trafficking to melanosomes, thus suppressing their normal maturation (Ni-Komatsu and Orlow, 2006; Toyofuku et al., 2002).

Mutations at the *OCA2* (*P*) locus in humans cause reductions in eumelanin production and null mutations lead to another form of oculocutaneous albinism, OCA2 (Suzuki and Tomita, 2008). Several SNPs of the *OCA2* gene are associated with hair or skin color variations in Australians and in East Asians (Edwards et al., 2010; Shekar et al., 2008b). A single SNP in the *HERC2* gene, which functions upstream of *OCA2* has been postulated to lead to decreased expression of OCA2 protein within melanocytes. It is a causative SNP for blue eye color with effects also apparent on skin and hair color (Branicki et al., 2009; Cook et al., 2009; Sturm, 2009).

Mutations in the *Slc24a5* gene have not been found in mice, probably because the mutant is indistinguishable from wild-type mice (Vogel et al., 2008). Instead, the mutation was originally found in *golden* zebrafish and in humans (Lamason et al., 2005). The *Slc24a5* gene encodes a potassium-dependent sodium–calcium exchanger denoted NCKX5 (Ginger et al., 2008). The evolutionarily conserved ancestral allele of a human coding polymorphism, Ala111Thr, predominates in African and in East Asian populations, while the variant allele is nearly fixed in European populations (Sturm, 2009). That polymorphism correlates with lighter skin pigmentation in admixed populations, accounting for 25–38% of the difference in skin color between Europeans and Africans (Lamason et al., 2005).

The *Slc45a2* (*underwhite*) locus in mice encodes a membrane-associated transporter protein (Matp) that has a 12-transmembrane-spanning structure (Newton et al., 2001). The mutations (*uw*, uw^{d}, and UW^{dbr}) at the *underwhite* locus reduce the production of eumelanin >90% compared to wild-type mice (Lehman et al., 2000). The hypopigmentary effect of the *underwhite* mutation is independent of *p*, because double mutant mice at *uw* and *p* have an *albino* appearance. The pigmentary function of Matp is not fully clarified. However, Costin et al. (2003) reported that processing and trafficking of tyrosinase to melanosomes is disrupted and tyrosinase is abnormally secreted from *underwhite* mouse melanocytes, a process similar to that seen in *p* melanocytes.

The orthologue of *underwhite* in mice was identified in medaka as the *b/Aim1* gene (Du and Fisher, 2002; Fukamachi et al., 2001) and then in humans as the *MATP* gene, mutations of which cause severe hypopigmentation, named OCA4 (Inagaki et al., 2004; Newton et al., 2001). The ancestral allele of the *MATP* coding polymorphism is also known; the 374Leu allele predominates in Africans and in East Asians, while the 374Phe allele is found at high frequency in Europeans (Sturm, 2009). The 374Leu polymorphism is highly associated with black hair (Branicki et al., 2008; Graf et al., 2005).

Pigmentary effects of mutations in the *OCA2* (*P*), *SLC45A2* (*MATP* or *OCA4*), and *SLC24A5* (*NCKX5*) genes in humans have been studied extensively in recent years. Most of those studies were aimed at identifying polymorphisms that cause the diversity in normal pigmentation in the skin, hair, and eyes between and within human populations (McEvoy et al., 2006; Stokowski et al., 2007; Sturm, 2006, 2009). However, biochemical studies that correlate those genotypes to chemical phenotypes are limited (Cook et al., 2009; Valenzuela et al., 2010).

Mutations at the *silver* (*Pmel17*) locus affect eumelanin production only slightly (20% reduction) on *nonagouti* background. However, the effects become more pronounced (40–50% reduction) when interacting with the *brown* locus (Lamoreux et al., 2001). Thus, the effects of mutations at the *brown* and *silver* loci are additive. The silver protein is important to the biogenesis of early stage melanosomes (Kobayashi et al., 1994a; Theos et al., 2005) and is required for and is the primary component of the matrix fibrils in eumelanosomes (Theos et al., 2006). In humans, the homologue *SILV* (*PMEL17*) has not been linked with hair pigmentation.

A/ASIP, Atrn/ATRN, Mc1r/MC1R, Pomc/POMC, and *Slc7a11/SLC7A11* loci

The pigment type switching between eumelanogenesis (black in mice and black to dark brown in humans) and pheomelanogenesis (yellow in mice and red in humans) has been studied extensively in the past 15 yr since human MC1R polymorphisms that cause the red hair color (RHC) phenotype were discovered (Rees, 2003; Valverde et al., 1995).

The master regulator of pigment-type switching is the melanocortin-1 receptor (Mc1r/MC1R). Briefly, when α-MSH binds MC1R on the plasma membrane of melanocytes, adenyl cyclase is activated through the stimulatory G-protein, raising levels of the second messenger cAMP, thereby activating the melanogenic transcription factor MITF (Bertolotto et al., 1998; Walker and Gunn, 2010). This leads to upregulation of many genes required for pigment production such as *TYR, TYRP1, DCT, OCA, SLC24A5,* and many others (Le Pape et al., 2009; Levy et al., 2006; Spry et al., 2009). Agouti signaling protein (Asip/ASIP), encoded by the *A/ASIP* gene, is a competitive antagonist that inhibits the eumelanogenic effect of α-MSH by competing with it for binding to the MC1R. Using cDNA microarrays, Le Pape et al. (2009) demonstrated that ASIP blocks the transcriptional effects of α-MSH.

Burchill et al. (1986) examined the effects of α-MSH on mixed melanogenesis in *viable yellow* mice. When pubertal mice producing a mixed-type melanin were injected with α-MSH, tyrosinase activity increased twofold and more eumelanic hair was produced with a concomitant increase in total melanin. When those pubertal mice were injected with bromocriptine (which reduces α-MSH secretion), tyrosinase activity was greatly reduced and pheomelanic hair was produced along with a decrease in total melanin. These results clearly indicate a significant role of tyrosinase activity in controlling mixed melanogenesis; higher tyrosinase activities favor eumelanogenesis, while lower activities favor pheomelanogenesis.

Asip requires two accessory proteins for pigment-type switching; the products of the *mahogany (mg)* and *mahoganoid (md)* loci (Walker and Gunn, 2010). The *mahogany* locus was identified as the mouse orthologue of the human *attractin (ATRN)* gene, and the *mahoganoid* locus encodes a novel RING-domain-containing protein. Mice homozygous for *mahogany* and heterozygous for *lethal yellow* produce a mixed-type melanin with a low level of eumelanin (<15% of *nonagouti black*) and have a reduced level of pheomelanin (~60% of *lethal yellow*). Similarly, Gunn et al. (2001) found that three *Atrn* mutants, either homozygous or compound heterozygous, showed a pheomelanin content 5- to 10-fold lower than wild-type agouti C3H/HeJ mice. The chemical phenotype of *mahoganoid* mice has not been studied. In humans, pigmentary effects of mutations in the human orthologues, ATRN and MGRN1, are not known.

Another control point in mixed melanogenesis is the concentration of cysteine in melanosomes (del Marmol et al., 1996). Chintala et al. (2005) demonstrated that the subtle gray (*sut*) mutation in mice arose because of a mutation in the *Slc7a11* gene that encodes the plasma membrane cystine/glutamate exchanger xCT. The resulting low rate of extracellular cystine transport into *sut* melanocytes reduces pheomelanin production with minimal or no effect on eumelanin production. In fact, the effect of the *sut* mutation on pheomelanin production was markedly accentuated on the A^y/a background, reducing pheomelanin levels in hair to one-sixth of the control level. In humans, no mutations in SLC7A11 are known to affect pigmentation.

In humans, several polymorphisms of the *MC1R* gene cause the RHC phenotype (Rees, 2003; Valverde et al., 1995). We have shown that RHC polymorphisms in *MC1R* can account for 67% variance of log values of eumelanin to pheomelanin ratio (Naysmith et al., 2004). Polymorphisms in other genes such as *OCA2, SLC45A2,* and *SLC24A5* may also contribute to the RHC phenotype in an additive manner (King et al., 2003; Valenzuela et al., 2010). A polymorphism in *ASIP* gene shows strong association with red hair (Sulem et al., 2008).

Mutations in the *proopiomelanocortin* (*POMC*) gene, which encodes the propeptide for α-MSH, cause red hair in humans, although only in rare cases (Krude et al., 1998). Interestingly, we found a case of a POMC mutation in a patient of African ancestry who did not exhibit the RHC phenotype or hypopigmentation (Clément et al., 2008). It was suggested that MC1R in this patient has a constitutive activity exhibited in the absence of its ligand α-MSH. The constitutive activity of Mc1r has been observed in mice; hairs of *nonagouti* mice lacking *Pomc* are indistinguishable from those of $Pomc^+$ mice (Slominski et al., 2005), although on *agouti* background (in the presence of agouti signaling protein) *Pomc*-null mice are more yellowish than $Pomc^+$ mice (Walker and Gunn, 2010; Yaswen et al., 1999).

What we have learned and have not learned from mouse coat color pigmentation

More than 300 pigmentary genes are known in mice. Mutations in those coat color genes have provided mice

with a diverse spectrum of colors, ranging from black, brown, light brown, gray, to yellow (Silvers, 1979). As have been discussed earlier, most of pigmentary genes in humans find the corresponding orthologues in mice that have been identified prior to the human orthologues. Thus, the progress in pigmentation research in the past 30 yr enjoyed the benefits from using those mutant mice or melanocytes isolated from them. However, the rapid upsurge in genetic studies has found pigmentary genes in humans that do not have precedents in mice. Typical examples are *SLC24A5* (Lamason et al., 2005) and *TPCN2* (Calcraft et al., 2009). The lack of mouse models of those mutants appears to stem from the fact that mutations in those genes would not have caused dramatic changes in mouse coat color. Another problem in relying on mouse models seems that coat color mutations in mice often lead to a complete loss-of-function with a drastic reduction in melanin content or a distinct change in eumelanin to pheomelanin ratio. On the contrary, diversity in normal human pigmentation is likely to result from the interaction of multiple genes with only a partial loss-of-function (Branicki et al., 2009; Cook et al., 2009; Sturm, 2009; Valenzuela et al., 2010). However, studies with double mutant or even triple to quadruple mutant mice would give valuable information regarding interaction of the coat color genes involved (Chintala et al., 2005; Gautam et al., 2006; Lamoreux et al., 2001; Lehman et al., 2000). Also, a caution should be given to the fact that proteins encoded by the same genes in mice and humans do not necessarily function in the same way; an example is found in Tyrp1/TYRP1 (Boissy et al., 1998).

Perspective for the diversity in human hair pigmentation

Effects of acidic pH on mixed melanogenesis

The role of pH in controlling mixed melanogenesis is now receiving much attention because it was recently found that melanosomes in melanocytes from White/fair skin are acidic, while those from Black/dark skin are near neutral (Smith et al., 2004). Also, tyrosinases from White and Black melanocytes have the same optimum at pH 7.4 with greatly reduced activities at acidic pHs; activities at pH 5.8 were ~20% those at pH 6.8 (Fuller et al., 2001). Furthermore, the great diversity in normal human skin pigmentation appears to stem from polymorphisms in only several genes, including *OCA2*, *MATP*, and *SLC24A5* (Lamason et al., 2005; Lao et al., 2007; Norton et al., 2007). Available evidence suggests that polymorphisms in those genes may result in the acidification of melanosomes. However, the significance of pH in controlling mixed melanogenesis has only been addressed directly in a study by Ancans et al. (2001). They showed by neutralizing intramelanosomal pH in cultured human melanocytes and in melanoma cells that cells with greatly increased tyrosinase activity after neutralization had a preferential increase in the production of eumelanin with an increase in the eumelanin to pheomelanin ratio. A recent study has shown that activation of the cAMP pathway by α-MSH or forskolin leads to an alkalization (neutralization) of melanosomes and a fourfold increase in melanin content (Cheli et al., 2009). Although that study links the pH of melanosomes to the cAMP pathway for the first time, the issue of mixed melanogenesis was not examined.

A hypothetical model for roles of transporters for melanin precursors and ions

Human hair pigmentation is very diverse, especially among Europeans. The genetic basis for the RHC phenotype has been largely attributed to mutations in *MC1R*. Biochemically, as shown in Figure 1, the presence of a minimal level of cysteine is essential for pheomelanogenesis as seen in red hair. However, much less is known about the genetic and biochemical basis for the blond phenotype. The chemical phenotype of blond brown to blond hair indicates that those light-colored hairs are essentially eumelanic, similar to black, but with only 25% the eumelanin content. Thus, the blond phenotype requires not only low levels of tyrosinase activity but also low (to trace) levels of cysteine to avoid the production of pheomelanin. This could be achieved through the acidification of melanosomes and the efflux of cysteine (or cystine) from them.

Tyrosinase is transferred to stage II melanosomes together with Tyrp1 and Tyrp2/DCT (Costin et al., 2003) and is involved in the production of pheomelanin and eumelanin. Melanogenesis also requires the melanin precursors, tyrosine and cysteine, which are actively taken up into melanosomes through specific transport systems (Potterf et al., 1996, 1999; Figure 2).

An acidic pH should be considered to explain the lower levels of pigmentation produced in Europeans. Lamason et al. (2005) proposed that SLC24A5 is involved in hypopigmentary effects through its activity as the potassium-dependent sodium–calcium exchanger NCKX5. Neutralization of melanosomal pH can be achieved through a cascade of ion transporters as shown in Figure 2. Thus, several vacuolar proton ATPases are known to be located in melanosomes (Tabata et al., 2008). Protons taken up are then effluxed through sodium–proton exchangers. Actually, several sodium–proton exchangers are present both in Black and in White melanocytes, and some are found in melanosomes (Smith et al., 2004). It would be interesting to determine whether SLC45A2 (MATP) and/or OCA2 (P) proteins act as sodium–proton exchanger(s) (Sturm, 2006), because those melanosomal proteins are believed to be transporters with 12-membrane-spanning structures. Sodium ions taken up by the sodium–proton exchangers are then effluxed through SLC24A5 (NCKX5). It should be noted that SLC45A2 and SLC24A5 are actually present in melanosomes, as

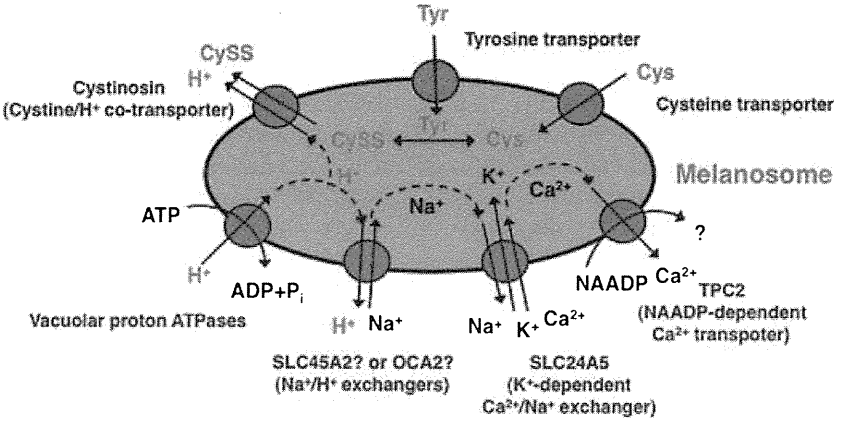


Figure 2. Roles of transporters for melanin precursors and various ions. Tyrosine (Tyr) and cysteine (Cys), precursors of eu- and pheomelanin, are actively transported into melanosomes. Cysteine and its oxidized dimer cystine (CySS) are convertible through redox exchange. Cystine is pumped out by cystinosin (CTNS), a cystine/H^+ cotransporter. Proton levels (acidity) of melanosomes are regulated through a cascade of four different ion transporters, vacuolar proton ATPases (H^+ influx), Na^+/H^+ exchangers (H^+ efflux and Na^+ influx), SLC24A5 (Na^+ efflux and Ca^{2+}, K^+ influx), and TPC2 (Ca^{2+} efflux). The net results of maximal activity of those transporters would be a low acidity (neutral pH), a high tyrosine level, a low cysteine level (and a high K^+ level), leading to proeumelanogenic conditions (dark hair phenotype). Polymorphisms of ion transporters would make melanosomes acidic, leading to the suppression of eumelanogenesis (light hair phenotype). Polymorphisms of MC1R would result in the downregulation of tyrosinase as well as these transporters, leading to a switch to pheomelanogenesis (red hair phenotype). For more details, see the Text.

proved by a proteomics study (Chi et al., 2006). Thus, acidification of melanosomes would result from several polymorphisms in the *SLC45A2* (and *OCA2*) and *SLC24A5* genes. It should be added that tyrosinase is properly trafficked to melanosomes only when intracellular organelles are neutralized (Watabe et al., 2004).

The above-mentioned hypothetical model requires calcium (and potassium) ions to be accumulated in melanosomes. In this regard, it should be noted that a newly identified TPC2 (two-pore segment channel 2), the product of the *TPCN2* gene, mediates nicotinic acid adenine dinucleotide phosphate (NAADP)-dependent calcium ion transport from lysosome-related acidic compartments (Calcraft et al., 2009). It is possible that TPC2 acts in concert with SLC24A5 to lower calcium levels in melanosomes (Figure 2). In this regard, it should be added that two polymorphisms of *TPCN2* are associated with blond hair color compared to brown hair color (Sulem et al., 2008).

Once the acidification of melanosomes is achieved, the levels of cysteine (or cystine) need to be regulated. We propose that this could be achieved through the activity of cystinosin/CTNS, a cotransporter of cystine/proton in lysosome-related organelles (Kalatzis et al., 2001). Cystinosis is an inherited lysosomal storage disease characterized by the defective transport of cystine, because of mutations of the *cystinosin/CTNS* gene (Town et al., 1998). The efflux of cystine from lysosomes by cystinosin is coupled to an efflux of protons. It has been shown that CTNS is localized in melanosomes and controls their pH (Ortonne, 2010; C. Chiaverini, R. Ballotti, and J.P. Ortonne, in submission). The chemical phenotypes of 27 cystinosis patients have now been analyzed. In patients, eumelanin levels are reduced 50%, while pheomelanin levels are increased twofold compared with normal family members. Together, the eumelanin to pheomelanin ratio is reduced fivefold (our unpublished results). Thus, the acidification of melanosomes together with cystine accumulation in cystinosis patients causes hypopigmentation with a partial shift to pheomelanogenesis. Conversely, it appears likely that cystine levels (hence cysteine levels through redox exchange with GSH) in melanosomes would be low as long as cystinosin functions properly. It should be added that a high potassium concentration, which can be achieved through the cascade of ion transporters (Figure 2), is favorable for the maximal efflux of cystine (and protons) (Smith et al., 1987).

In light of these considerations of melanosomal pH and cysteine levels, we propose a hypothesis for the control of human hair pigmentation based on the activities of melanosomal transporters (Table 2). The 'dark' hair phenotype, black to dark brown hair, is characterized by high levels of MC1R signaling and full activities of ion transporters and cystinosin, the cystine transporter. Under these promelanogenic conditions, melanosomes become neutral and cysteine deficient, leading to the production of high levels of eumelanin.

The 'light' hair phenotype, light brown to blond hair, is characterized by high levels of MC1R signaling, reduced activities of ion transporters because of polymorphisms, and full activity of cystinosin. Under these suppressed, but proeumelanogenic conditions, melanosomes become acidic and cysteine deficient, leading to the production of lower levels of eumelanin. One may be

Table 2. Biochemical characteristics of three major phenotypes of human hair pigmentation

Characteristic	Dark hair	Light hair	Red hair
Color of hair	Black to dark brown	Light brown to blond	Red
MC1R	Functional	Functional	Not functional
SLC24A5/SLC45A2?/OCA2?/TPC2	Functional	Not functional	Functional (low)
CTNS	Functional	Functional	Functional (low)
Melanosomal pH	Near neutral	Acidic	Acidic
Melanosomal Cys (CySS)	Low	Low	High
Melanin	High eumelanin	Low eumelanin	Low pheomelanin (+ low eumelanin)

Note: Neutral pH in the strict sense is pH 7.0 and 6.8 at 25°C and 37°C, respectively, as the dissociation constant of water is 10^{-14} at 25°C and $10^{-13.6}$ at 37°C.

surprised to know that blond hair contain mostly eumelanin, considering the rather yellowish tint of its hair color. It would be possible that eumelanin polymers produced under these 'suppressed' conditions may be smaller in molecular size thus providing lighter, yellowish color to 'blond' hair.

Lastly, the 'red' hair phenotype is characterized by low levels of MC1R signaling, leading to the downregulation of various genes through *MITF*, the master gene that controls melanogenesis (Walker and Gunn, 2010). Those genes include *TYR, TYRP1, DCT, OCA2, SLC24A5*, and *SLC45A2* (Le Pape et al., 2009). The *CTNS* gene should also be downregulated, leaving high levels of cystine (hence cysteine) inside melanosomes. Under these suppressed, but propheomelanic conditions, melanosomes become acidic but cysteine rich, leading to the production of low levels of pheomelanin (and eumelanin).

Future directions to a better understanding of human hair pigmentation

One important unsolved problem in melanogenesis is the precise mechanism of switching between eumelanogenesis and pheomelanogenesis in vivo (Walker and Gunn, 2010). Biochemically, that switching appears to depend on tyrosinase activity as well as on the availability of cysteine (or cystine) in melanosomes. In this connection, the role of the cystine transporter Slc7a11 (Chintala et al., 2005) in regulating mixed melanogenesis deserves more attention. It would be interesting to determine whether SLC7A11 (or another transporter) acts as a transporter taking cystine into melanosomes. Also, a recent study suggests the presence of additional factor(s) controlling pheomelanogenesis (Hida et al., 2009). Certainly, further studies are needed to understand how cysteine (and cystine) levels in melanosomes are regulated.

The roles of transporters in regulating melanosomal pH and cysteine level, as proposed in Figure 2 and Table 2, requires several confirmations. SLC45A2 and/or OCA2 proteins need to be shown to act as sodium–proton exchangers. Biochemical studies on the TPC2 protein will be required to confirm the calcium ion transporter activity in melanosomes. The roles of calcium ions in melanosomes also need to be studied. Finally, the involvement of cystinosin as a cotransporter for cystine/proton in melanosomes awaits clarification. Another interesting problem that needs to be clarified is how the yellowish color of eumelanin in blond hair can be produced in acidic environments.

In addition to the suppression of tyrosinase activity, pheomelanogenesis is kinetically favored under a more acidic environment because the cyclization of dopaquinone (the first step in eumelanogenesis) proceeds much slower at lower pHs, while the cysteinyldopa quinone cyclization (yielding the first bicyclic intermediate in pheomelanogenesis) proceeds faster (Thompson et al., 1985). Future studies will no doubt further elucidate the biochemical control of mixed melanogenesis in an acidic environment.

Acknowledgements

The authors acknowledge valuable comments by Dr. Vincent J. Hearing, NIH, USA. They also thank Prof. Jean-Paul Ortonne, University of Nice, Sophia Antipolis, France for the permission to cite their unpublished data. This work was supported, in part, by a Japan Society for the Promotion of Science (JSPS) grant (No. 20591357).

References

Ancans, J., Tobin, D.J., Hoogduijn, M.J., Smit, N.P., Wakamatsu, K., and Thody, A.J. (2001). Melanosomal pH controls rate of melanogenesis, eumelanin/phaeomelanin ratio and melanosome maturation in melanocytes and melanoma cells. Exp. Cell Res. *268*, 26–35.

Bennett, D.C., and Lamoreux, M.L. (2003). The color loci of mice – a genetic century. Pigment Cell Res. *16*, 333–344.

Bertolotto, C., Abbe, P., Hemesath, T.J., Bille, K., Fisher, D.E., Ortonne, J.P., and Ballotti, R. (1998). Microphthalmia gene product as a signal transducer in cAMP-induced differentiation of melanocytes. J. Cell Biol. *142*, 827–835.

Boissy, R.E., Zhao, H., Oetting, W.S. et al. (1996). Mutation in and lack of expression of tyrosinase-related protein-1 (TRP-1) in mela-

nocytes from an individual with brown oculocutaneous albinism: a new subtype of albinism classified as "OCA3". Am. J. Hum. Genet. *58*, 1145–1156.

Boissy, R.E., Sakai, C., Zhao, H., Kobayashi, T., and Hearing, V.J. (1998). Human tyrosinase-related protein-1 (TRP-1) does not function as a DHICA oxidase activity in contrast to murine TRP-1. Exp. Dermatol. *7*, 198–204.

Branicki, W., Brudnik, U., Draus-Barini, J., Kupiec, T., and Wojas-Pelc, A. (2008). Association of the *SLC45A2* gene with physiological human hair colour variation. J. Hum. Genet. *53*, 966–971.

Branicki, W., Brudnik, U., and Wojas-Pelc, A. (2009). Interactions between *HERC2, OCA2* and *MC1R* may influence human pigmentation phenotype. Ann. Hum. Genet. *73*, 160–170.

Burchill, S.A., Thody, A.J., and Ito, S. (1986). Melanocyte-stimulating hormone, tyrosinase activity and the regulation of eumelanogenesis and phaeomelanogenesis in the hair follicular melanocytes of the mouse. J. Endocrinol. *109*, 15–21.

Calcraft, P.J., Ruas, M., Pan, Z. et al. (2009). NAADP mobilizes calcium from acidic organelles through two-pore channels. Nature *459*, 596–600.

Cheli, Y., Luciani, F., Khaled, M., Beuret, L., Bille, K., Gounon, P., Ortonne, J.P., Bertolotto, C., and Ballotti, R. (2009). Alpha-MSH and cyclic-AMP elevating agents control melanosome pH through a protein kinase A-independent mechanism. J. Biol. Chem. *284*, 18699–18706.

Chi, A., Valencia, J.C., Hu, Z.Z. et al. (2006). Proteomic and bioinformatic characterization of the biogenesis and function of melanosomes. J. Proteome Res. *5*, 3135–3144.

Chintala, S., Li, W., Lamoreux, M.L., Ito, S. et al. (2005). *Slc7a11* gene controls production of pheomelanin pigment and proliferation of cultured cell. Proc. Natl Acad. Sci. USA *102*, 10964–10969.

Clément, K., Dubern, B., Mencarelli, M., Czernichow, P., Ito, S., Wakamatsu, K., Barsh, G.S., Vaisse, C., and Leger, J. (2008). Unexpected endocrine features and normal pigmentation in a young adult patient carrying a novel homozygous mutation in the POMC gene. J. Clin. Endocrinol. Metab. *93*, 4955–4962.

Commo, S., Gaillard, O., Thibaut, S., and Bernard, B.A. (2004). Absence of TRP-2 in melanogenic melanocytes of human hair. Pigment Cell Res. *17*, 488–497.

Cook, A.L., Chen, W., Thurber, A.E. et al. (2009). Analysis of cultured human melanocytes based on polymorphisms with the *SLC45A2*/MATP, *SLC24A5*/NCKX5, and *OCA2*/P loci. J. Invest. Dermatol. *129*, 392–405.

Costin, G.E., Valencia, J.C., Vieira, W.D., Lamoreux, M.L., and Hearing, V.J. (2003). Tyrosinase processing and intracellular trafficking is disrupted in mouse primary melanocytes carrying the underwhite (uw) mutation. A model for oculocutaneous albinism (OCA) type 4. J. Cell Sci. *116*, 3203–3212.

Du, J., and Fisher, D.E. (2002). Identification of *Aim-1* as the *underwhite* mouse mutant and its transcriptional regulation by MITF. J. Biol. Chem. *277*, 402–406.

Edwards, M., Bigham, A., Tan, J., Li, S., Gozdzik, A., Ross, K., Jin, L., and Parra, E.J. (2010). Association of the *OCA2* polymorphism His615Arg with melanin content in east Asian populations: further evidence of convergent evolution of skin pigmentation. PLoS Genet. *6*, e1000867.

Fukamachi, S., Shimada, A., and Shima, A. (2001). Mutations in the gene encoding B, a novel transporter protein, reduce melanin content in medaka. Nat. Genet. *28*, 381–385.

Fuller, B.B., Spaulding, D.T., and Smith, D.R. (2001). Regulation of the catalytic activity of preexisting tyrosinase in black and Caucasian human melanocyte cell cultures. Exp. Cell Res. *262*, 197–208.

Gautam, R., Novak, E.K., Tan, J., Wakamatsu, K., Ito, S., and Swank, R.T. (2006). Interaction of Hermansky-Pudlak syndrome genes in the regulation of lysosome-related organelles. Traffic *7*, 779–792.

Gerstenblith, M.R., Shi, J., and Landi, M.T. (2010). Genome-wide association studies of pigmentation and skin cancer: A review and meta-analysis. Pigment Cell Melanoma Res. Accepted Article; doi: 10.1111/j.1755-148et al.X.2010.00730.x.

Ginger, R.S., Askew, S.E., Ogborne, R.M. et al. (2008). SLC24A5 encodes a *trans*-Golgi network protein with potassium-dependent sodium-calcium exchange activity that regulates human epidermal melanogenesis. J. Biol. Chem. *283*, 5486–5495.

Graf, J., Hodgson, R., and van Daal, A. (2005). Single nucleotide polymorphisms in the *MATP* gene are associated with normal human hair pigmentation variation. Hum. Mutat. *25*, 278–284.

Gunn, T.M., Inui, T., Kitada, K., Ito, S., Wakamatsu, K., He, L., Bouley, D.M., Serikawa, T., and Barsh, G.S. (2001). Molecular and phenotypic analysis of attractin mutant mice. Genetics *158*, 1683–1695.

Han, J., Kraft, P., Nan, H. et al. (2008). A genome-wide association study identifies novel alleles associated with hair color and skin pigmentation. PLoS Genet. *4*, e1000074.

Hida, T., Wakamatsu, K., Sviderskaya, E.V. et al. (2009). Agouti protein, mahogunin and attractin in pheomelanogenesis and melanoblast-like alteration of melanocytes: a cAMP-independent pathway. Pigment Cell Melanoma Res. *22*, 623–634.

Hirobe, T., Wakamatsu, K., Ito, S., Abe, H., Kawa, Y., and Mizoguchi, M. (2002). Stimulation of the proliferation and differentiation of mouse pink-eyed dilution epidermal melanocytes by excess tyrosine in serum-free primary culture. J. Cell. Physiol. *191*, 167–172.

Inagaki, K., Suzuki, T., Shimizu, H. et al. (2004). Oculocutaneous albinism type 4 is one of the most common types of albinism in Japan. Am. J. Hum. Genet. *74*, 466–471.

Ito, S. (2003). A chemist's view of melanogenesis. Pigment Cell Res. *16*, 230–236.

Ito, S., and Fujita, K. (1985). Microanalysis of eumelanin and pheomelanin in hair and melanomas by chemical degradation and liquid chromatography. Anal. Biochem. *144*, 527–536.

Ito, S., and Prota, G. (1977). A facile one-step synthesis of cysteinyldopas using mushroom tyrosinase. Experientia *33*, 1118–1119.

Ito, S., and Wakamatsu, K. (1998). Chemical degradation of melanins: application to identification of dopamine-melanin. Pigment Cell Res. *11*, 120–126.

Ito, S., and Wakamatsu, K. (2003). Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: a comparative review. Pigment Cell Res. *16*, 523–531.

Ito, S., and Wakamatsu, K. (2008). Chemistry of mixed melanogenesis – Pivotal roles of dopaquinone. Photochem. Photobiol. *84*, 582–592.

Jackson, I.J., Chambers, D.M., Tsukamoto, K., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., and Hearing, V.J. (1992). A second tyrosinase-related protein, TRP-2, maps to and is mutated at the mouse slaty locus. EMBO J. *11*, 527–535.

Jiménez-Cervantes, C., Solano, F., Kobayashi, T., Urabe, K., Hearing, V.J., Lozano, J.A., and García-Borrón, J.C. (1994). A new enzymatic function in the melanogenic pathway. The 5,6-dihydroxyindole-2-carboxylic acid oxidase activity of tyrosinase-related protein-1 (TRP1). J. Biol. Chem. *269*, 17993–18000.

Kalatzis, V., Cherqui, S., Antignac, C., and Gasnier, B. (2001). Cystinosin, the protein defective in cystinosis, is a H^+-driven lysosomal cystine transporter. EMBO J. *20*, 5940–5949.

King, R.A., Willaert, R.K., Schmidt, R.M., Pietsch, J., Savage, S., Brott, M.J., Fryer, J.P., Summers, C.G., and Oetting, W.S. (2003). *MC1R* mutations modify the classic phenotype of oculo-

cutaneous albinism type 2 (OCA2). Am. J. Hum. Genet. *73*, 638–645.

Kobayashi, T., Urabe, K., Orlow, S.J., Higashi, K., Imokawa, G., Kwon, B.S., Potterf, S.B., and Hearing, V.J. (1994a). The Pmel17/*silver* locus protein. Characterization and investigation of its melanogenic function. J. Biol. Chem. *269*, 29198–29205.

Kobayashi, T., Urabe, K., Winder, A.J., Jiménez-Cervantes, C., Imokawa, G., Brewington, T., Solano, F., García-Borrón, J.C., and Hearing, V.J. (1994b). Tyrosinase related protein 1 (TRP1) functions as a DHICA oxidase in melanin biosynthesis. EMBO J. *13*, 5818–5825.

Kobayashi, T., Imokawa, G., Bennett, D.C., and Hearing, V.J. (1998). Tyrosinase stabilization of Tyrp1 (the *brown* locus protein). J. Biol. Chem. *273*, 31801–31805.

Kroumpouzos, G., Urabe, K., Kobayashi, T., Sakai, C., and Hearing, V.J. (1994). Functional analysis of the *slaty* gene product (TRP2) as dopachrome tautomerase and the effect of a point mutation on its catalytic function. Biochem. Biophys. Res. Commun. *202*, 1060–1068.

Krude, H., Biebermann, H., Luck, W., Horn, R., Brabant, G., and Grüters, A. (1998). Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. Nat. Genet. *19*, 155–157.

Lamason, R.L., Mohideen, M.-A.P.K., Mest, J.R. et al. (2005). SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans. Science *310*, 1782–1786.

Lamoreux, M.L., Wakamatsu, K., and Ito, S. (2001). Interaction of major coat color gene functions in mice as studied by chemical analysis of eumelanin and pheomelanin. Pigment Cell Res. *14*, 23–31.

Land, E.J., and Riley, P.A. (2000). Spontaneous redox reactions of dopaquinone and the balance between the eumelanic and phaeomelanic pathways. Pigment Cell Res. *13*, 273–277.

Land, E.J., Ito, S., Wakamatsu, K., and Riley, P.A. (2003). Rate constants for the first two chemical steps of eumelanogenesis. Pigment Cell Res. *16*, 487–493.

Lao, O., de Gruijter, J.M., van Duijin, K., Navarro, A., and Kayser, M. (2007). Signatures of positive selection in genes associated with human skin pigmentation as revealed from analyses of single nucleotide polymorphisms. Ann. Hum. Genet. *71*, 354–369.

Le Pape, E., Passeron, T., Glubellino, A., Valencia, J.C., Wolber, R., and Hearing, V.J. (2009). Microarray analysis sheds light on the dedifferentiating role of agouti signal protein in murine melanocytes via the Mc1r. Proc. Natl Acad. Sci. USA *106*, 1802–1807.

Lehman, A.L., Silvers, W.K., Puri, N., Wakamatsu, K., Ito, S., and Brilliant, M.H. (2000). The underwhite (uw) locus acts autonomously and reduces the production of melanin. J. Invest. Dermatol. *115*, 601–606.

Levy, C., Khaled, M., and Fisher, D.E. (2006). MITF: master regulator of melanocyte development and melanoma oncogene. Trends Mol. Med. *12*, 406–414.

del Marmol, V., Ito, S., Bouchard, B., Libert, A., Wakamatsu, K., Ghanem, G., and Solano, F. (1996). Cysteine deprivation promotes eumelanogenesis in human melanoma cells. J. Invest. Dermatol. *107*, 698–702.

McEvoy, B., Beleza, S., and Shriver, M.D. (2006). The genetic architecture of normal variation in human pigmentation: an evolutionary perspective and model. Hum. Mol. Genet. *15*, R176–R181.

Mengel-From, J., Wong, T.H., Morling, N., Rees, J.L., and Jackson, I.J. (2009). Genetic determinants of hair and eye colours in the Scottish and Danish populations. BMC Genet. *10*, 88 (online).

Müller, G., Ruppert, S., Schmid, E., and Schütz, G. (1988). Functional analysis of alternatively spliced tyrosinase gene transcripts. EMBO J. *7*, 2723–2730.

Naysmith, L., Waterston, K., Ha, T., Flanagan, N., Bisset, Y., Ray, A., Wakamatsu, K., Ito, S., and Rees, J.L. (2004). Quantitative measures of the effect of the melanocortin 1 receptor on human pigmentary status. J. Invest. Dermatol. *122*, 423–428.

Newton, J.M., Cohen-Barak, O., Hagiwara, N., Gardner, J.M., Davisson, M.T., King, R.A., and Brilliant, M.H. (2001). Mutations in the human orthologue of the mouse *underwhite* gene *(uw) underlie a new form of oculocutaneous albinism, OCA4*. Am. J. Hum. Genet. *69*, 981–988.

Ni-Komatsu, L., and Orlow, S.J. (2006). Heterologous expression of tyrosinase recapitulates the misprocessing and mistrafficking in oculocutaneous albinism type 2: effects of altering intracellular pH and pink-eyed dilution gene expression. Exp. Eye Res. *82*, 519–528.

Norton, H.L., Kittles, R.A., Parra, E., McKeigue, P., Mao, X., Cheng, K., Canfield, V.A., Bradley, D.G., McEvoy, B., and Shriver, M.D. (2007). Genetic evidence for the convergent evolution of light skin in Europeans and East Asians. Mol. Biol. Evol. *24*, 710–722.

Oetting, W.S., and King, R.A. (1999). Molecular basis of albinism: mutations and polymorphisms of pigmentation genes associated with albinism. Hum. Mutat. *13*, 99–115.

Olivares, C., Jiménez-Cervantes, C., Lozano, J.A., Solano, F., and García-Borrón, J.C. (2001). The 5,6-dihydroxyindole-2-carboxylic acid (DHICA) oxidase activity of human tyrosinase. Biochem. J. *354*, 131–139.

Ortonne, J.P. (2010). Hypopigmentation in cystinosis: clinical aspects and molecular mechanisms. Pigment Cell Melanoma Res. *23*, 457 (abstract).

Ozeki, H., Ito, S., Wakamatsu, K., and Hirobe, T. (1995). Chemical characterization of hair melanins in various coat-color mutants of mice. J. Invest. Dermatol. *105*, 361–366.

Ozeki, H., Ito, S., Wakamatsu, K., and Thody, A.J. (1996). Spectrophotometric characterization of eumelanin and pheomelanin in hair. Pigment Cell Res. *9*, 265–270.

Ozeki, H., Wakamatsu, K., Ito, S., and Ishiguro, I. (1997). Chemical characterization of eumelanins with special emphasis on 5,6-dihydroxyindole-2-carboxylic acid content and molecular size. Anal. Biochem. *248*, 149–157.

Pawelek, J.M., Körner, A.M., Bergstrom, A., and Bolognia, J. (1980). New regulators of melanin biosynthesis and the autodestruction of melanoma cells. Nature *286*, 617–619.

Potterf, S.B., Muller, J., Bernardini, I., Tietze, F., Kobayashi, T., Hearing, V.J., and Gahl, W.A. (1996). Characterization of a melanosomal transport system in murine melanocytes mediating entry of the melanogenic substrate tyrosine. J. Biol. Chem. *271*, 4002–4008.

Potterf, S.B., Furumura, M., Sviderskaya, E.V., Santis, C., Bennett, D.C., and Hearing, V.J. (1998). Normal tyrosinase transport and abnormal tyrosinase routing in *pink-eyed dilution* melanocytes. Exp. Cell Res. *244*, 319–326.

Potterf, S.B., Virador, V., Wakamatsu, K., Furumura, M., Santis, C., Ito, S., and Hearing, V.J. (1999). Cyteine transport in melanosomes from murine melanocytes. Pigment Cell Res. *12*, 4–12.

Rees, J.L. (2003). Genetics of hair and skin color. Annu. Rev. Genet. *37*, 67–90.

Rooryck, C., Roudaut, C., Robine, E., Müsebeck, J., and Arveiler, B. (2006). Oculocutaneous albinisms with TYRP1 gene mutations in a Caucasian patient. Pigment Cell Res. *19*, 239–242.

Rosemblat, S., Durham-Pierre, D., Gardner, J.M., Nakatsu, Y., Brilliant, M.H., and Orlow, S.J. (1994). Identification of a melanosomal membrane protein encoded by the *pink-eyed dilution* (type II oculocutaneous albinism) gene. Proc. Natl Acad. Sci. USA *91*, 12071–12075.

Shekar, S.N., Duffy, D.L., Frudakis, T., Montogomery, G.W., James, M.R., Sturm, R.A., and Martin, N.G. (2008a). Spectrophotometric methods for quantifying pigmentation in human hair – influence of MC1R genotype and environment. Photochem. Photobiol. *84*, 719–726.

Shekar, S.N., Duffy, D.L., Frudakis, T., Sturm, R.A., Zhao, Z.Z., Montgomery, G.W., and Martin, N.G. (2008b). Linkage and association analysis of spectrophotometrically quantified hair color in Australian adolescents: the effect of *OCA2* and *HERC2*. J. Invest. Dermatol. *128*, 2807–2814.

Silvers, W.K. (1979). The Coat Colors of Mice: A Model for Mammalian Gene Action and Interaction (Basel: Springer-Verlag).

Simon, J.D., Peles, D., Wakamatsu, K., and Ito, S. (2009). Current challenges in understanding melanogenesis: bridging chemistry, biological control, morphology, and function. Pigment Cell Melanoma Res. *22*, 563–579.

Slominski, A., Plonka, P.M., Pisarchik, A., Smart, J.L., Tolle, V., Wortsman, J., and Low, M.J. (2005). Preservation of eumelanin hair pigmentation in proopiomelanocortin-deficient mice on a nonagouti (*a/a*) genetic background. Endocrinology *146*, 1245–1253.

Smith, M.L., Green, A.A., Potashnik, R., Mendoza, S.A., and Schneider, J.A. (1987). Lysosomal cystine transport. Effect of intralysosomal pH and membrane potential. J. Biol. Chem. *262*, 1244–1253.

Smith, D.R., Spaulding, D.T., Glenn, H.M., and Fuller, B.B. (2004). The relationship between Na^+/H^+ exchanger expression and tyrosinase activity in human melanocytes. Exp. Cell Res. *298*, 521–534.

Spry, M.L., Vanover, J.C., Scott, T., Abona-Ama, O., Wakamatsu, K., Ito, S., and D'Orazio, J.A. (2009). Prolonged treatment of fair-skinned mice with topical forskolin causes persistent tanning and UV protection. Pigment Cell Melanoma Res. *22*, 219–229.

Stokowski, R.P., Pant, P.V., Datdd, T. et al. (2007). A genomewide association study of skin pigmentation in a South Asia polulation. Am. J. Hum. Genet. *81*, 1119–1132.

Sturm, R.A. (2006). A *golden* age of human pigmentation genetics. Trends Genet. *22*, 464–468.

Sturm, R.A. (2009). Molecular genetics of human pigmentation diversity. Hum. Mol. Genet. *18*, R9–R17.

Sturm, R.A., Duffy, D.L., Zhao, Z.Z. et al. (2008). A single SNP in an evolutionary conserved region within intron 86 of the *HERC2* gene determines human blue-brown eye color. Am. J. Hum. Genet. *82*, 424–431.

Sulem, P., Gudbjartsson, D.F., Stacey, S.N. et al. (2008). Two newly identified genetic determinants of pigmentation in Europeans. Nat. Genet. *40*, 835–837.

Suzuki, .T., and Tomita, Y. (2008). Recent advances in genetic analyses of oculocutaneous albinism types 2 and 4. J. Dermatol. Sci. *51*, 1–9.

Tabata, H., Kawamura, N., Sun-Wada, G.H., and Wada, Y. (2008). Vacuolar-type H^+-ATPase with the *a3* isoform is the proton pump on premature melanosomes. Cell Tissue Res. *332*, 447–460.

Theos, A.C., Truschel, S.T., Raposo, G., and Marks, M.S. (2005). The *silver* locus product Pmel17/gp100/Silv/ME20: controversial in name and in function. Pigment Cell Res. *18*, 322–336.

Theos, A.C., Berson, J.F., Theos, S.C. et al. (2006). Dual loss of ER export and endocytic signals with altered melanosome morphology in the *silver* mutation of Pmel17. Mol. Biol. Cell *17*, 3598–3612.

Thompson, A., Land, E.J., Chedekel, M.R., Subbarao, K.V., and Truscott, T.G. (1985). A pulse radiolysis investigation of the oxidation of the melanin precursors 3,4-dihydroxyphenylalanine (dopa) and the cysteinyldopas. Biochim. Biophys. Acta *843*, 49–57.

Town, M., Jean, G., Cherqui, S. et al. (1998). A novel gene encoding an integral membrane protein is mutated in nephropathic cystinosis. Nat. Genet. *18*, 319–324.

Toyofuku, K., Valencia, J.C., Kushimoto, T., Costin, G.E., Virador, V., Vieira, W.D., Ferrans, V.J., and Hearing, V.J. (2002). The etiology of oculocutaneous albinism (OCA) type II: the pink protein controls the transport and processing of tyrosinase. Pigment Cell Res. *15*, 217–224.

Tsukamoto, K., Jackson, I.J., Urabe, K., Montague, P.M., and Hearing, V.J. (1992). A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase. EMBO J. *11*, 519–526.

Valenzuela, R.K., Henderson, M.S., Walsh, M.H. et al. (2010). Predicting phenotype from genotype: normal pigmentation. J. Forensic Sci., *55*, 315–322.

Valverde, P., Healy, E., Jackson, L., Rees, J.L., and Thody, A.J. (1995). Variants of the melanocyte-stimulating hormone receptor gene are associated with red hair and fair skin in humans. Nat. Genet. *11*, 328–330.

Vogel, P., Read, R.W., Vance, R.B., Platt, K.A., Troughton, K., and Rice, D.S. (2008). Ocular albinism and hypopigmentation defects in *Slc24a5-/-* mice. Vet. Pathol. *45*, 264–279.

Wakamatsu, K., and Ito, S. (2002). Advanced chemical methods in melanin determination. Pigment Cell Res. *15*, 174–183.

Wakamatsu, K., Ito, S., and Rees, J.L. (2002). The usefulness of 4-amino-3-hydroxyphenylalanine as a specific marker of pheomelanin. Pigment Cell Res. *15*, 225–232.

Wakamatsu, K., Fujikawa, K., Zucca, F., Zecca, L., and Ito, S. (2003). The structure of neuromelanin as studied by chemical degradative methods. J. Neurochem. *86*, 1015–1023.

Wakamatsu, K., Hirobe, T., and Ito, S. (2007). High levels of melanin-related metabolites in plasma from pink-eyed dilution mice. Pigment Cell Res. *20*, 222–224.

Wakamatsu, K., Ohtara, K., and Ito, S. (2009). Chemical analysis of late stages of pheomelanogenesis: conversion of dihydrobenzothiazine to a benzothiazole structure. Pigment Cell Melanoma Res. *22*, 474–486.

Walker, W.P., and Gunn, T.M. (2010). Shades of meaning: the pigment-type switching system as a tool for discovery. Pigment Cell Melanoma Res. *23*, 485–495.

Watabe, H., Valencia, J.C., Yasumoto, K., Kushimoto, T., Ando, H., Muller, J., Vieira, W.D., Mizoguchi, M., Appella, E., and Hearing, V.J. (2004). Regulation of tyrosinase processing and trafficking by organellar pH and by proteasome activity. J. Biol. Chem. *279*, 7971–7981.

Yaswen, L., Diehl, N., Brennan, M.B., and Hochgeschwender, U. (1999). Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. Nat. Med. *5*, 1066–1070.

[Correction added after online publication October 2010: a dash was incorrectly present in Table 2, third row of column 1, after 'TPC2' that has now been removed.]

Pigment Cell Melanoma Res. **24**; 605–613

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

Shosuke Ito[1], Yukiko Nakanishi[1], Robert K. Valenzuela[2], Murray H. Brilliant[3], Ludger Kolbe[4] and Kazumasa Wakamatsu[1]

1 Department of Chemistry, Fujita Health University School of Health Sciences, Toyoake, Aichi, Japan **2** Graduate Interdisciplinary Program in Genetics, University of Arizona, Tucson, AZ, USA **3** Center for Human Genetics, Marshfield Clinic, Marshfield, WI, USA **4** Beiersdorf AG, R&D, Skin Research Center, Hamburg, Germany

CORRESPONDENCE K. Wakamatsu, e-mail: kwaka@fujita-hu.ac.jp

KEYWORDS eumelanin pheomelanin melanin hair oxidation hydrogen peroxide

PUBLICATION DATA Received 11 April 2011, revised and accepted for publication 21 April 2011, published online 27 April 2011

doi: 10.1111/j.1755-148X.2011.00864.x

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

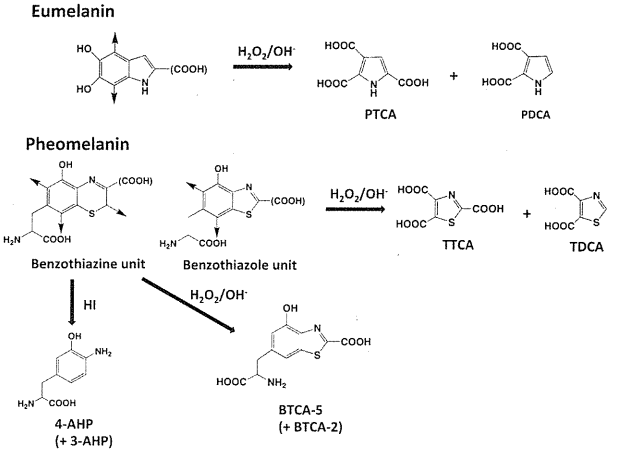


Figure 1. Chemical degradation of eumelanin and pheomelanin. Upon H_2O_2 oxidation, DHICA-derived units in eumelanin give PTCA while DHI-derived units gave PDCA. Upon H_2O_2 oxidation, benzothiazole units in pheomelanin give TTCA and TDCA. Upon H_2O_2 oxidation, benzothiazine units in pheomelanin give 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

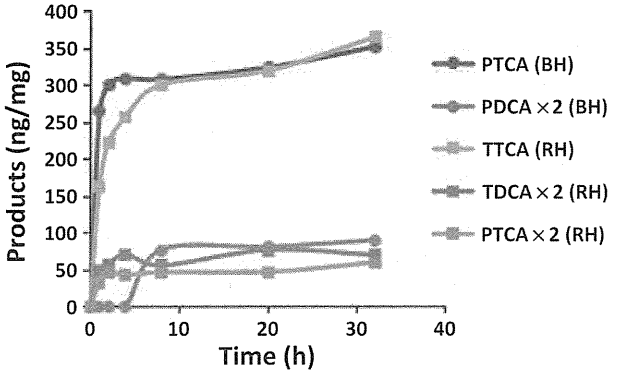


Figure 2. Time course of alkaline H_2O_2 oxidation of human black hair and human red hair. The production of PTCA and PDCA in black hair and of TTCA, TDCA, and PTCA in red hair was followed in duplicate. BH and RH denote black hair and red hair, respectively.

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]

Sample	A500	Alkaline H_2O_2 Oxidation (ng)						Acidic $KMnO_4$ Oxidation (ng)				HI Hydrolysis (ng)	
		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

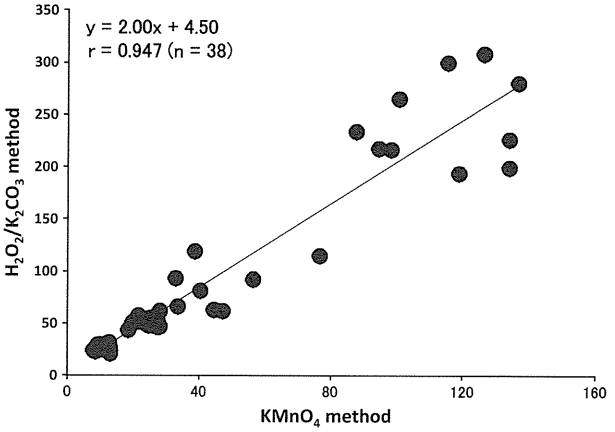


Figure 4. Correlation of PTCA levels between alkaline H_2O_2 oxidation and acidic $KMnO_4$ oxidation in human hair samples. Correlation was significant at $P < 0.0001$.

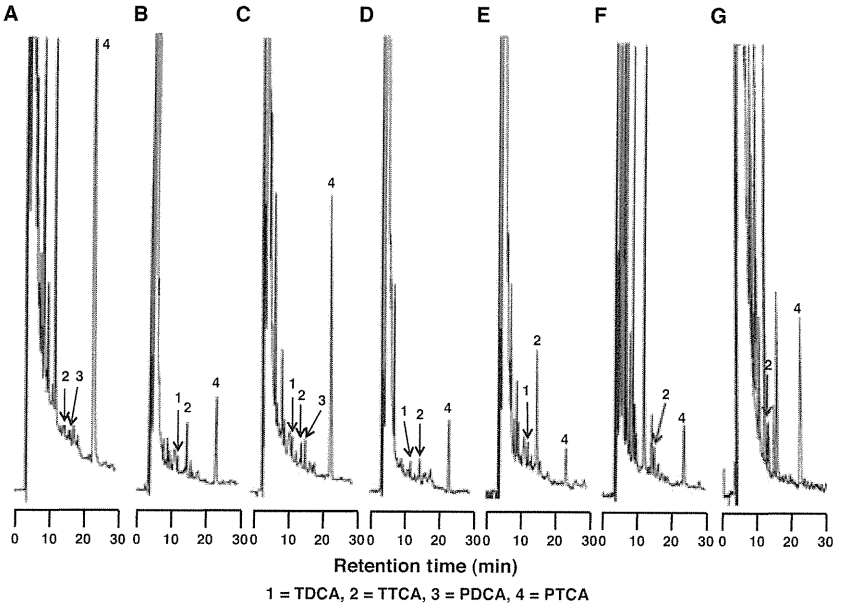


Figure 3. HPLC chromatograms of alkaline H_2O_2 degradation products. (A) mouse black (*a a*) hair, (B) mouse yellow (*e e*) hair, (C) human black hair, (D) human blond hair, (E) human red hair, (F) human melanocytes, (G) human epidermis. Amounts of samples used were 1 mg for A – E, 0.75 10^6 cells for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

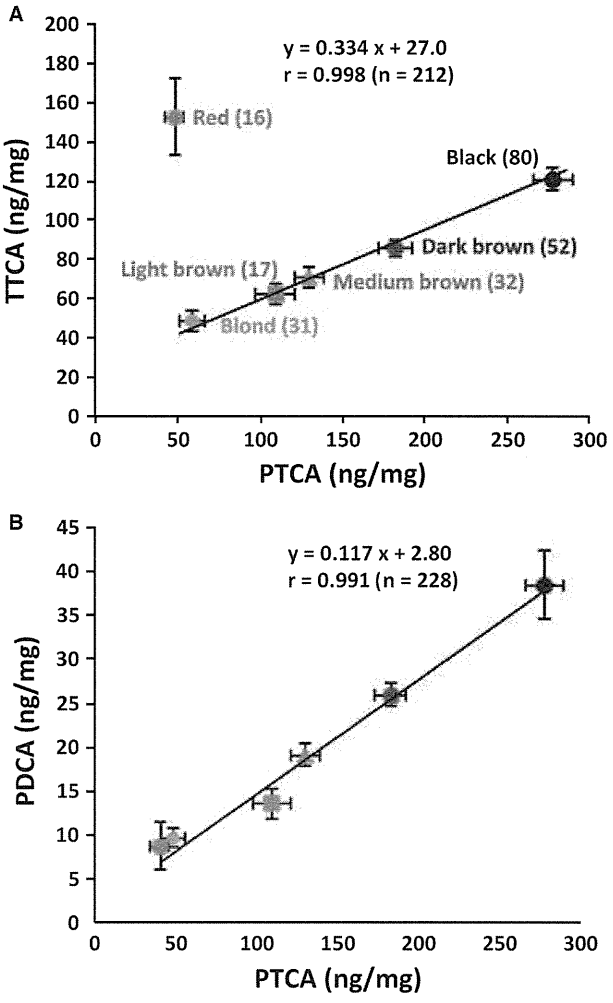


Figure 5. Correlation of PTCA levels with TTCA levels (A) and PDCA levels (B) in alkaline H_2O_2 oxidation of human hair samples. Correlations are significant with $P < 0.0001$ in A (excluding red hairs) and in B. Bars represent SEM.

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

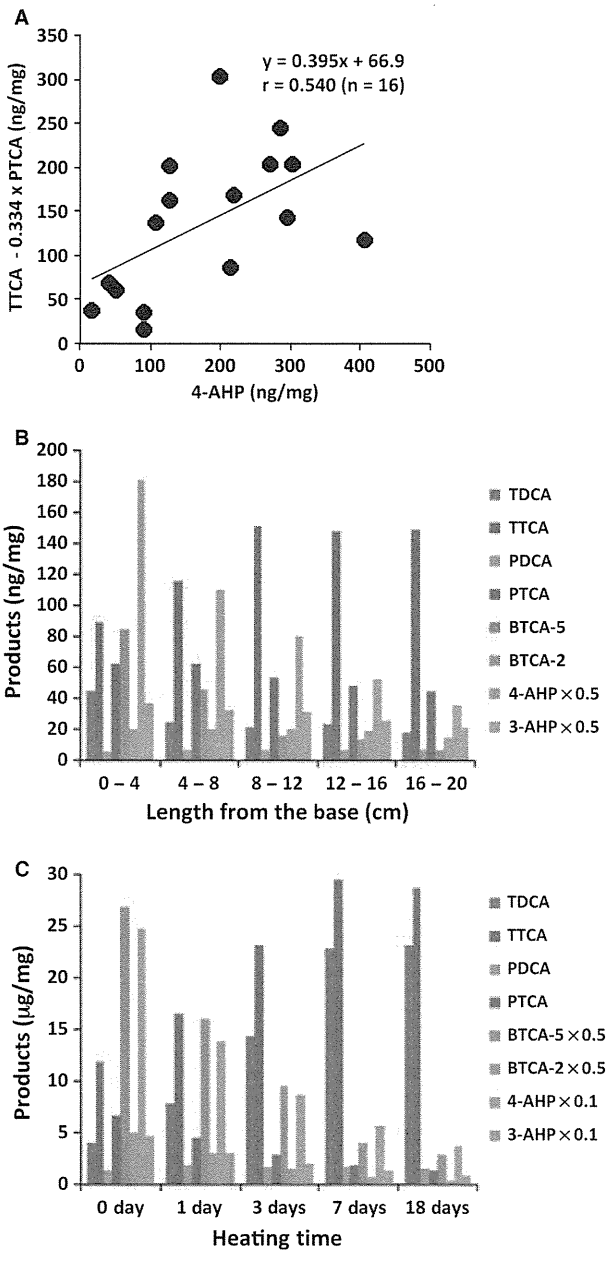


Figure 6. Evaluation of pheomelanin markers, TTCA, BTCA-5, and 4-AHP. (A) Correlation of TTCA levels with 4-AHP levels in human red hair. Correlation is significant at $P = 0.0313$. (B) (Photo)degradation of pheomelanin in vivo. Red hair sample was cut to 4 cm length. Similar trend of results were obtained 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.

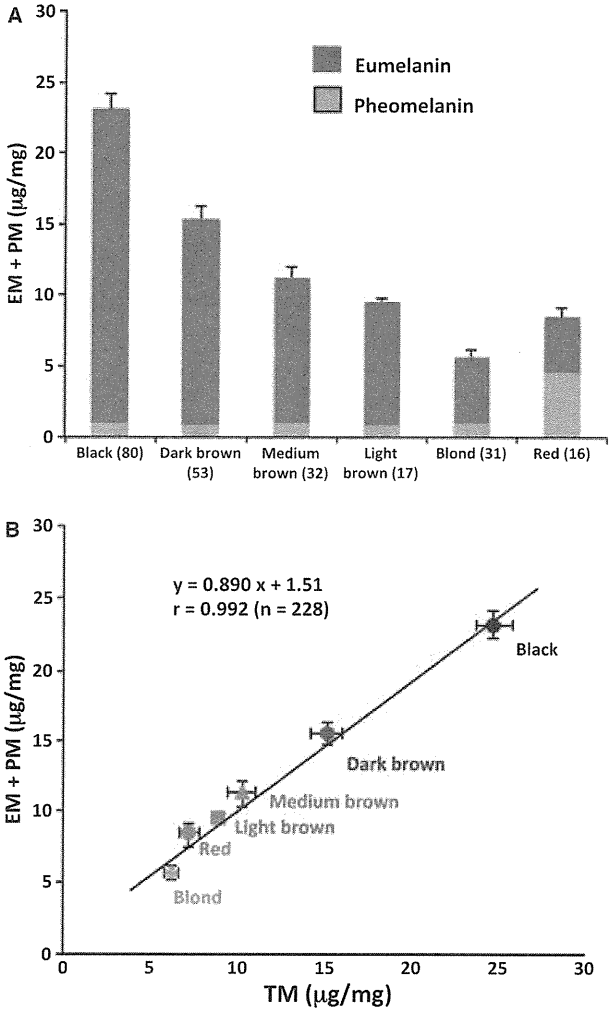


Figure 7. Chemical analysis of human hair melanin. (A) Contents of eumelanin and pheomelanin (chemical phenotypes) in human hair samples of various colors (visual phenotypes). For the conversion factors, see Results. (B) Correlation of combined amounts of eumelanin and pheomelanin with total melanin (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).

Acknowledgements

This work was supported, in part, by a Japan Society for the Promotion of Science (JSPS) grant (No. 20591357, No. 21500358) given to SI and KW.

References

Commo, S., Gaillard, O., Thibaut, S., and Bernard, B.A. (2004). Absence of TRP-2 in melanogenic melanocytes of human hair. Pigment Cell Res. *17*, 488–497.

Cooksey, C.J., Garrant, P.J., Land, E.J., Pavel, S., Ramsden, C.A., Riley, P.A., and Smit, N.P. (1997). Evidence of the indirect formation of the catecholic intermediate substrate responsible for the

autoactivation kinetics of tyrosinase. J. Biol. Chem. *272*, 26226–26235.

Greco, G., Wakamatsu, K., Panzella, L., Ito, S., Napolitano, A., and d'Ischia, M. (2009). Isomeric cysteinyldopas provide a (photo)degradative bulk component and a robust structural element in red human hair pheomelanin. Pigment Cell Melanoma Res. *22*, 319–327.

Hirobe, T., Ito, S., and Wakamatsu, K. (2011). The mouse pink-eyed dilution allele of the P-gene greatly inhibits eumelanin, but not pheomelanin synthesis as studied by chemical analysis. Pigment Cell Melanoma Res. *24*, 241–246.

Hunt, G., Kyne, S., Ito, S., Wakamatsu, K., Todd, C., and Thody, A.J. (1995). Eumelanin and pheomelanin contents of human epidermis and cultured melanocytes. Pigment Cell Res. *8*, 202–208.

Ito, S., and Fujita, K. (1985). Microanalysis of eumelanin and pheomelanin in hair and melanomas by chemical degradation and liquid chromatography. Anal. Biochem. *144*, 527–536.

Ito, S., and Prota, G. (1977). A facile one-step synthesis of cysteinyldopas using mushroom tyrosinase. Experientia *33*, 1118–1119.

Ito, S., and Wakamatsu, K. (1994). An improved modification of permanganate oxidation of eumelanin that gives a constant yield of pyrrole-2,3,5-tricarboxylic acid. Pigment Cell Res. *7*, 141–144.

Ito, S., and Wakamatsu, K. (1998). Chemical degradation of melanins: application to identification of dopamine-melanin. Pigment Cell Res. *11*, 120–126.

Ito, S., and Wakamatsu, K. (2003). Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: a comparative review. Pigment Cell Res. *16*, 523–531.

Ito, S., and Wakamatsu, K. (2008). Chemistry of mixed melanogenesis – Pivotal roles of dopaquinone. Photochem. Photobiol. *84*, 582–592.

Ito, S., Fujita, K., Takahashi, H., and Jimbow, K. (1984). Characterization of melanogenesis in mouse and guinea pig hair by chemical analysis of free and protein-bound dopa and 5-S-cysteinyldopa. J. Invest. Dermatol. *83*, 12–14.

Lamoreux, M.L., Wakamatsu, K., and Ito, S. (2001). Interaction of major coat color gene functions in mice as studied by chemical analysis of eumelanin and pheomelanin. Pigment Cell Res. *14*, 23–31.

McCapra, F., and Razavi, Z. (1975). A model for firefly luciferin biosynthesis. J. Chem. Soc. Chem. Commun. 43–48.

Napolitano, A., Vincensi, M.R., d'Ischia, M., and Prota, G. (1996). A new benzothiazole derivative by degradation of pheomelanins with alkaline hydrogen peroxide. Tetrahedron Lett. *37*, 6799–6802.

Napolitano, A., Vincensi, M.R., Di Nonato, P., Monfrecola, G., and Prota, G. (2000). Microanalysis of melanins in mammalian hair by alkaline hydrogen peroxide degradation: identification of a new structural marker of pheomelanins. J. Invest. Dermatol. *114*, 1141–1147.

Nezirević Dernroth, D., Årstrand, K., Greco, G., Panzella, L., Napolitano, A., and Kågedal, B. (2010). Phemelanin-related benzothiazole isomers in the urine of patients with diffuse melanosis of melanoma. Clin. Chim. Acta *411*, 1195–1203.

Ozeki, H., Ito, S., Wakamatsu, K., and Hirobe, T. (1995). Chemical characterization of hair melanins in various coat-color mutants of mice. J. Invest. Dermatol. *105*, 361–366.

Ozeki, H., Ito, S., Wakamatsu, K., and Thody, A.J. (1996). Spectrophotometric characterization of eumelanin and pheomelanin in hair. Pigment Cell Res. *9*, 265–270.

Panzella, L., Manini, P., Monfrecola, G., d'Ischia, M., and Napolitano, A. (2007). An easy-to-run method for routine analysis of eumelanin and pheomelanin in pigmented tissues. Pigment Cell Res. *20*, 128–133.

Prota, G. (1992). Melanin and Melanogenesis. (San Diego: Academic Press).

Simon, J.D., and Peles, D.N. (2010). The red and the black. Acc. Chem. Res. *16*, 1452–1460.

Simon, J.D., Peles, D., Wakamatsu, K., and Ito, S. (2009). Current challenges in understanding melanogenesis: bridging chemistry, biological control, morphology, and function. Pigment Cell Melanoma Res. *22*, 563–579.

Tsukamoto, K., Jackson, I.J., Urabe, K., Montague, P.M., and Hearing, V.J. (1992). A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase. EMBO J. *11*, 519–526.

Valenzuela, R.K., Henderson, M.S., Walsh, M.H. et al. (2010). Predicting phenotype from genotype: normal pigmentation. J. Forensic Sci., *55*, 315–322.

Wakamatsu, K., and Ito, S. (2002). Advanced chemical methods in melanin determination. Pigment Cell Res. *15*, 174–183.

Wakamatsu, K., Ito, S., and Rees, J.L. (2002). The usefulness of 4-amino-3-hydroxyphenylalanine as a specific marker of pheomelanin. Pigment Cell Res. *15*, 225–232.

Wakamatsu, K., Fujikawa, K., Zucca, F., Zecca, L., and Ito, S. (2003). The structure of neuromelanin as studied by chemical degradative methods. J. Neurochem. *86*, 1015–1023.

Wakamatsu, K., Kavanagh, R., Kadekaro, A.L., Terzieva, S., Sturm, R.A., Leachman, S., Abdel-Malek, Z., and Ito, S. (2006). Diversity of pigmentation in cultured melanocytes is due to differences in the type as well as quantity of melanin. Pigment Cell Melanoma Res. *19*, 154–162.

Wakamatsu, K., Hu, D.-N., McCormick, S.A., and Ito, S. (2008). Characterization of melanin in human iridal and choroidal melanocytes from eyes with various colored irides. Pigment Cell Melanoma Res. *21*, 97–105.

Wakamatsu, K., Ohtara, K., and Ito, S. (2009). Chemical analysis of late stages of pheomelanogenesis: conversion of dihydrobenzothiazine to a benzothiazole structure. Pigment Cell Melanoma Res. *22*, 474–486.

Wolber, R., Schlenz, K., Wakamatsu, K., Smuda, C., Nakanishi, Y., Hearing, V.J., and Ito, S. (2008). Pigmentation effects of solar-simulated radiation as compared with UVA and UVB radiation. Pigment Cell Melanoma Res. *21*, 487–491.

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.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

機能性磁性ナノ粒子の開発と医療への応用

井藤　彰

九州大学大学院工学研究院化学工学部門

akira@chem-eng.kyushu-u.ac.jp

1. 緒論

磁性ナノ粒子は磁力に引き寄せられる性質をもつことから、磁性ナノ粒子を結合させて磁気標識した細胞は磁力で遠隔操作することが可能となり、ティッシュエンジニアリングのプロセスにおける細胞操作（「細胞の分離」「細胞の増幅」「三次元組織構築」）に利用可能である。さらに、磁性ナノ粒子は高周波磁場中で発熱する性質をもつことから、ガンの温熱療法（ハイパーサーミア）における発熱体として利用できる。我々は Fe_3O_4 で標記される酸化鉄であるマグネタイトで 10 nm というナノサイズの粒子径に調製した磁性ナノ粒子を、様々なバイオマテリアルで修飾することで、標的細胞に集積させることができる機能を持った磁性ナノ粒子（機能性磁性ナノ粒子）を作製し（図 1）、ティッシュエンジニアリングやガン治療といった医療分野への応用を行ってきた（1）。

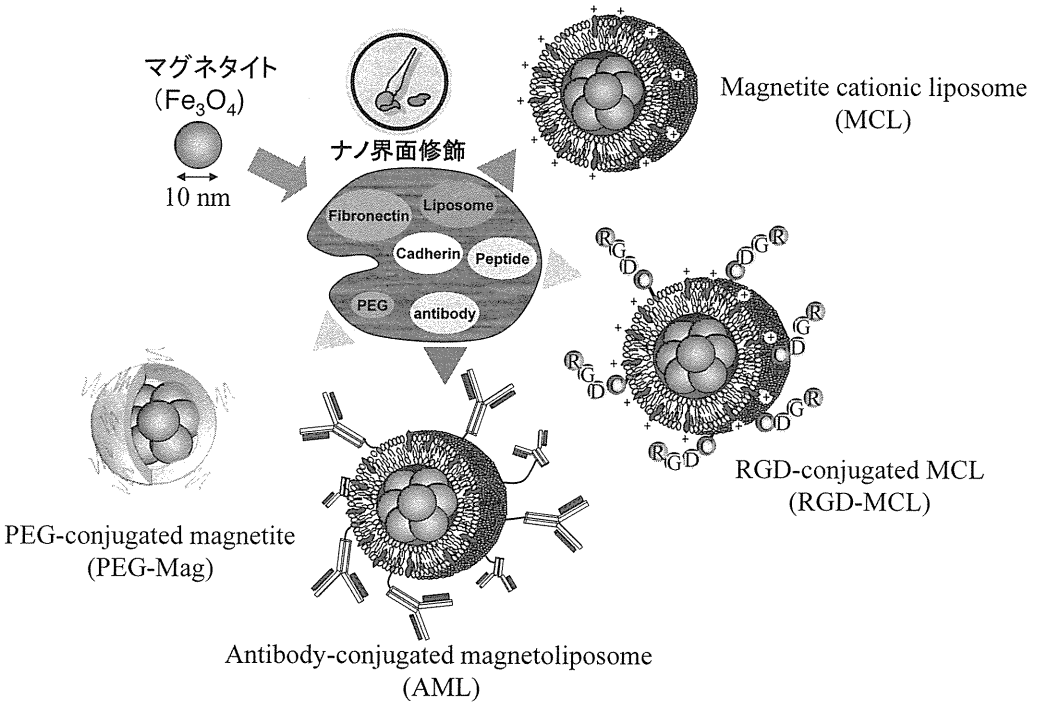


図 1　機能性磁性ナノ粒子の概略

2. 実験方法

ティッシュエンジニアリング分野において、細胞を磁力で操作するためには、標的細胞に能動的かつ大量に磁性ナノ粒子を結合する必要がある。そこで、正電荷脂質からなるリポソームに磁性ナノ粒子を包埋することで、正電荷脂質包埋型磁性ナノ粒子（MCL）を作製した。さらに、リポソーム表面に RGD ペプチドが結合した細胞接着性磁性ナノ粒子（RGD-MCL）を作製した。また、全く逆の発想で、細胞が接着しない機能性磁性ナノ粒子を開発すれば、細胞の非接着領域をつくり出すことが可能であり、また、その領域は磁力で制御できる。筆者らは、磁性ナノ粒子をアミノシランで被覆し、その表面に PEG を共有結合することで、細胞非接着性磁性ナノ粒子(PEG-Mag)を作製した。

一方、ガン治療においては、ガン細胞特異的なターゲティングが必要である。我々は、磁性ナノ粒子を封入したリポソームにガン細胞に特異的な抗体を結合させ、抗体固定化磁性ナノ粒子(AML)を作製した。また、DDS として、メラニン形成酵素チロシナーゼの基質である NPrCAP を磁性ナノ粒子表面に結合させた NPrCAP 結合型磁性ナノ粒子(NPrCAP/M)を合成し、皮膚がん（メラノーマ）への標的指向性を調べた。

3. 結果

図2に示す磁力を用いたティッシュエンジニアリング技術“Mag-TE”法の開発を行った。AMLを用いた骨髄液からの間葉系幹細胞の分離法を開発した。目的細胞の培養プロセスにおいて、MCLを用いた共培養法の開発やRGD-MCLおよびPEG-Magを用いた細胞のパターニング法の開発を行った。さらに磁力を用いた多孔性足場担体への細胞播種法および細胞を磁力で積み上げていくことによる三次元組織構築法の開発を行った。

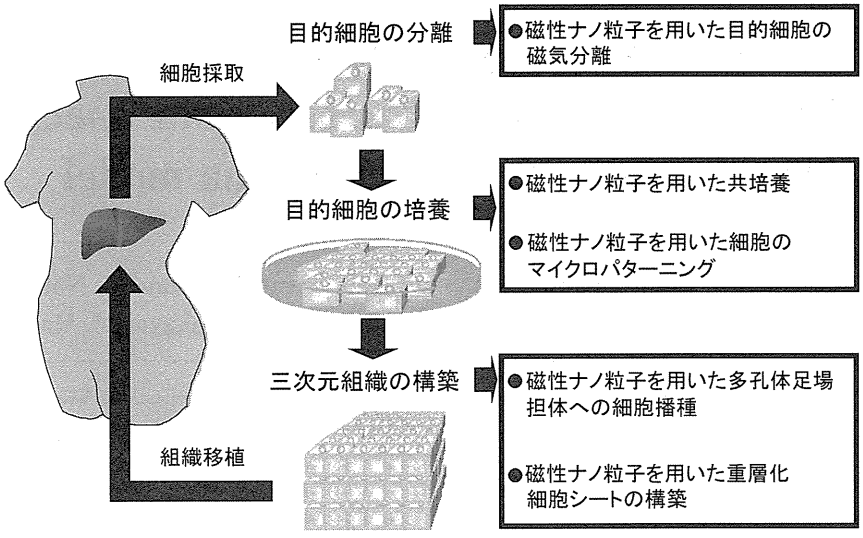


図2　磁性ナノ粒子を用いたティッシュエンジニアリング

図3に示す磁性ナノ粒子を発熱体としたガン温熱療法の開発を行った。MCLを腫瘍に直接投与して、磁場照射を行ったところ、腫瘍特異的に発熱し、高い治療効果が得られた。この治療効果には腫瘍免疫が活性化されることを示した（2）。また、NPrCAP/Mを用いたマウスメラノーマに対する温熱療法において、メラノーマ特異的な磁性ナノ粒子の取り込みと、高い治療効果がみられた。

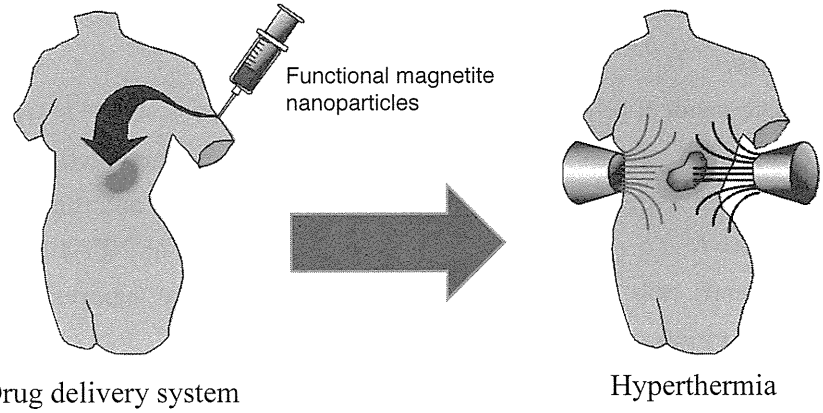


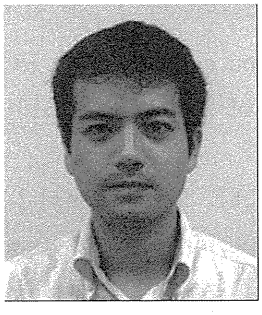
図3　磁性ナノ粒子を用いたハイパーサーミア

4. 結論

磁性ナノ粒子は他の物質では得難い特長を備えており、それらの特長を利用した医療技術の開発は有用であると考えられる。一日でも早く患者さんのお役にたてるように、努力している。

5. 参考文献

(1) Ito A, Shinkai M, Honda H, Kobayashi T. Medical application of functionalized magnetic nanoparticles. J Biosci Bioeng. 2005, 100(1):1-11. Review.
(2) Ito A, Honda H, Kobayashi T. Cancer immunotherapy based on intracellular hyperthermia using magnetite nanoparticles: a novel concept of "heat-controlled necrosis" with heat shock protein expression. Cancer Immunol Immunother. 2006, 55(3):320-8. Review.



略歴

2006.3-　　九州大学大学院工学研究院　准教授
2002.2-2006.2　名古屋大学大学院工学研究科　助手
2002.1-2002.1　日本学術振興会特別研究員（PD）
2000.4-2001.12　日本学術振興会特別研究員（DC1）

N-propionyl-4-*S*-cysteaminylphenol induces apoptosis in B16F1 cells and mediates tumor-specific T-cell immune responses in a mouse melanoma model

Yasue Ishii-Osai[1], Toshiharu Yamashita[1*], Yasuaki Tamura[2], Noriyuki Sato[2], Akira Ito[3], Hiroyuki Honda[4], Kazumasa Wakamatsu[5], Shosuke Ito[5], Eiichi Nakayama[6], Masae Okura[1] and Kowichi Jimbow[1]

[1]Department of Dermatology, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan ; [2]First Department of Pathology, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan ; [3]Department of Chemical Engineering, Faculty of Engineering, Kyushu University, Fukuoka 819-0395, Japan ; [4]Department of Biotechnology, School of Engineering, Nagoya University, Nagoya 464-8603, Japan ; [5]Department of Chemistry, Fujita Health University School of Health Sciences, Toyoake 470-1192, Japan ; [6]Faculty of Health and Welfare, Kawasaki University of Medical Welfare, Kurashiki 701-0193, Japan

*Correspondence: Toshiharu Yamashita, Department of Dermatology, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan. Phone: +81-11-611-2111, FAX: +81-11-613-3739, yamasita@sapmed.ac.jp

Abstract

Background: N-propionyl-4-*S*-cysteaminylphenol (NPr-4-*S*-CAP) is selectively incorporated into melanoma cells and degrades them. However, it remains unclear whether NPr-4-S-CAP can induce cell death associated with the induction of host immune responses and tumor suppression in vivo.

Objective: To examine the molecular mechanism of NPr-4-*S*-CAP-mediated cytotoxicity toward melanoma cells and to test whether NPr-4-*S*-CAP can suppress transplanted primary and secondary B16F1 melanomas.

Methods: Cytotoxicity and apoptosis of melanoma cells were assessed by cell counting, flow cytometry, and detection of reactive oxygen species (ROS) and apoptotic molecules. NPr-4-*S*-CAP-associated host immunity was studied using a B16F1 mouse melanoma model through the application of CD4- and CD8-specific antibodies and tetramer assay.

Results: NPr-4-*S*-CAP suppressed growth of pigmented melanoma cells in a dose-dependent manner associated with an increase of intracellular ROS, activation of caspase 3 and DNA fragmentation, suggesting that NPr-4-*S*-CAP mediated ROS production, eliciting apoptosis of melanoma cells. Growth of transplanted B16F1 melanomas was inhibited after the consecutive intratumoral injection of NPr-4-*S*-CAP, and the tumor growth after rechallenge with B16F1 was significantly suppressed in the treated mice. It was suggested that B16F1-specific CD8+ T cell immunity was induced in the NPr-4-*S*-CAP-treated mice by the tumor formation in the presence of anti-CD8 antibody and TRP-2-specific tetramer assay.

Conclusions: It is suggested that NPr-4-*S*-CAP induces apoptosis in melanoma cells through ROS production and generates $CD8^+$ cell immunity resulting in the suppression of rechallenge B16F1 melanoma.

Abbreviations: NPr-4-*S*-CAP, N-propionyl-4-*S*-cysteaminylphenol; NPr-2-*S*-CAP, N-propionyl-2-S-cysteaminylphenol; AMF, alternating magnetic field; ROS, reactive oxygen species; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DMEM, Dulbecco's modified Eagle's medium; FBS,