研究成果の刊行に関する一覧表

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Shosuke Ito, Yukiko Nakanishi, Robert K. Valenzuela, Murray H. Brilliant, Ludger Kolbe and Kazumasa Wakamatsu	Usefulness of alkaline hydrogen peroxide oxidation to analyze eumelanin and pheomelanin in various tissue samples: application to chemical analysis of human hair melanins	Pigment Cell Melanoma Res.	24	605-613	2011
井藤　彰	機能性磁性ナノ粒子の開発と医療への応用				
Yasue Ishii-Osai, Toshiharu Yamashita, Yasuaki Tamura, Noriyuki Sato, Akira Ito, Hiroyuki Honda, Kazumasa Wakamatsu, Shosuke Ito, Eiichi Nakayama, Masae Okura and Kowichi Jimbow	N-propionyl-4-S-cysteaminylphenol induces apoptosis in B16F1 cells and mediates tumor-specific T-cell immune responses in a mouse melanoma model	J Dermatol Sci		印刷中	2012

Ⅳ. 研究成果の刊行物・別刷

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Scientific Research

Conjugation of magnetite nanoparticles with melanogenesis substrate, NPrCAP provides melanoma targeted, *in situ* peptide vaccine immunotherapy through HSP production by chemo-thermotherapy*

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ABSTRACT

In order to develop melanoma-targeted *in situ* peptide vaccine immunotherapy, magnetite nanoparticles were conjugated with a melanogenesis substrate, N-propionyl cysteaminylphenol (NPrCAP). Magnetite nanoparticles introduced thermotherapy which caused non-apoptotic cell death and generation of heat shock protein (HSP) upon exposure to alternating magnetic field (AMF). NPrCAP was expected to develop a melanoma-targeted therapeutic drug because of its selective incorporation into melanoma cells and production of highly reactive free radicals, that result in not only oxidative stress but also apoptotic cell death by reacting with tyrosinase.

Keywords: melanogenesis; chemo-thermo-immunotherapy; nanomedicine; melanoma; magnetite nanoparticles

1. Introduction

Management of metastatic melanoma is extremely difficult challenge for physicians and scientists. Currently only 10% with metastatic melanoma patients survive for five years because of the lack of effective therapies [1]. There is, therefore, an emerging need to develop innovative therapies for the control of advanced metastatic melanoma.

Intracellular hyperthermia using magnetite nanoparticles (10-100 nm-sized, Fe_3O_4) has been shown to be effective for treating cancers in not only primary but also metastatic lesions [2-4]. Incorporated magnetite nanoparticles generate heat within the cells after exposure to AMF due to hysteresis loss [5]. In this treatment, there is not only the heat-mediated cell death but also immune reaction due to the generation of HSPs [6-15]. HSP expression induced by hyperthermia has been shown to be involved in tumor immunity, providing the basis for developing a cancer thermo-immunotherapy.

Exploitation of biological properties unique to cancer cells may also provide a novel approach to overcome difficult challenge to the melanoma treatment. Melanogenesis is inherently cytotoxic and uniquely occurs in melanocytic cells; thus, tyrosine analogs that are tyrosinase substrates can be good candidates for developing drugs to melanoma-targeting therapies [16]. N-propionyl and N-acetyl derivatives of 4S-cysteaminylphenol (NPr- and NAcCAP) were synthesized, and found to possess cytotoxic effects on *in vivo* and *in vitro* melanomas through the oxidative stress that derives from production of cytotoxic free radicals [17-21].

Based upon these rationales, we now provide evidence that melanoma-targeted *in situ* peptide vaccine immunotherapy has been successfully developed by conjugation of magnetite nanoparticles with a chemically modified melanogenesis substrate, and that a novel melanoma-targeted chemo-thermo-immunotherapy (CTI Therapy) is provided for patients suffering from advanced metastatic melanoma.

2. Chemical modification of melanogenesis substrate as a potential source for development of selective drug delivery system and melanoma toxicity

2.1. Melanogenesis cascade in melanoma cells

The major advance of drug discovery for targeted therapy to cancer cells can be achieved by exploiting their unique biological property. The biological property unique to the melanocyte and melanoma cell resides the biosynthesis of melanin pigments within specific compartments, melanosomes. Melanogenesis begins with the conversion of amino acid, tyrosine to dopa and subsequently to dopaquinone in the presence of tyrosinase. This pathway is unique to all of melanocytes and melanoma cells including "amelanotic" melanoma. With the interaction of melanocyte-stimulating hormone (MSH)/ melanocortin 1 receptor (MC1R), the melanogenesis cascade begins from activation of microphthalmia transcription factor (MITF) for induction of either eu- or pheomelanin biosynthesis. Tyrosinase is the major player in this cascade. Tyrosinase is a glycoprotein and its glycosylation process is regulated by a number of molecular chaperons, including calnexin in the endoplasmic reticulum [22, 23]. Vesicular transport then occurs to carry tyrosinase and its related proteins from trans-Golgi network to melanosomal compartments. In this process a significant number of transporters, such as small GTP-binding protein, adaptor proteins and PI3-kinase are involved, and early and late endosomes are closely associated with the production of these compartments. Once melanin biosynthesis is completed to conduct either eu- or pheomelanogenesis within melanosomal compartments, they will move along dendritic processes and transferred to surrounding keratinocytes in normal skin [24-26]. In metastatic melanoma cells, however, there will be no melanosome transfer inasmuch as there will be no receptor cells such as keratinocytes, and melanosomes synthesized are aggregated in autophagic vacuoles of melanoma cells. Thus a chemically modified melanogenesis substrate can be retained in melanoma cells once they are incorporated into their melanogenesis cascade, hence providing a unique drug delivery system (DDS) to melanoma cells.

2.2. Synthesis of sulfur analogs (amine and amide) of tyrosine, cysteaminylphenols and their melanocyte toxicity

In order to utilize melanin biosynthesis pathway for developing cytocidal compounds in controlling melanoma growth, N-acetyl and N-propionyl derivatives of cysteaminylphenols (CAPs) have been synthesized [27, 28] (Fig. 1, 2). These compounds are found to possess cytocidal effect on *in vivo* and *in vitro* melanocytes through the oxidative stress. For example, both NPrCAP and NAcCAP can selectively disintegrate follicular melanocytes after single or multiple *ip* administration to new-born or adult C57 black mice [3, 29]. In the case of adult mice after repeated *ip* administration of NPrCAP, white follicles with 100% success can be seen at the site where hair follicles were plucked to stimulate new melanocyte growth and to activate tyrosinase synthesis. A single *ip* injection of NPrCAP into a new born mouse resulted in the development of silver follicles in the entire body coat. The selective disintegration of melanocytes can be seen as early as in 12 hr after a single *ip* administration. None of surrounding keratinocytes or fibroblasts showed such membrane degeneration and cell death.

A high, specific uptake of NAcCAP was seen by melanoma cell lines compared to non-melanoma cells. A melanoma-bearing mouse showed, on the whole body autoradiogram, the selective uptake and covalent binding of NAcCAP in melanoma tissues of lung and skin. The specific cytotoxicity of NPrCAP and NAcCAP was examined on various types of culture cells by MTT assay [30], showing that only melanocytic cells except HeLa possessed the low IC50. The cytotoxicity on DNA synthesis inhibition was time-dependent and irreversible on melanoma cells, but was transient on HeLa cells[21].

We also examined to what extent the melanoma growth can be blocked in both *in vitro* culture and *in vivo* lung metastasis assays by administration of NAcCAP combined with buthionine sulfoximine (BSO), which blocks the effect of anti-oxidants through reducing glutathione levels. There was a marked growth inhibition of cultured melanoma cells in the presence of BSO, indicating that the selective cytotoxicity by our NPr- and NAc-CAP is mediated by the production of cytotoxic free radicals. The *in vivo* lung metastasis experiment also showed the decreased number of lung melanoma colonies [17]. The problem was, however, that a fairly large number of amelanotic melanoma lesions were seen to grow in the lung. NPrCAP has been developed and conjugated with magnetite nanoparticles in the hope of increasing the cytotoxicity and overcoming the problem.

3. Conjugation of magnetite nanoparticles with N-propionyl 4S-cysteaminylphenol, NPrCAP for melanoma targeted chemo-thermo-immunotherapy

3.1.Synthesis for conjugates of magnetite nanoparticles and NPrCAP, and their selective aggregation in melanosomal compartments

Magnetite nanoparticles have been employed for thermotherapy in a number of cancer treatments including human gliomas and prostate cancers [31-33]. They consist of 10-100 nm-sized iron oxide (Fe_3O_4) with a surrounding polymer coating and become magnetized when

placed in AMF [3]. We expected the combination of NPrCAP and magnetite nanoparticles to be a potential source for developing not only anti-melanoma pharmacologic but also immunogenic agent. It was expected that NPrCAP/magnetite nanoparticles complex could be selectively incorporated into melanoma cells because NPrCAP possesses a specific effect on DDS. The degradation of melanoma tissues occurs from oxidative and heat stresses by exposure of NPrCAP to tyrosinase and by exposure of magnetite nanoparticles to AMF. These two stress processes produce the synergistic or additive effect for generating tumor-infiltrating lymphocytes (TIL) that will kill melanoma cells in distant metastases (Fig. 3).

We synthesized, in our initial study, the conjugates of NPrCAP with magnetite nanoparticles, NPrCAP/ML and NPrCAP/CML, in which NPrCAP was embedded in neutral and cationic liposomes respectively (Fig. 4). There were, however, non-specific electrostatic interaction between cationic magneto-liposomes (NPrCAP/CML) and various non-target cells [33] as well as non-specific aggregations in neutral magneto-liposomes (NPrCAP/ML). A promising technique is the use of tumor-targeted magnetite nanoparticles, and this approach was extended by synthesizing another type of magnetite nanoparticles, NPrCAP/M and NPrCAP/PEG/M, on which NPrCAP is superficially and directly bound on the surface of magnetite nanoparticles without using liposomes. They are chemically stable, can be produced in large quantities and may develop effective melanoma-targeted chemotherapy (by NPrCAP) and thermo-immunotherapy (by magnetite nanoparticles with HSP), hence providing a basis for a novel CTI therapy. Most of the experiments described below were carried out by employing NPrCAP/M. A preliminary clinical trial, however, used NPrCAP/PEG/M to which polyethylene glycol (PEG) was employed to conjugate NPrCAP and magnetite nanoparticles.

In our studies, we could see that NPrCAP/M nanoparticle conjugates were selectively aggregated in melanoma cells compared to non-melanoma cells (Fig.5-a). Specifically NPrCAP/M nanoparticles were found to be incorporated into and aggregated in melanosomal compartments after *ip* administration by electron microscopy (Fig.5-b). After AMF exposure, there will be selective disintegration of melanoma tissues as can be seen by Berlin Blue staining [34, 35] (Fig. 5-c). The conjugates of NPrCAP and magnetite nanoparticles will be selectively aggregated on the cell surface of melanoma cells through still unknown surface receptor and then incorporated into melanoma cells by early and late endosomes. The conjugates will then be incorporated into melanosomal compartment as the stage I melanosomes and late endosomes share common compartments, to which tyrosinase will be transported from trans-Golgi network by vesicular transport (Fig.6).

3.2. *In vivo* chemo-thermo-immunotherapy in mouse melanoma by conjugates of magnetite nanoparticles and melanogenesis substrate, NPrCAP.

In this study, we employed three cell lines of B16 melanoma, *i.e.*, B16F1, B16F10 and B16-ovalbmine (OVA) cells and compared the thermotherapeutic protocols in detail by evaluating the growth of the re-challenge melanoma as well as the duration and rates of survival of melanoma- bearing mice (Fig. 7).

By employing B16F1 and F10 cells, we first evaluated the chemotherapeutic effect of NPrCAP/M with or without AMF exposure which generates heat. NPrCAP/M without heat inhibited growth of primary transplants to the same degree as did NPrCAP/M with heat, indicating that NPrCAP/M alone has a chemotherapeutic effect. However, there was a significant difference in the melanoma growth inhibition of re-challenge transplants between the groups of NPrCAP/M with and without heat. NPrCAP/M with AMF exposure showed the most significant growth inhibition in re-challenge melanoma and increased life span of the host animals, *i.e.*, almost complete rejection of re-challenge melanoma growth whereas NPrCAP/M without heat was much less, *i.e.*, 30-50%, indicating that NPrCAP/M with heat possesses a thermo-immuno- therapeutic effect (Fig. 8-a, b. c).

Specifically our study indicated that the most effective thermo-immunotherapy for re-challenge B16 F1 and F10 melanoma cells can be obtained at a temperature of 43°C for 30 min with the treatment repeated three times on every other day intervals without complete degradation of the primary melanoma (Fig. 8-b). Our therapeutic conditions and their effects differ from those of magnetically mediated hyperthermia on the transplanted melanomas reported previously by Suzuki *et al.* [36]. Cationic magneto-liposomes-mediated hyperthermia for B16 melanoma showed that hyperthermia at 46°C once or twice led to regression of 40-90% of primary tumors and to 30-60% survival of mice, whereas their hyperthermia at 43°C failed to induce regression of the secondary tumors with 0% survival of mice [36].

3.3. Production of heat shock protein by chemo-thermo-immunotherapy using NPrCAP/ magnetite nanoparticle conjugates

It has been reported that the intracellular hyperthermia using magnetic nanoparticles is effective for treating certain types of cancer in not only primary but also metastatic lesions [37-43]. Incorporated magnetic nanoparticles generate heat within the cells after exposure to the AMF due to hysteresis loss or relaxational loss [44, 45].

Hyperthermic treatment using CMLs, which are cationic liposomes containing 10-nm magnetite nanoparticles, induced antitumor immunity by enhancement of HSP expression [38, 46-48].

In our animal study, those animals bearing B16F1 and B16F10 melanoma cells showed, to certain degree, rejection of second re-challenge melanoma transplantation by administration of both NPrCAP alone and NPrCAP/M minus AMF exposure [49]. Our working hypothesis for this finding is that there is a difference in the cytotoxic mechanism and immunogenic property of NPrCAP/M between experimental groups with and without AMF exposure. The animals with NPrCAP/M without AMF exposure resulted in non- necrotic, apoptotic cell death. The animals with NPrCAP/M plus AMF exposure, on the other hand, resulted in non-apoptotic, necrotic cell death with immune complex production of melanoma peptide as well as Hsp70 and a small amount of Hsp 90. The latter group of NPrCAP/M plus AMF exposure showed the most significant growth inhibition of the re-challenged melanoma growth which resulted in the almost complete survival of the host animals as long as for 3 months that we have conducted our experimental protocol [34]. In the latter study [34], we also found that repeated hyperthermia (3 cycles of NPrCAP/M administration and AMF irradiation) was required to induce the maximal antitumor immune response.

In the study using B16-OVA cells, the hyperthermia of melanoma cells using NPrCAP/M with AMF exposure further showed antitumor immune responses via cross-presentation of HSP-chaperoned antigen (Fig.9-a, b) (Fig.10-a, b, c, d). Moreover, the HSPs-antigen peptide complex released from melanoma cells treated with this intracellular hyperthermia was taken-up by dendritic cells (DCs) and cross-presented HSP-chaperoned peptide in the context of MHC class I molecules [49]. As stated above, our CTI therapy with AMF exposure induced NPrCAP- as well as heat-mediated melanoma cell necrosis to NPrCAP/M incorporated cells. If melanoma cells escaped from necrotic cell death, repeated hyperthermia should produce necrotic cell death of previously heat shocked-melanoma cells in which HSPs were induced. In addition, our data of CTI therapy with AMF exposure using B16-OVA cells suggested that Hsp72/Hsc73, Hsp90, and ER-resident HSPs participated in the induction of $CD8^+$ T cell response. In particular, among HSPs, Hsp72 was largely responsible for the augmented antigen presentation to $CD8^+$ T cells. As Hsp72 is known to up-regulate in response to hyperthermia or heat shock treatment [46], newly synthesized Hsp72 has a chance to bind to the heat-denatured melanoma-associated antigen.

3.4. Melanocytotoxic and immunogenic properties of NPrCAP compared to hydroquinone

Monobenzyl ether of hydroquinone has long been known to produce the skin depigmentation at both drug-applied area by direct chemical reaction with tyrosinase and non-applied distant area by immune reaction with still unknown mechanism [50-53]. The melanogenesis-related cytotoxicity primarily derives from tyrosinase-mediated formation of dopaquinone and other quinone intermediates, which produce reactive oxygen species such as superoxide and H_2O_2 [19, 54-56]. This unique biological property of melanin intermediates not only causes cell death, but also may produce immunogenic properties. We postulated that the cytotoxic action of NAcCAP and NPrCAP appears to involve two major biological processes. One is cytostatic process which derives from the DNA synthesis inhibition through the interaction of quinone and free radicals with SH-enzymes and thymidine synthase. Another is the cytocidal process by damage of DNA and mitochondrial ATP through oxidative stress and interaction with SH-enzyme [21]. They bind protein disulphide isomerise [57]. Although we have not yet studied which one of these two processes is responsible for the immune reaction, it is likely that the cyotocidal process of NPrCAP is involved in the induction of immune reaction.

Monobenzy ether of hydroquinone was shown to produce a reactive ortho-quinone generated by tyrosinase-catalyzed oxidation and self-coupling and thiol conjugation reactions [58]. It was also shown to induce cell death without activating the caspase cascade or DNA fragmentation, indicating that the death pathway is non-apoptotic [58, 59]. It was further reported that monobenzyl ether of hydroquinone induced the immunogenicity to melanocytes and melanoma cells by forming quinone-haptens to tyrosinase protein and by inducing the release of tyrosinase- and melanoma antigen recognized by T cells-1 (MART-1) containing $CD63^+$ exosomes following melanosome oxidative stress induction. The drug further augmented the processing and shedding of melanocyte differentiation antigens by inducing melano- some autophagy and enhanced tyrosinase ubiquitination, ultimately activating DCs, which induced cytotoxic human melanoma-reactive cells. These T cells eradicated melanoma *in vivo* [59]. It is necessary to examine if NPrCAP will also take an immune-biological process similar to that reported in the case of mono- benzyl ether of hydroquinone.

4. DISCUSSION

The immune system can respond to cancer cells in two ways: by reacting against tumor-specific antigens (molecules that are unique to cancer cells) or against tumor-associated antigens (molecules that are expressed differentially by cancer cells and normal cells) [60]. The immunotherapy for cancer cells is further subdivided into

immunotherapy with antibodies and T cells, therapeutic cancer vaccines, therapeutic vaccines combined with chemotherapy and immunoprevention of cancer cells. Several clinical trials using melanoma peptides or an antibody that blocks cytotoxic T-lymphocyte-associated antigen on lymphocytes have been shown to improve overall melanoma survival [61-63]. Exploitation of a specific biological property to cancer cells may, however, be another approach for developing novel cancer-targeted drugs. Promising oncogene-targeted melanoma therapy has successfully introduced recently [64].

Hyperthermia increases the expression of intracellular HSPs which is important in and necessary for the induction of antitumor immunity [46, 65]. Over expression of HSPs, such as Hsp 70, increases tumor immunogenicity by augmenting the chaperoning ability of antigenic peptides and presentation of antigenic peptides in MHC class I molecules [66, 67]. In this process professional antigen presenting DCs play unique and important roles in taking up, processing and presenting exogenous antigens in association with MHC class I molecules. Our working hypothesis for induction of *in situ* vaccination immunotherapy is that CTI therapy causes degradation of melanoma tissues which results in the release of HSP/melanoma antigen complex. This complex is taken up by professional antigen-presenting DCs through HSP receptor (Fig. 11).

Based upon these animal experiments, a preliminary human clinical trial has been carried out by employing NPrCAP/PEG/M plus AMF after we obtained the approval of our human clinical trial for a limited number of stage III and IV melanoma patients (Clinical Trial Research No. 18-67, Sapporo Medical University). The therapeutic protocol followed the basically identical experimental schedule as that of animal experiments. In the clinical trials, however, we utilized NPrCAP/PEG/M which was made by conjugating polyethylene glycol between NPrCAP and magnetite nanoparticles. Among two of four patients showed complete and partial responses to our treatment and have been able to carry out normal daily activities after CTI therapy. In one patient, for example, four distant cutaneous metastasis sites were evaluated and either significant regression or shrinkage of all of these four melanoma lesions was seen. The patient was able to survive 30 months after several trials of CTI therapy. The pathological and immunological specimens revealed dense aggregation of lymphocytes and macrophages at the site of CTI therapy. Importantly there was a trend to have an almost identical distribution of $CD8^+$ T cells and MHC class 1 positive cells. Another patient had many lymph node metastases, but still has been surviving more than 32 months. In order to evaluate the overall therapeutic effect to advanced melanoma, it is important to have larger-scaled clinical trials and define concisely the molecular interaction between chemo-therapeutic and thermo-immunotherapeutic effect in our CTI therapy.

5. SUMMARY AND CONCLUSION

In this study, we examined to what extent the conjugates of magnetite nanoparticles and melanogenesis substrate can generate melanoma-targeted vaccines through the chemotherapeutic and thermotherapeutic effects on primary transplant of B16 mouse melanoma cells with and without AMF exposure (heat generation). Specifically we evaluated the immunotherapeutic effect on the second, re-challenge transplant of the same melanoma cells to see if the growth of distant metastatic melanomas can be inhibited. We also investigated the possible association of HSP production, $CD8^+$ T cell activation and MHC expression along with rejection of the re-challenge melanoma.

Our approach using melanogenesis substrate and magnetite nanoparticles is based upon the expectation that the combination of (1) direct killing of melanoma cells by chemotherapeutic and thermo-therapeutic effect of melanogenesis-targeted drug (NPrCAP/M) and (2) indirect killing by immune reaction (*in situ* peptide vaccine) after exposure to AMF. It is hoped from these rationales that a tumor-specific DDS is developed by NPrCAP and selective cell death can be achieved by exposure of conjugates of NPrCAP/M nanoparticles to AMF, which then can induce HSP expression through either necrotic or non-necrotic process or combination of the two, without damaging non-cancerous tissues. Finally a novel immunotherapy targeted to metastatic melanoma lesions is achieved through "*in situ* peptide vaccine".

6. ABBREVIATIONS

AMF= alternating magnetic field
BSO= buthionine sulfoximine
CML= cationic magneto-liposome
CTI therapy= chemo-thermo-immunotherapy
DC= dendritic cell
DDS= drug delivery system
HSP/Hsp= heat shock protein
mAb=monoclonal antibody
MC1R= melanocortin 1 receptor
MITF= microphthalmia transcription factor
ML=non-cationic magneto-liposome
MSH= melanocyte stimulating hormone
NAcCAP= N-acetyl 4S-cysteaminylphenol
NPrCAP= N-propionyl 4S cysteaminylphenol
NPrCAP/M= N-propionyl 4S-cysteaminylphenol/ magnetite nanoparticle
NPrCAP/PEG/M= N-propionyl 4S-cysteaminylphenol/ polyethylene glycol/ magnetite nanoparticle
OVA= ovalbumin
PEG= polyethylene glycol
TIL= tumor infiltrating lymphocytes

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Figures

Fig.1
Selective Drug Delivery System (DDS)

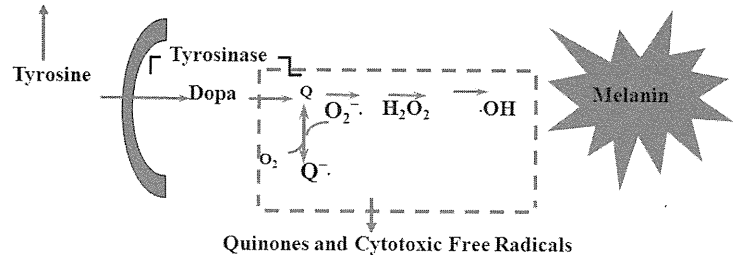


Fig.2

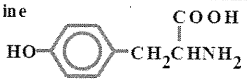
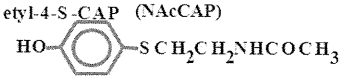
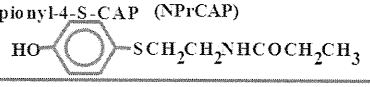
	Km (μM)	Vmax (μmole/min/mg)
Tyrosine 	0.3	1.80
N-Acetyl-4-S-CAP (NAcCAP) 	375.0	9.28
N-Propionyl-4-S-CAP (NPrCAP) 	340.9	5.43

Fig.3
Selective Drug Delivery (DDS)

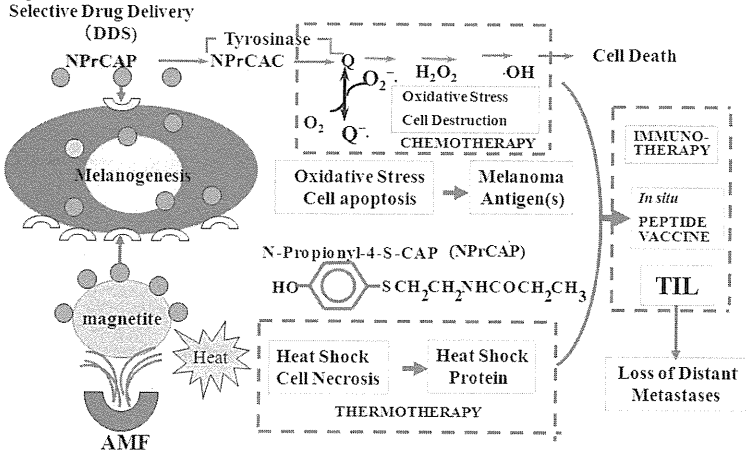
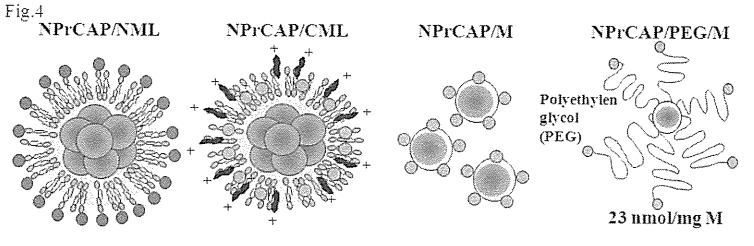


Fig.4



NPrCAPis conjugated on surface of neutral ML (NML)	NPrCAPis embedded in cationic ML (CML)	NPrCAPis directly conjugated with magnetite (M)	NPrCAPis conjugated with magnetite (M) by PEG

Fig.5

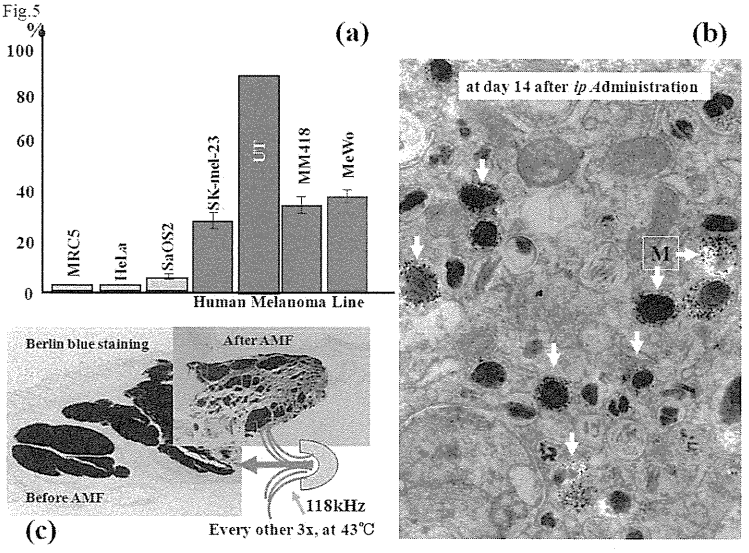


Fig.6

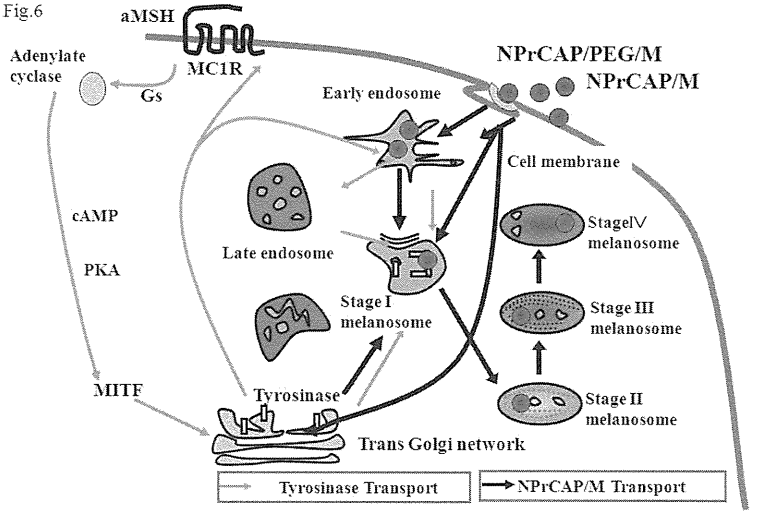


Fig.7

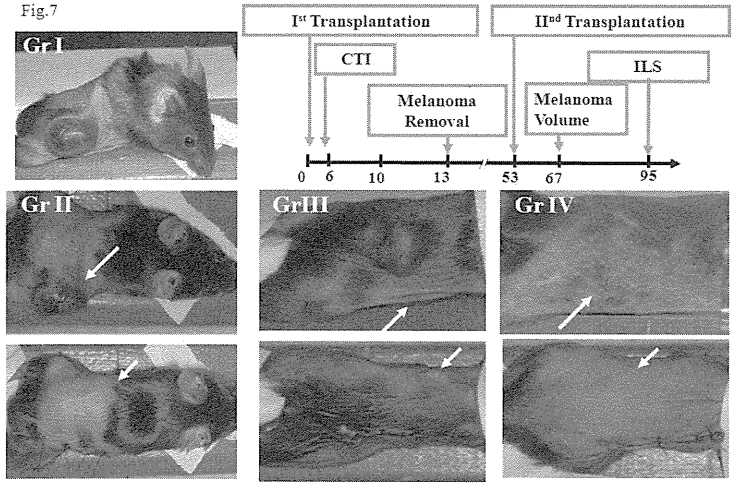


Fig. 9-a, b

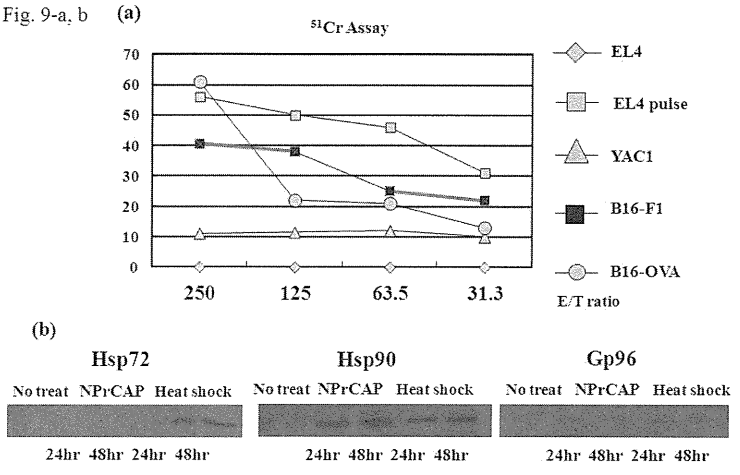


Fig. 10-a, b, c, d

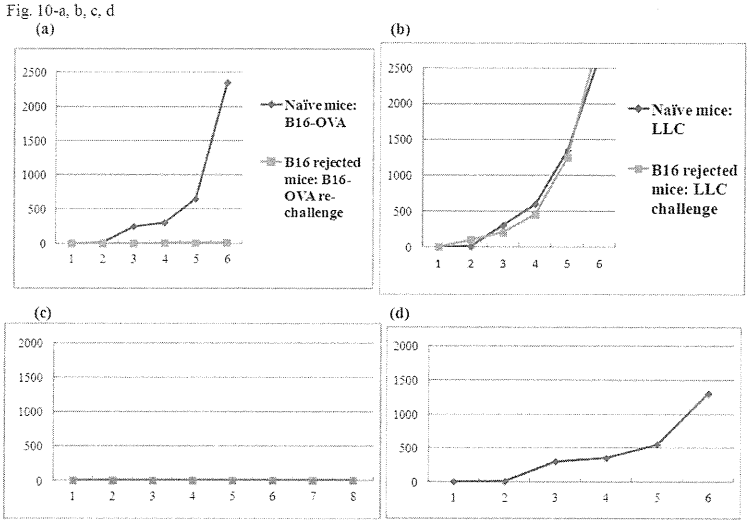


Fig.11

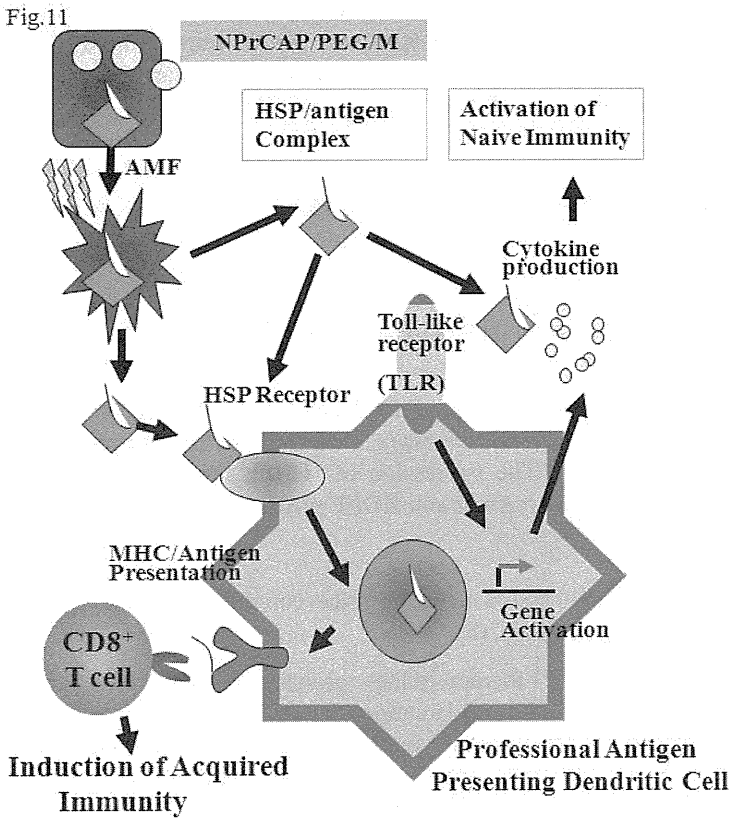


Figure Legends

Figure 1: Exploitation of melanogenesis cascade for better management of melanoma.

Figure 2: Synthesis and chemical structures of NAcCAP and NPrCAP and their tyrosinase kinetics.

Figure 3: Strategy for melanogenesis-targeted CTI therapy by conjugates of NPrCAP and magnetite nanoparticles with AMF exposure.

Figure 4: Conjugates of NPrCAP/magnetite nanoparticles for developing melanogenesis-targeted melanoma nano-medicine.

Figure 5: Selective aggregation of NPrCAP/M nanoparticles in human melanoma cell lines (a), their selective accumulation in melanosomal compartments of melanoma cells as can be seen in electron micrograph (see arrows) (b) and selective disintegration of melanoma tissues after exposure to AMF as can be seen in Berlin Blue iron staining (c).

Figure 6: Selective accumulation of NPrCAP/M in melanoma cells. Conjugates of NPrCAP and magnetite nanoparticles (NPrCAP/M and NPrCAP/PEG/M) are aggregated on the cell surface of melanoma cells and incorporated into melanosomal compartments through early and late endosomes inasmuch as the stage I melanosomes derive from the specific late endosomes, to which tyrosinase will be transported from trans-Golgi network by vesicular transport.

Figure 7: Growth inhibition of melanoma cells by primary first transplant and secondary re-challenge transplant in C57 black mice. Gr.I; control without NPrCAP/M treatment and second re-challnege melanoma transplant. Gr.II: control

without NPrCAP/M treatment, but received second re-challenge of melanoma; Gr.III: control with NPrCAP/M treatment without AMF exposure, but received second re-challenge melanoma transplant. Gr.IV: mice with NPrCAP/M and AMF exposure as well as second re-challenge melanoma transplant. After removal of the first melanoma transplant, all mice (except Gr.I) received the second re-challenge melanoma transplant on the opposite site of trunk.

Figure 8: Melanoma growth and survival of melanoma-bearing mice by CTI therapy using NPrCAP/M with and without AMF exposure.

Figure 9. Hyperthermia of melanoma cells using B16-OVA cells for induction of CTL (a) and generation of HSPs (b) in CTI therapy.

(a) Cytotoxic activity of spleen cells after CTI therapy against B16-OVA cells, B16F1 cells, EL4 cells, EL4 cells pulsed with SL8 peptide or YAC-1 cells was determined by standard ^{51}Cr-release assay. B16-OVA cells were subjected to hyperthermia using NPrCAP/M with AMF exposure *in vitro*. (b)The expression of Hsp72, Hsp90 and Gp96 was determined by western blotting with an anti-Hsp72 mAb, anti-Hsp90 mAb or anti KDEL mAb.

Figure 10. Tumor-specific immunity induced by CTI therapy. Naïve mice or mice cured by CTI therapy were re-challenged with B16-OVA melanoma cells (a) or lung carcinoma LLC (b).

Failure of the melanoma growth by secondary re-challenge after CTI therapy to the primary melanoma transplant (a). There was no growth inhibition of LLC cells in mice which received transplantation of B16-OVA melanoma cells and received CTI therapy (b). Antitumor immunity is dependent on $CD8^+$ T cells. Mice cured by CTI therapy were depleted of $CD4^+$ or $CD8^+$ T cells by an intraperitoneal injection of rat IgG (c) or anti-CD4 mAb (c) or anti-CD8 mAb (d). The growth inhibition of secondary re-challenge melanoma transplant failed after pretreatment with anti-CD8 mAb, but not by anti-CD4 mAb.

Figure 11. Scheme of intracellular hyperthermia using NPrCAP/PEG/M with AMF exposure. NPrCAP/PEG/M nanoparticles are selectively incorporated into melanoma cells. Intracellular hyperthermia can induce necrotic cell death and adjacent live melanoma cells suffer heat shock, resulting in increased level of intracellular HSP-peptide complexes. Repeated hyperthermia turns heat-shocked cells to necrotic cells, leading to the release of HSPs-peptide complexes into extracellular milieu. The released HSPs-peptide complexes are taken-up by DCs. Then, DCs migrate into regional lymph nodes and cross-present HSP chaperoned antigenic peptides to $CD8^+$ T cells in the context of MHC class I molecules, thereby inducing anti-melanoma cytotoxic $CD8^+$ T cells.

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PERSPECTIVE

Human hair melanins: what we have learned and have not learned from mouse coat color pigmentation

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Summary

Hair pigmentation is one of the most conspicuous phenotypes in humans. Melanocytes produce two distinct types of melanin pigment: brown to black, indolic eumelanin and yellow to reddish brown, sulfur-containing pheomelanin. Biochemically, the precursor tyrosine and the key enzyme tyrosinase and the tyrosinase-related proteins are involved in eumelanogenesis, while only the additional presence of cysteine is necessary for pheomelanogenesis. Other important proteins involved in melanogenesis include P protein, MATP protein, α-MSH, agouti signaling protein (ASIP), MC1R (the receptor for MSH and ASIP), and SLC7A11, a cystine transporter. Many studies have examined the effects of loss-of-function mutations of those proteins on mouse coat color pigmentation. In contrast, much less is known regarding the effects of mutations of the corresponding proteins on human hair pigmentation except for MC1R polymorphisms that lead to pheomelanogenesis. This perspective will discuss what we have/have not learned from mouse coat color pigmentation, with special emphasis on the significant roles of pH and the level of cysteine in melanosomes in controlling melanogenesis. Based on these data, a hypothesis is proposed to explain the diversity of human hair pigmentation.

Introduction

Pigmentation of hair, skin, and eyes in animals is mainly a manifestation of the presence of melanin that is synthesized in melanocytes, within membrane-bound organelles termed melanosomes. Melanosomes in follicular or epidermal melanocytes are then transferred to surrounding keratinocytes to afford surprisingly diverse colors to hair and a wide range of color from light to dark in skin (Sturm, 2009). Melanocytes in mammals (and birds) produce two chemically distinct types of melanin, black to brown eumelanin and yellow to reddish brown pheomelanin (Ito, 2003; Ito and Wakamatsu, 2008). 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).

Hair pigmentation is one of the most conspicuous phenotypes in humans. It is highly variable in color, ranging from black, dark brown, light brown, and blond to red. The underlying genetic basis for this diversity in human hair color has been a subject of extensive studies in recent years (Gerstenblith et al., 2010; McEvoy et al., 2006; Sturm, 2006, 2009). Those studies, especially genome-wide association studies (Han et al., 2008; Sulem et al., 2008), led to the identification of several genes involved in the normal variation of human hair color, including some previously unrecognized genes such as *TPCN2* (Sulem et al., 2008), *HERC2* (Han et al., 2008; Sturm et al., 2008), *IRF4* (Han et al., 2008), and *SLC24A4* (Han et al., 2008). In contrast, variations in mouse coat color have a long history of genetic as well as biochemical studies, as summarized in a classical treatise by Silvers (1979) and a review by Bennett and Lamoreux (2003). Increased interest in pigment research has resulted in the identification of a large number of new coat color genes. At present, 169

mouse coat color genes have been cloned, although 206 genes still remain to be cloned (http://www.espcr.org/micemut/). Among those 169 cloned mouse coat color genes, the majority are involved in the development of melanocytes and/or the construction and transport of melanosomes. However, fewer than 20 genes are directly involved in the production of melanin or in the regulation of the ratio of eumelanin to pheomelanin (Table 1). It should be stressed that congenic mice are available to study pigmentary effects of many specific coat color genes. Homologues of most of those genes are also involved in the variation of human pigmentation. Therefore, comparison of pigmentary effects of mouse coat color genes to those of human hair color genes should be informative. The effects of interactions between two (even three) coat color genes can be precisely analyzed in mice, but is not so straightforward in humans.

Biochemical/chemical background

Biochemical pathway for the production of eumelanin and pheomelanin

The biochemical pathway of melanogenesis has been described in detail in our recent reviews (Ito and Wakamatsu, 2008; Simon et al., 2009), and a brief summary of the scheme follows: both eumelanin and pheomelanin derive from the common precursor dopaquinone that is produced from tyrosine by the action of tyrosinase, the product of the *albino/Tyr* locus (Figure 1). Dopaquinone, an *ortho*-quinone, is a highly reactive intermediate, and in the absence of thiol compounds, it undergoes intramolecular cyclization to give cyclodopa (Land et al., 2003). Once formed, cyclodopa is rapidly oxidized to give dopachrome, a relatively stable intermediate. When not accelerated by any additional factors, dopachrome undergoes a mostly decarboxylative rearrangement to form 5,6-dihydroxyindole (DHI). However, a tyrosinase-related protein (Tyrp), called dopachrome tautomerase (Dct) or Tyrp2, the product of the *Dct/slaty* locus, catalyzes the tautomerization of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Kroumpouzos et al., 1994; Pawelek et al., 1980; Tsukamoto et al., 1992). The dihydroxyindoles DHI and DHICA are then further oxidized to produce the eumelanin polymer. Oxidative polymerization of DHI is catalyzed directly by tyrosinase or indirectly by dopaquinone, while oxidation of DHICA appears to be catalyzed by Tyrp1, the *brown* locus protein, at least in mice (Jiménez-Cervantes et al., 1994; Olivares et al., 2001). However, the human homologue TYRP1 may not act in the same way as in mice (Boissy et al., 1998), and its precise enzymatic function in humans is not clear.

In contrast to eumelanogenesis, the production of pheomelanin appears to proceed spontaneously after the production of dopaquinone as long as cysteine is present (Ito and Wakamatsu, 2008; Land and Riley, 2000). Cysteinyldopa isomers are rapidly formed among which 5-*S*-cysteinyldopa is the major isomer (Ito and Prota, 1977). Oxidation of cysteinyldopas proceeds by redox exchange with dopaquinone to form the quinone form. Cyclization and rearrangement afford benzothiazine intermediates, which in turn gives rise to pheomelanin pigment (Wakamatsu et al., 2009). This whole

Table 1. Pigmentary functions of mouse color genes and the corresponding human genes involved in melanosome structure and function

Mouse symbol (synonym)	Gene name (locus)	Pigmentary function	Human symbol	Human disease or phenotype in loss-of-function mutation
(a) Components of melanosomes				
Tyr (c)	Tyrosinase (albino)	Melanogenesis	*TYR*	OCA1; dilution of skin/hair/eye color
Tyrp1 (b)	Tyrosinase-related protein 1 (brown)	Eumelanogenesis	*TYRP1*	OCA3; dilution of skin/hair/eye color
Dct (slt)	Dopachrome tautomerase (slaty)	Eumelanogenesis	*DCT*	Not known
Oca2 (p)	Pink-eyed dilution	Tyrosinase trafficking; Neutralization of MS?	*OCA2/P*	OCA2; dilution of skin/hair/eye color
Slc24a5	Solute carrier family 24, member 5	Neutralization of MS	*SLC24A5/NCKX5*	Dilution of skin/hair/eye color
Slc45a2	Matp (underwhite)	Tyrosinase trafficking; Neutralization of MS?	*SLC45A2/MATP*	OCA4; dilution of skin/hair/eye color
Si	Silver	Melanosome genesis	*SILV/PMEL17*	Not known
(b) Eumelanin and pheomelanin switch				
A	Agouti	Switch to pheomelanogenesis	*ASIP*	Red hair (gain-of-function)
Atrn (mg)	Attractin (mahogany)	Switch to pheomelanogenesis	*ATRN*	Not known
Mc1r (e)	Melanocortin 1 receptor (extension)	Switch to eumelanogenesis	*MC1R*	Red hair
Pomc	Pro-opiomelanocortin	Switch to eumelanogenesis	*POMC*	Red hair
Slc7a11	Subtle gray (sut)	Cystine transport	*SLC7A11*	Not known

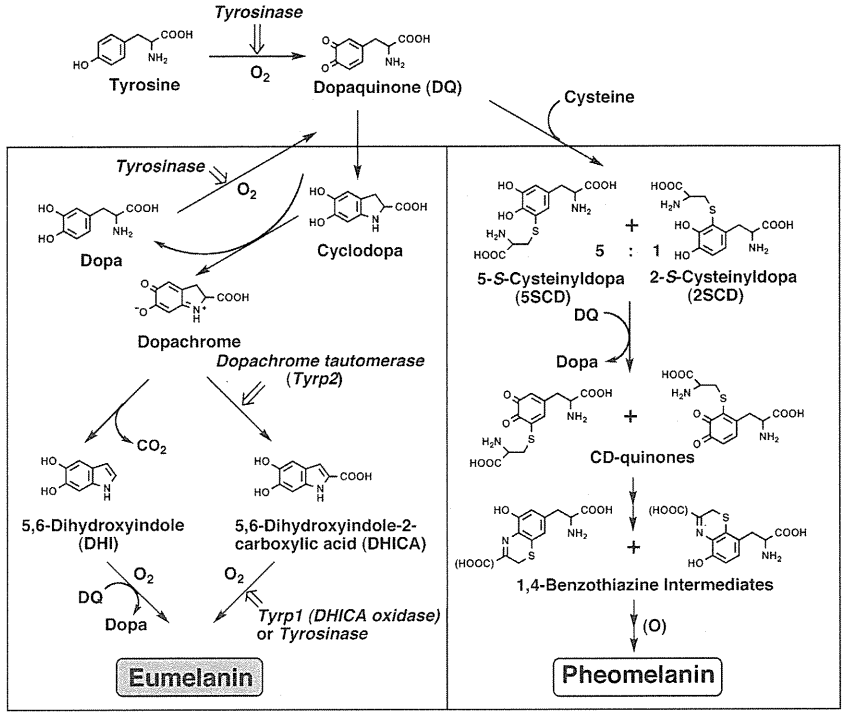

Figure 1. Biosynthetic pathways leading to eumelanin and pheomelanin production (adopted from Ito and Wakamatsu, 2008). Note that the activities of tyrosinase, Tyrp1 and Dct/Tyrp2 (and the precursor tyrosine) are involved in the production of eumelanin, while only tyrosinase (and the precursors tyrosine and cysteine) are necessary for the production of pheomelanin. (Figure used with permission, Blackwell Publishing.)

process of pheomelanogenesis requires only the activity of tyrosinase, in addition to the precursors, tyrosine and cysteine.

A three-step pathway for mixed melanogenesis

Melanogenesis in vivo produces mixtures (or copolymers) of eumelanin and pheomelanin. Therefore, melanogenesis should be considered as 'mixed melanogenesis'. We previously proposed a three-step pathway for mixed melanogenesis (Ito, 2003; Ito and Wakamatsu, 2008). In short, the total amount of melanin produced is proportional to dopaquinone production, which is in turn proportional to tyrosinase activity. Melanogenesis proceeds in three distinct stages: kinetic studies have shown that the initial stage is the production of cysteinyldopa isomers, which continues as long as the cysteine concentration is above 0.13 μM. The second stage is the oxidation of cysteinyldopas to produce pheomelanin, which is preferred over the production of eumelanin as long as cysteinyldopas are present at concentrations above 9 μM. The last stage is the production of eumelanin that begins only after most cysteinyldopas and cysteine are depleted. Therefore, the ratio of eumelanin to pheomelanin is determined by tyrosinase activity and the availability of tyrosine and cysteine in melanosomes, the branching point being 0.8 μM at pH 7.4 (Land and Riley, 2000; Land et al., 2003). More detailed discussion is presented by Ito and Wakamatsu (2008).

Chemical phenotype

Natural melanin pigments are mixtures (or copolymers) of eumelanin and pheomelanin. Although in most dark hairs of mammals, eumelanin is the predominant component, pheomelanin is a significant component in yellow/red hair in mice and in humans. Therefore, it is preferable to measure both eumelanin and pheomelanin so that the genotype can be correlated to the 'chemical phenotype' in a quantitative manner. Many studies use a melanin index measured with reflectometry as a quantitative marker of phenotype in the skin (e.g. Lamason et al., 2005; Stokowski et al., 2007) and in the hair (e.g. Mengel-From et al., 2009; Shekar et al., 2008a). Although this approach is quite useful to quantitatively evaluate pigmentation in large populations, such as the subjects in epidemiological studies, the method is somewhat limited because it is only an indirect measure of pigmentation.

We have previously established chemical methods to directly analyze eumelanin and pheomelanin contents based on the production of specific markers, pyrrole-2,3,5-tricarboxylic acid (PTCA) and aminohydroxyphenylalanine (AHP), respectively (Ito and Fujita, 1985; Wakamatsu and Ito, 2002). Our original methods used acidic permanganate oxidation and hydroiodic acid hydrolysis to produce PTCA and AHP isomers, respectively. That method was later improved so that AHP isomers are separated to 4-amino-3-hydroxyphenylalanine

(4-AHP) and its isomer 3-AHP, the former isomer being specific for 5-*S*-cysteinyldopa-derived pheomelanin so that the specificity for pheomelanin was increased (Wakamatsu et al., 2002). The acidic permanganate oxidation has now been replaced with alkaline hydrogen peroxide oxidation in which the yield of PTCA is doubled, and other markers can be simultaneously analyzed in a more simplified method (Wakamatsu et al., 2003). Notably, pyrrole-2,3-dicarboxylic acid (PDCA) can now be analyzed, and PTCA and PDCA can be used as markers to estimate DHICA- and DHI-derived units, respectively (Ito and Wakamatsu, 1998). As an independent parameter of pigmentation, we use absorbance at 500 nm after solubilization in aqueous Soluene-350 (Ozeki et al., 1995, 1996).

We have used these chemical degradation methods to evaluate the diversity of human hair pigmentation. Chemical phenotypes were analyzed for 209 University of Arizona students (our unpublished results). Except for red hair, human hair melanins consist of approximately 99% eumelanin. Dark brown hair (n = 53) contains eumelanin at 64% the level of black hair (n = 82), medium to light brown hair (n = 43) at 46%, and blond brown to blond hair (n = 23) at 23%. On the other hand, red hair (n = 8) contains eumelanin at 31% the level of black hair and pheomelanin at a level comparable to eumelanin. Similar results with red hair have been reported in Naysmith et al. (2004). So, how is this diversity in human hair pigmentation biochemically controlled? This is the major question addressed in this Perspective.

Effects of mutations on mouse/human pigmentation

Tyr/ TYR locus

The enzyme tyrosinase is encoded at the *albino/Tyrosinase* (*C*) locus in mice. Tyrosinase is the key enzyme in melanogenesis, producing dopaquinone that is a common precursor for eumelanin and pheomelanin (Figure 1). Without functional tyrosinase, no melanin is produced in mice or in humans (in humans, this is termed oculocutaneous albinism type 1, OCA1) (Oetting and King, 1999; Suzuki and Tomita, 2008). The *chinchilla* allele at the *albino* locus encodes a partially functional tyrosinase whose activity is <50% wild type, because of a point mutation (Ala464Thr) that makes it susceptible to proteolytic cleavage (Müller et al., 1988). Thus, this mutant is an ideal model to examine the specific effects of tyrosinase activity on melanogenesis. Mice homozygous for the *chinchilla* allele have eumelanin contents about 50% that contained in *nonagouti* black hairs (Lamoreux et al., 2001). However, this suppression was not observed in *chinchilla* mice homozygous also for *slaty* (Dct^{slt}/Dct^{slt}) or *brown* ($Tyrp1^{b}/Tyrp1^{b}$) to significant extents (Table 1). Interestingly, homozygosity for the *chinchilla* allele greatly reduces the amount of pheomelanin both in *lethal yellow* (A^{y}/a) and in *recessive yellow* ($Mc1r^{e}/Mc1r^{e}$) mice by >80%.

These results with *chinchilla* mice indicate that functional Dct and Tyrp1 are also necessary, in addition to high levels of tyrosinase, for maximal production of eumelanin. On the other hand, the greatly reduced production of pheomelanin in *chinchilla* mice with *lethal yellow* or *recessive yellow* mutation can be explained by the three-step theory of melanogenesis noted earlier. It is likely that under conditions of low tyrosinase activity (and high cysteine concentration), cysteinyldopas are produced, but are not readily oxidized to pheomelanin (Ito and Prota, 1977; Ito and Wakamatsu, 2008). In *black* (*nonagouti*) *chinchilla* mice, the degree of eumelanogenesis can be directly proportional to tyrosinase activity under low cysteine concentration.

Mutations at the *TYR* locus in humans cause OCA1 with various degrees of pigmentation. SNPs in *TYR* genes are also known to affect normal pigmentation in humans (Sturm, 2009). However, those mutations do not seem to play major roles leading to the diversity of human hair pigmentation (Sturm, 2009; Valenzuela et al., 2010). It would be interesting to know whether human red hair caused by a mutation in *MC1R* would be affected by a mutation in tyrosinase that decreased its activity, a situation similar to *recessive yellow chinchilla* mice.

Dct/DCT and *Tyrp1/TYRP1* loci

The *slaty/Dct* (*Slt*) locus in mice encodes Tyrp2/Dct that catalyzes the tautomerization of dopachrome to DHICA, and thus wild-type mice produce DHICA-rich eumelanin. This is indicated by high values of PTCA/total melanin ratios in mice wild-type at the *slaty* locus, including *black*, *brown*, *black chinchilla*, and *brown chinchilla* mice (Lamoreux et al., 2001). Homozygosity for the mutant *slaty* allele results in Dct whose functional activity is reduced 3- to 10-fold (Jackson et al., 1992) or null (our unpublished observation). This mutant *slaty* genotype was found to greatly reduce the PTCA value with a mild reduction in total melanin, and hence, the PTCA/total melanin ratio was reduced four to sixfold, indicating the production of DHICA-poor eumelanin.

Polymorphisms in the *DCT* gene in humans are known and are believed to affect hair pigmentation, at least in Asians (McEvoy et al., 2006). However, it is not known whether such polymorphisms play a major role in producing the lighter color in the skin (and hair) of Asians compared to Africans. Interestingly, follicular melanocytes of brown or black hairs were found to lack expression of DCT protein irrespective of ethnic origin (Commo et al., 2004). This would result in the production of DHICA-poor eumelanin in human hair. However, systematic studies evaluating the ratio of DHI to DHICA in human hair remain to be carried out.

Tyrp1 is encoded at the *brown/Typr1* (*B*) locus and is believed to act as a DHICA oxidase in mice