Unrevealing the role of P-protein on melanosome biology and structure, using siRNA-mediated down regulation of OCA2

Sangjoo Park · V. K. Morya · Dong Hoang Nguyen · Birendra K. Singh · Hyang-Bok Lee · Eun-Ki Kim

Abstract The pink-eyed dilution protein (P-protein) plays a critical role in melanin synthesis in melanocytes and retinal pigment epithelium cells. Mutation in this protein may cause complete or partial albinism. Role of the P-protein ranges in melanin synthesis to maturation and trafficking of the melanosomes. The aim of the present study was to evaluate the effect of P-protein inhibition on melanosome biology by comparing the shape, size, count, and types of melanosomes in melan-a melanocytes. The cells were extensively examined by the transmission electron microscopy. The P-protein inhibition was carried by P-protein-siRNA transfection to melan-a melanocytes, B16F10 mouse melanoma, and melan-p1 cells. Measurement of melanin contents, cellular tyrosinase, and different tyrosinase related proteins were also determined to investigate the effect of P-protein siRNA transfection on melanocytes. Results suggested that the inhibition of P-protein can significantly change the melanosomal morphology, types and their respective numbers, and provided a novel strategy for the control of melanin synthesis.

Keywords Pink-eyed dilution (P) protein · Melanosome · siRNA transfection · Melanin · Transmission electron microscopy

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Introduction

Melanins are complex pigments; typically consist of co-polymers of black or brown eumelanins and red or yellow pheomelanins; and playing a major role in pigmentation of the skin, hair, and eyes of mammals. Its physiological role is to provide photoprotection against ionizing radiation. The color of the skin is primarily determined by the quantity, type, and distribution of melanin within the skin [1]. Melanosomes are membrane delimited structure of melanocyte which anchors melanin. Four morphologically distinct stages of melanosomes are reported [2]. The stages I and II lack pigments whereas, intraluminal proteinaceous fibrils are found in stage II melanosomes. The stage III melanosomes have melanin deposition on the fibrils whereas, stage IV is characterized by fully deposited melanin in the lumen of melanosome, which completely masked the internal structure [3]. The synthesis of melanin is a multistep process and about 192 known gens (e.g., MC1R, TYR, OCA2, SLC24A5, MATP, ASIP, and etc.) are involved in this process [1, 4–6]. Thus, alteration in any gene or their expression highly influences the skin pigmentation and resulting in either hyperpigmentation or hypopigmentation disease.

The murine pink-eyed dilution (p) locus codes for 'P-protein,' which is a melanosome membrane protein, control melanin synthesis up to certain extant [7, 8]. This protein has 12 trans-membrane domain peptides and resembling as a channel or transporter. Mutation or deletion of this protein causes Oculocutaneous albinism type II (OCA2), one of the most common types of albinism [9, 10]. People with this type of albinism are generally had more pigment and better vision than those with OCA1 [10].

Function and structure of the P-protein are still unclear; however, some proposed functions have been reported by

indirect method of investigations, like pH regulation in melanosome [11], melanosomal protein complex stabilization [12], sorting of tyrosinase to melanosomes [13], membrane transport [14], arsenic sensitivity [15], control of tyrosinase processing [16, 17], and disorder in glutathione metabolism [15].

Present study was designed to gain insights into function of P-protein on melanosomal morphology, physiology, and types using electron microscopy. The small interfering RNA (siRNA)-mediated down regulation of P-protein was performed in melan-a melanocytes, B16F10 mouse melanoma, and melan-p1 cells (null at the p-locus) in order to find the involvement of P-protein in the melanosome maturation and melanin synthesis. In order to evaluate the effect of P-protein down regulation on melanosome specific proteins (cellular tyrosinase, TRP-1, and TRP-2), the Western blot analysis was also performed.

Materials and methods

All chemicals and media constituents used in this study were of analytical grade. RPMI medium, 0.5 % Trypsin–EDTA solution (10X), phosphate buffered saline (PBS, pH 7.4), Penicillin/Streptomycin (10,000U/mL), and 2-mercaptoethanol were purchased from Invitrogen Corp. (CA, USA). Dulbecco's Modified Eagle's medium (DMEM) (Gibco BRL, Eggenstein, Germany). 12-*O*-tetradecanoylphorbol-13-acetate (TPA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT), and L-tyrosine were procured from Sigma Chemical Co. (St.Louis, MO, USA). Lipofectamine^{TM} RNAiMAX reagent was obtained from Invitrogen Corp. (CA, USA). P-protein-siRNA was purchased from Bioneer Corporation (Bioneer, Daejeon, Korea). PVDF membrane (Invitrolon, USA) was purchased from Sigma. Antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Cell lines and cell culture

Melan-a cell line, a highly pigmented, immortal, non-tumorigenic mouse melanocyte cell line, B16F10 mouse melanoma cell line, melan-p1 cell line (which is null at p-locus), and HeLa cells were used for the present study. The melanocytes and melanoma cells were generously provided by Prof. Dorothy C. Bennett (St. George's Hospital Medical School, London, UK). Culture procedures were carried out as described in literature [12]. In brief, the melan-a murine melanocytes were cultured in RPMI supplemented with 10 % (v/v) fetal bovine serum, 1 % (v/v) penicillin/streptomycin (100 units/mL), and 200 nM TPA at 37 °C in a humidified atmosphere with 10 % CO_2. In

addition to this, melan-p1 was supplemented with 100 μM of 2-mercaptoethanol during the time of subculture to improve the cell growth. B16F10 murine melanoma cells were cultured in DMEM supplemented with 10 % (v/v) fetal bovine serum, 1 % (v/v) penicillin/streptomycin (100 units/mL) at 37 °C in a humidified atmosphere in a 5 % CO_2. The HeLa cells (ATCC No. CCL 185) were cultured in DMEM supplemented with 10 % (v/v) fetal calf serum, 1 % (v/v) penicillin/streptomycin (100units/mL). Subculture of the cells was carried out at fourth day of incubation until a passage number 20 was achieved.

Preparation of small interfering RNA (siRNA)

For targeted knockdown of P-protein, one pair of P-protein- siRNA was designed according to an OCA2 gene transcript (NCBI GenBank accession number, NM_021879.2), which is synthesized by Invitrogen, Carlsbad, CA, USA (Purchased form Bioneer Korea). Nucleotide sequences of P-protein-siRNA were as follows: OCA2-MSS276194 (RNA)-2280 CACACTATCAGGA-CAATGGCAGCTG 2304 (sense strand) and OCA2-MSS276194 (RNA)- 2280 CAGCTGCCATTGTCCTGA-TAGTGTG 2304 (antisense strand). The oligoribonucleotide duplex (NC) used as Negative control was purchased from Bioneer (Daejeon, Korea), and the sequences were as follows: NC, 5′-CCU ACG CCA CCA AUU UCG U(dTdT)-3′ (sense strand) and 5′-ACG AAA UUG GUG GCG UAG G(dTdT)-3′ (antisense strand).

P-protein- siRNA transfection

For transfections, the melan-a cells, B16F10 cells, and melan-p1cells were treated with siRNA-Lipofectamine RNAiMAX mixture as described in standard protocol provided by the manufacturer. In brief, melan-a cells, B16F10 cells, and melan-p1 cells were seeded (6.5 × 10^5, 6.5 × 10^5 and 7.5 × 10^{5}cells/dish, respectively) on 100-mm dishes separately. After 24 h of incubation (cell monolayers at approximately 30 % confluence), the cells were subjected to P-protein siRNA and negative siRNA transfection separately. Initially, cells were washed with Opti-MEM GlutaMax medium (Invitrogen, Carlsbad, CA, USA) and further treated with transfection mixture (diluted siRNA (separate): diluted Lipofectamine RNAiMAX::1:1) which was prepared in Opti-MEM GlutaMax medium with siRNA and Lipofectamine RNAiMAX, separately according to the manufacturer's protocol. Different concentration of P-protein-siRNA from 1 to 50 nM and final volume was 5 mL. The cells were cultured in the presence of transfection mixture for 24 h, and then the transfection mixture was replaced by fresh medium without antibiotics (RPMI for melan-a cells and melan-p1 and DMEM for B16F10).

The melan-a and melan-P1 cell cultures were pursued for an additional 48 h incubation in culture media containing no antibiotics. After 24 h of incubation in fresh media, the B16F10 cells were harvested. Negative siRNA transfection was carried with 20 nM of concentrations.

Toxicity assay

Toxicity assay or MTT assay was used to determine cell toxicity in response to siRNA transfection. The melan-a (5×10^3 cells/well), B16F10 (2×10^4 cells/well), and melan-p1 (5×10^3 cells/well) cells were seeded in 96-well plate system at their respective cell density and treated with different concentration of P-protein siRNA from 1 to 50 nM. To determine the toxic effect on other cell lines from melanocytes, HeLa cells (non-melanogenic in nature, 1×10^4 cells/well) were also cultured in a 96-well plate and exposed to various concentrations of P-protein siRNA. Negative siRNA transfection was carried at 20-nM concentration. After treatment, cells were incubated at appropriate conditions for 24 h. Further, 20 µL of MTT solution (5 mg/mL PBS) and 80 µL of medium were then added to each well and incubated at 37 °C for **3** h. Then, the MTT solution was removed and the blue crystalline precipitate in each well was dissolved in 200 µL of DMSO. Toxicity of siRNA was assessed by measuring absorbance at 540 nm using a molecular device "Spectra Max 340 microplate-reader" [18]. Untreated cells were used as control group.

Measurement of melanin content

Melan-a cells (2.5×10^4 cells/well), melan-p1 (2.5×10^4 cells/well) cells, and B16F10 (2.5×10^4 cells/well) mouse melanoma cells were seeded into 12-well plate incubated for 24 h, and then transfected with P-protein siRNA and negative control siRNA separately. After 24 h, cultures were fed with fresh media, and incubated for further 48 h (24 h for B16F10 cells). Then cells were detached by trypsin/EDTA solution and harvested. The harvested cells were washed twice with PBS for 5 min at 5,000 rpm, suspended in 100 µL of extraction buffer (1 N NaOH, 10 % DMSO), heated at 80 °C for 1 h, and transferred to 96-well plate. Melanin content was determined by absorbance at 405 nm in ELISA microplate reader [18]. Untreated cells were used as a control.

Cellular tyrosinase assay

For the cellular tyrosinase assay, melan-a, melan-p1, and B16F10 cells were cultured and treated as described in previous section. The cells were lysed in lysis buffer (0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 %

Triton-100X). Cells were disrupted by sonication at full cycle, for 30 min at 4 °C, and lysates were cleared by centrifugation at 13,000 rpm for 20 min at 4 °C. The protein content of lysate was quantified by protein assay kit (Bio-Rad, USA). All the similar reaction mixtures consisting of 40 µg of protein, 40 µL of 5 mM L-DOPA and 0.1 M PBS (pH 6.8), was assayed on a 96-well plate at 37 °C. Absorbance was measured at 475 nm using an ELISA reader [5]. Extracts from normal cells and lipo-factamine-treated cells were used as a control.

Expression profile of tyrosinase, TRP-1, and TRP-2 by Western blotting

The effect of P-protein-siRNA on the expression of tyrosinase, TRP-1, and TRP-2 was also examined by Western blotting. The transfected and control cells were lysed, using lysis buffer (40 mM Tris (pH 8.0), 120 mM NaCl, 0.5 % Nonidet P-40 (NP-40), 0.1 mM sodium orthovanadate, 2 µg/mL aprotinin, 2 µg/mL leupeptin, and 100 µg/mL phenylmethylsulfonyl fluoride (PMSF)). The lysates were centrifuged at $10,000 \times g$ for 5 min 4 °C. The supernatants were collected, and protein concentrations were determined by protein assay kit (Bio-Rad, USA). For the Western blotting, equal amount of proteins were boiled for 5 min in loading dye, then chilled in ice, subjected to 10 % sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to a PVDF membrane. The membrane was incubated in a blocking solution (5.0 gm of nonfat dry milk in 100 ml of TBS) for 2 h at room temperature on rocker. The membrane was then incubated for 2 h with goat polyclonal Primary antibody, and further incubated with an anti-goat horseradish peroxidase-conjugated secondary antibody. Excess of antibody was removed by three time washing with TBS-T buffer (TBS consisting 1 % Tween-20). The protein bands were visualized by the enhanced chemiluminescence (ECL) detection system (GE Healthcare Biosciences, Piscataway, NJ, USA) [13]. β-actin was used as loading control.

Electron microscopy

After 48 h of incubation, the transfected and control cells were harvested, and washed three times with 0.1 M phosphate buffer. These cells were primarily fixed by fixing solution containing 4 % paraformaldehyde, 1 % gluteraldehyde in 0.1 M Phosphate buffer for 4 h followed by treatment of 1 % osmium tertroxide at 4 °C for 1 h, for secondary fixation. After that dehydration was performed, using an ascending concentration of ethyl alcohol i.e., 10 min each in 50, 70, 80, and 90 %, while two times treatment in 95 % for 15 min, and finally 1 h in 100 %.

After this step, the sample was treated with propylene oxide for 15 min. For the embedding of sample, the Embed 812 was used [19], which is a commercial quick-setting epoxy glue having Epoxy resin (mixture A, and mixture B mixed according to manufacturer with 1.4 % DMP-30). The Mixture A contained Embed 812® (EMS) and dodecyl succinic anhydride, while Mixture B had Embed 812® (EMS) and nadic methyl anhydride. The resin (R) and hexylene glycol (HG) was combined in a 1:2 ratios, add to dish, and mix on rotor for 2 h. Then resin, and hexylene glycol at 2:1 ratio, for 2 h and finally replaced with 100 % resin and place on rotor to mix for 6 h. It was allowed to polymerize at 60 °C for 3 days. Then ultrathin sections of sample were obtained by Ultramicrotome (MTX ULTRA-MICROTOME). The specimen was treated with Uranyl acetate and lead citrate for 30 and 15 min, respectively. This specimen was examined by transmission electron microscope (TEM). Cells from each lattice grid were observed and recorded.

Statistical analysis

Student's t test was used to assess the differences between the results obtained and a p value of <0.05 was accepted as significant. All determination was run in triplicate on at least two independent experiment and data shown are mean ± SD. The p value was interpreted as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$: statistically significant from control.

Results

This study was aimed to investigate the effect of P-protein down regulation on melanosome biology. To achieve the goal, the P-protein siRNA (20nM)-mediated transfection was optimized for different time intervals and 24 h of siRNA treatment was found to be more effective (data not shown). The dose-dependent inhibition of P-protein and their effect on melanin synthesis was investigated on melan-a cell line as shown in Fig. 1. It was found that the 1 nM P-protein siRNA was sufficient to inhibit approximately 25 % of melanin content. However, a dose-dependent inhibition was observed from 1 to 50 nM siRNA treatment (Fig. 1). The P-protein siRNA concentration more than 20nM was found to be little toxic to melan-a cells; however, no further inhibition in melanin content was observed. At 10-nM concentration of the P-protein siRNA about 40 % inhibition in melanin synthesis was observed. Therefore, siRNA concentration of 10 nM was used for further study. The Western blot for expression profile of the P-protein in response to siRNA treatment was investigated and a clear dose-dependent pattern was observed (Fig. 1.).

The siRNA transfection experiments in melan-p1 (null at p-locus) cells were conducted in L-tyrosine (2 mM) supplemented RPMI media. It was shown in Fig. 2 that the melan-p1 cells were pigment less when cultured in RPMI media. However, the supplementation of L-tyrosine in RPMI media induced melanin synthesis in dose-dependent manner (Fig. 2). In order to compare the effect of the P-protein siRNA on other different cell lines i.e., the B16F10 and melan-p1 cells were also used. A comparison of siRNA effect on melan-a, melan-p1, B16F10, and HeLa (for toxicity evaluation) cells were shown in Fig. 3. Three different concentrations of siRNA (1, 10 and 20 nM) were used. The melan-a cells were used as a reference for other cells (Fig. 3a). Melanin synthesis in melan-p1 (cultured in L-tyrosine supplemented media) was not affected by siRNA treatment (Fig. 3b), whereas the B16 cells were showed similar pattern to melan-a cells (Fig. 3c). The antimelanogenic effect of siRNA on B16F10 cells was slightly less than the melan-a cells (Fig. 3c). The HeLa Cell was used in this study to ensure the cytotoxic effect of siRNA, and it was revealed that the siRNA was not toxic to the non-melanogenic cells also (Fig. 3d.).

To ensure the effect of siRNA on cell morphology and growth pattern of melan-a cells in culture condition the optical microscopic view of the cells were compared (Online resource 1). The results from cultured cells and embedded (In resin) cells revealed that there was no effect of siRNA on cellular morphology of melan-a cells (Online resource 1).

TEM was performed to analyze the structure, stages and number of melanosomes in response to P-protein siRNA. Figure 4 showing the normal melan-a cells and their ultrastructures revealed melanosomes at different magnifications or scale bar (Fig. 4). Figure 4a represented the grid view (Bar; 20 μm) and Fig. 4b explained the normal melan a cells (Bar; 2 μm) having different stages of melanosomes. Mature melanosomes were mostly present on the peripheral region of the cells. Figure 4c–f described the different stages of melanosomes (Bar; 0.05 μm). Premelanosomes contain two different stages (Stage I and II) which were clarified by Fig. 4c, d using high resolution (Bar; 0.05 μm), whereas other two stages i.e., III and IV belong to mature melanosomes (Fig. 4e, f, Bar; 0.05 μm). Vesicular structures with internal membranes were observed in stage I (Fig. 4c). In stage II, differentiation appeared such as extended structures with interior striations. However, in both stages (Stage I and II) melanin deposition was absent. In stage III melanosomes, striations became thick and black in color due to deposition of melanin (Fig. 4e), whereas melanosome fully packed with melanin designated as stage IV melanosomes (Fig. 4f).

Further, comparisons of cellular morphologies were done between transfected and normal cells which found to be
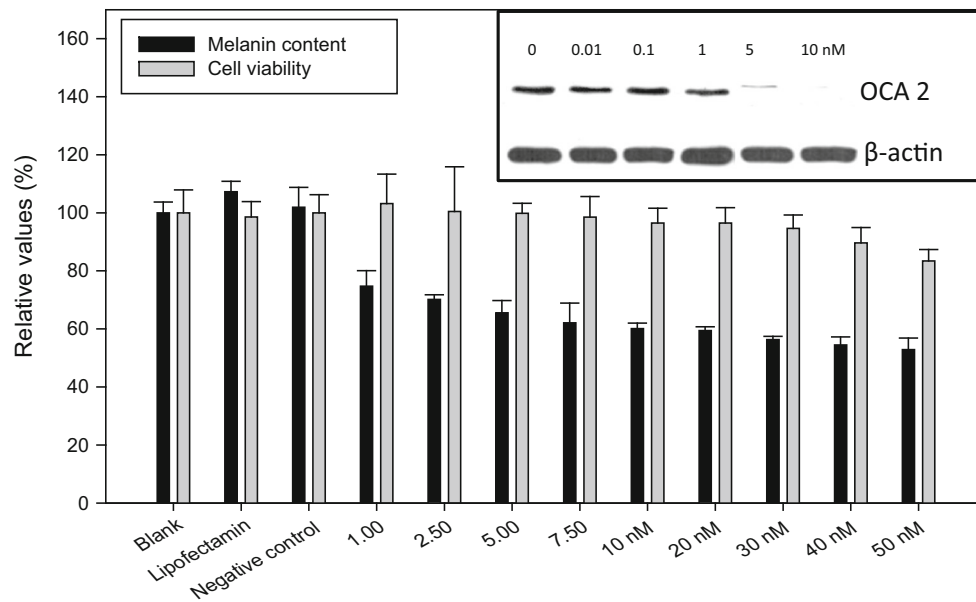
Fig. 1 Effect of the P-protein siRNA on melanin synthesis and toxicity assessment of melan-a cells in a dose-dependent manner. *Inset* showing the Western blot for P-protein expression in response to P-protein siRNA in a dose-dependent manner
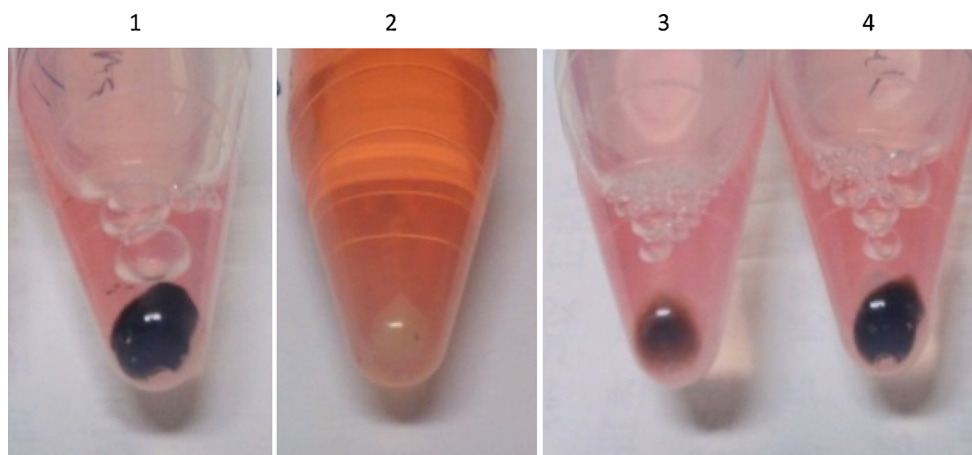

Fig. 2 Effect of L-tyrosine on melan-p1 cells (*Lane, 1* Melan-a cells, *2* melan-p1 cells in RPMI media, *3* melan-p1 cells in RPMI media supplemented with 1 mM L-tyrosine, *4* melan-p1 cells in RPMI media supplemented with 2 mM L-tyrosine)

similar in both type of cells (Fig. 5). Results also explained that stage IV melanosomes were predominantly observed in untreated or normal melan-a cell than the siRNA-transfected melan-a cell and normal melan-p1 cells. In transfected melan-a cells, the stage II and III of melanosomes were higher than the stage IV (Fig. 5a, b). Stage II melanosomes had elongated vesicle and distinct fibrillar structures, whereas in stage III melanosomes melanin synthesis was initiated and the pigment was deposited uniformly (Fig. 5b). Significant reduction in the number of stage IV melanosomes was also found in siRNA-transfected cells. However, the melanosomes were found to be slightly lowered and spherical in the transfected cells (Fig. 5). In melan-p1 cells only

vesicles were observed; there was lack of differentiated melanosomes (Fig. 5c). The number of melanosomes at different stages were counted (per unit area) in both normal and transfected melan-a cells. It was found that the average number of the melanosomes were less in P-protein siRNA-transfected cells then normal melan-a cells (Fig. 6). When stage I and stage II melanosomes were counted together, it was revealed that the transfected cells have higher percentage than the non-treated cells. The percentage of melanosome stages III was found to be slightly decreased in transfected cells. Whereas, the percentage of stage IV melanosomes were significantly low in transfected cells (Table 1).
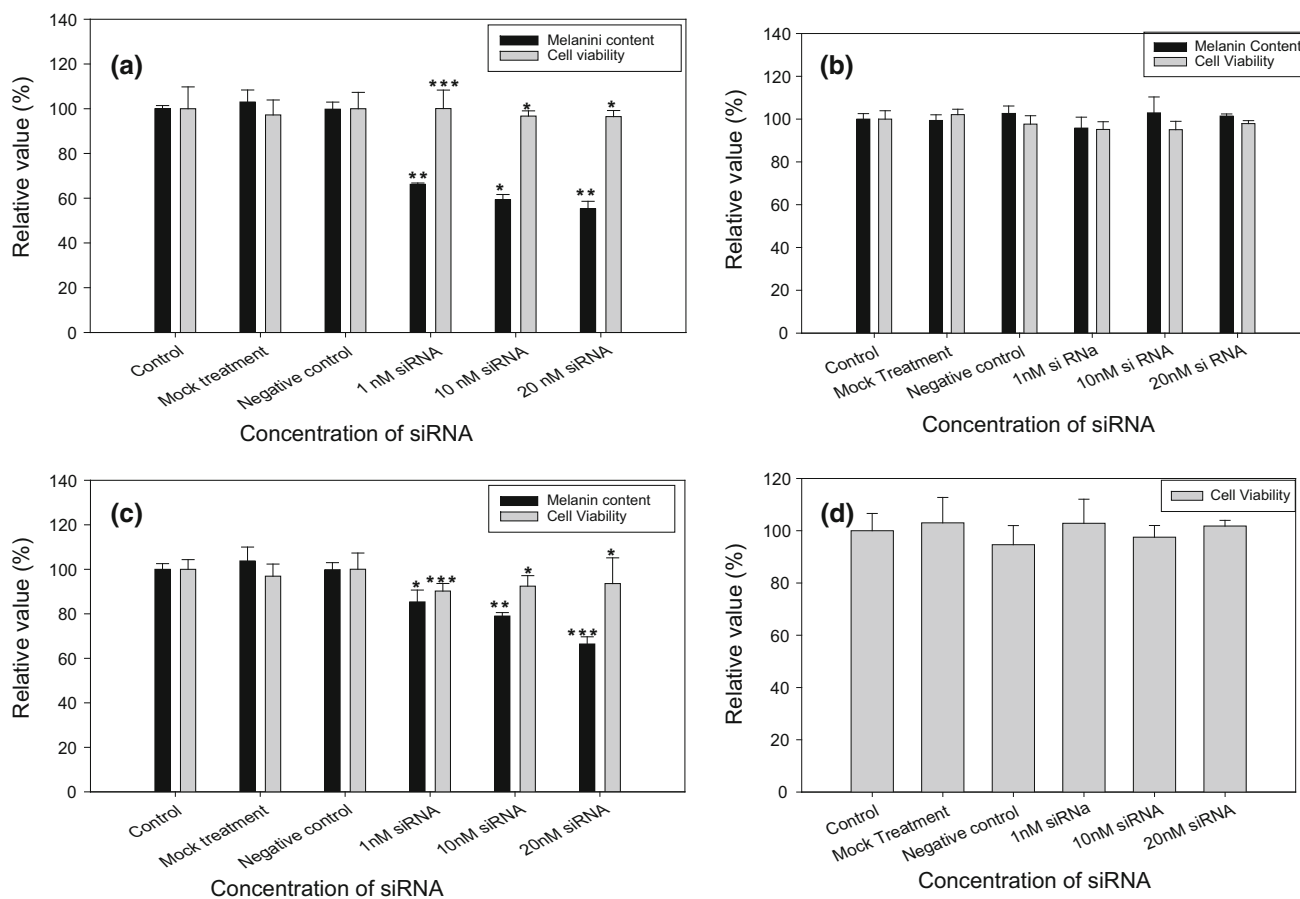
Fig. 3 Effect of P-protein-siRNA on different cell line **a** Melan-a cells, **b** Melan-p1 cells, **c** B16F10 cells, **d** Hela cells

Total cellular tyrosinase activity was measured to evaluate the effect of P-protein siRNA on different cells. The results from cellular tyrosinase assay showed that cellular tyrosinase activity was significantly inhibited after treatment with P-protein siRNA at the concentration of 10 nM (Fig. 7a). At this concentration, the cellular tyrosinase activity was inhibited up to 60 % in melan-a cell. The B16F10 melanoma cells were found to be more resistant against siRNA and about 80 % tyrosinase activity was retained at 10 nM siRNA (Fig. 7b). However, the tyrosinase activity was unaffected in melan-p1 irrespective to siRNA concentration (Fig. 7c).

Expression profiles of melanosomal key proteins were evaluated by Western blot. The OCA-2 expression was down regulated in dose dependent manner in melan-a cells in response to siRNA (Fig. 8). The OCA-2 protein was not detected at 10 nM siRNA-treated melan-a cells. The Tyrosinase was inhibited in a dose-dependent manner in response to P-protein siRNA, in melan-a cells (Fig. 8). The effect of P-protein on TRP-1 and TRP-2 was also examined. The results showed that P-protein siRNA did not show significant effect on the expression of these proteins (Fig. 8).

In B16F10 cells, the P-protein expression was down regulated in siRNA-treated cells (Fig. 9a). The expression of the tyrosinase was lowered in 5 nM siRNA-treated cells, whereas in 1nM expression of tyrosinase was approximately similar to control group (Fig. 9a). TRP-1 and TRP-2 was remained unaffected in B16F10 melanoma cells (Fig. 9a). There was no band detected for P-protein in melan-p1. The TRP-1 and TRP-2 was also unaffected in melan-p1 cells (Fig. 9b).

Discussion

The mutation and deletion in Pin- eyed dilution protein (P-protein) cause a significant reduction in melanin biogenesis, and its mechanism of action and effect on melanosomal biology was still unclear. However, some studies have been done to predict their involvement [11–14, 16, 17]. This study was crafted to examine the structural and numerical changes of melanosome, observed under TEM, in response to inhibition of P-protein. To inhibit P-protein a siRNA-mediated method was used. The consecutive effect on other
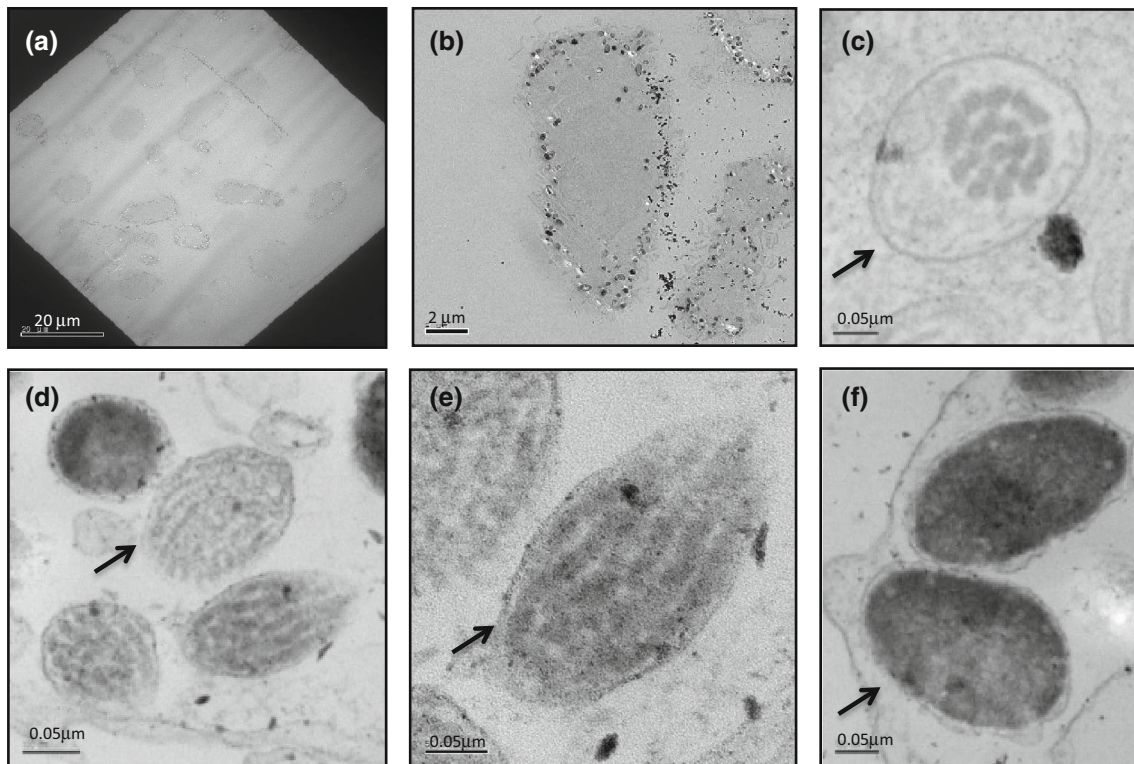
Fig. 4 Normal melan-a cell and their ultrastructures showing melanosomes viewed under different magnification, **a** Grid view **b** Normal melan-a cells **c** Stage I melanosome **d** Stage II melanosome **e** Stage III melanosome **f** Stage IV melanosome
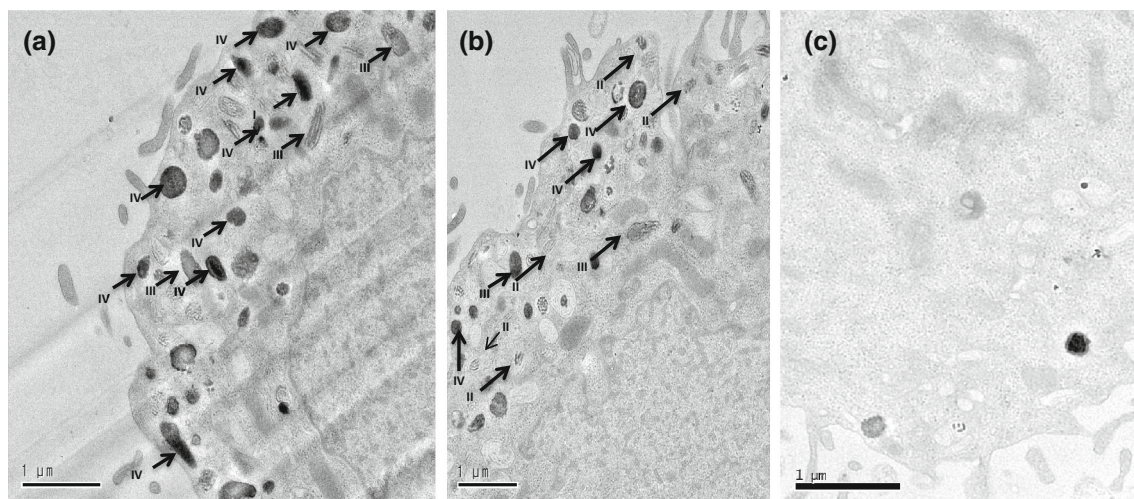

Fig. 5 Comparison of melanosome stage IV in between normal melan-a cells (**a**), P-protein- siRNA-transfected melan-a cells (**b**), Control Melan-p1 cells cultured in RPMI media (**c**)

melanosomal proteins like Tyrosinase, TRP-1, and TRP-2 were also examined.

Literature on siRNA-mediated inhibition of P-protein has been unavailable until now. However, it is well known that the mutation or deletion of P-protein in a melanocyte directly affects melanin synthesis [3, 14, 17]. It was observed that the OCA-2 gene expression was down

regulated in a dose-dependent manner; however, the melanin synthesis was reduced up to 40 %. The transfected cells were further cultured in normal media, which allow to secret the P-protein. The melan-a cell which showed a down regulation of P-protein in Western bolt also showed melanin inhibition (Fig. 1). This phenomenon could be explained by the previous report on an oculocutaneous
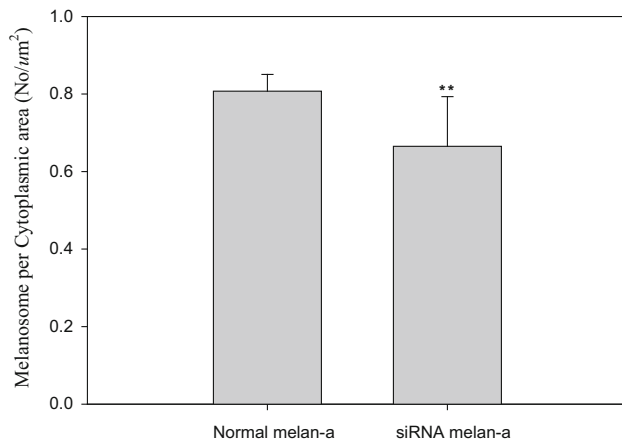
Fig. 6 Average number of melanosomes counted (per unit area) in normal and P-protein-siRNA-transfected melan-a cells

Table 1 Comparison of average (%) number of melanosome stages in normal and P-protein siRNA transfected melan-a cells

	Melanosome stage/s	Normal melan-a cells (average number of melanosomes) (%)	P-protein siRNA transfected melan-a cells (average number of melanosomes) (%)
1	I + II	16.77 ± 3.3	35.74 ± 10.1
2	III	32.21 ± 6.1	28.06 ± 7.8
3	IV	51.02 ± 1.1	36.19 ± 8.6

albinism type–II, the patients having OCA-2 albinism have increased pigmentation in the skin, hair, and eyes with age [19]. To ensure the effect of tyrosine on melanin synthesis in melan-p1 cells (null at p-locus), the RPMI media was supplemented with L-tyrosine. The result shown in Fig. 2 revealed that the production of melanin was enhanced, a similar result also reported by Rosemblat and co-worker in 1998 [14]. P-protein was reported as tyrosine transport facilitator and mutation or deletion in this protein may affect availability of tyrosine in melanosome, a limiting factor of melanin synthesis [14]. However, other reported functions of P-protein may also affect the gross melanin synthesis. Inhibition of the P-protein may alter the melanosome pH, tyrosinase processing, maturation, and trafficking [11, 13, 16, 17]. The P-protein siRNA-mediated down regulation of the P-protein caused significant inhibition of the melanin synthesis without affecting the growth of melan-a and B16F10 (Fig. 3a, c). However, the siRNA was not effective on melan-p1 cells cultured in 2 mM L-tyrosine supplemented RPMI media (Fig. 3b). It was reported that melan-p1 cells produce melanin in the presence of high concentration of tyrosine, and probably at higher concentration of tyrosine, the availability of tyrosine in melanosome was higher due to diffusion [14]. The

melanin produced in melan-p1 cells was not affected by P-protein siRNA, due to lack of P-protein. The P-protein siRNA was nontoxic to cells other than the melanogenic cells, like HeLa cells (Fig. 3d). Similar study conducted with tyrosinase siRNA-mediated knockdown of tyrosinase for inhibition of melanin synthesis showed that the siRNA itself did not cause toxicity to the cells [20]. The morphological behavior of the melan-a cell was not affected by siRNA and lipofectamine as revealed by microscopic examination of the cultured cells (Online resource 1). These results suggested that molecular approaches using siRNA targeting P-protein may provide a novel strategy for the control of cell pigmentation. The effect of P-protein down regulation, using siRNA, on structural and numerical alterations of melanosomes in melanocytes was analyzed by TEM. Results showed that siRNA significantly changed the number of melanosomes in transfected cells (Fig. 5, 6). Not only the average number of melanosome was found to be lowered, but the stages of melanosome were also varied in the transfected melan-a cell. The immature melanosome, i.e., stage I & II was found to be approximately double in transfected cells than the normal melan-a cell. The studies on melan-p1 (null at p-locus) cells stated that the numbers of immature melanosome were higher than the mature melanosomes [7, 14]. The melan-p1 cells have no P-protein and lowered number of mature melanosome reflects that the P-protein has a significant role in melanosome maturation. These results were supported by previous studies carried on mutant cells [7, 11, 14]. During the study of melanosome stages, it was found that the normal melan-a cell was highly pigmented with stage IV melanosomes, whereas transfected cells contained stage, I, II melanosomes, predominantly. The melanosome were slightly lowered and spherical in shape in transfected cells. The shape of melanosome was reported oval or elliptical in most of the previous studies [21, 22]. The percentage of stage IV melanosomes was significantly low in transfected cells. These results showed that P-protein siRNA affected the maturation and number of melanosomes. Study conducted on melanocyte form OCA-2 patient showed the abnormal shape of melanosome and a diminution in the number of melanized melanosomes [23]. As it was already published that melanosome number and differentiation levels are decreased, and melanosomal ultrastructure is abnormal in patients with mutations at the p-locus [24]. Present findings revealed that the biogenesis of melanosomes could be obstructed by down regulation of P-protein through silencing of OCA2 gene. The down regulation of the P-protein also affects the processing and trafficking of tyrosinase. In the present study, it was found the tyrosinase expression was down regulated in response to siRNA in melan a cells and B16F10 cells (Figs. 8, 9a). It was reported that the melan-p1 cells were secreted a large
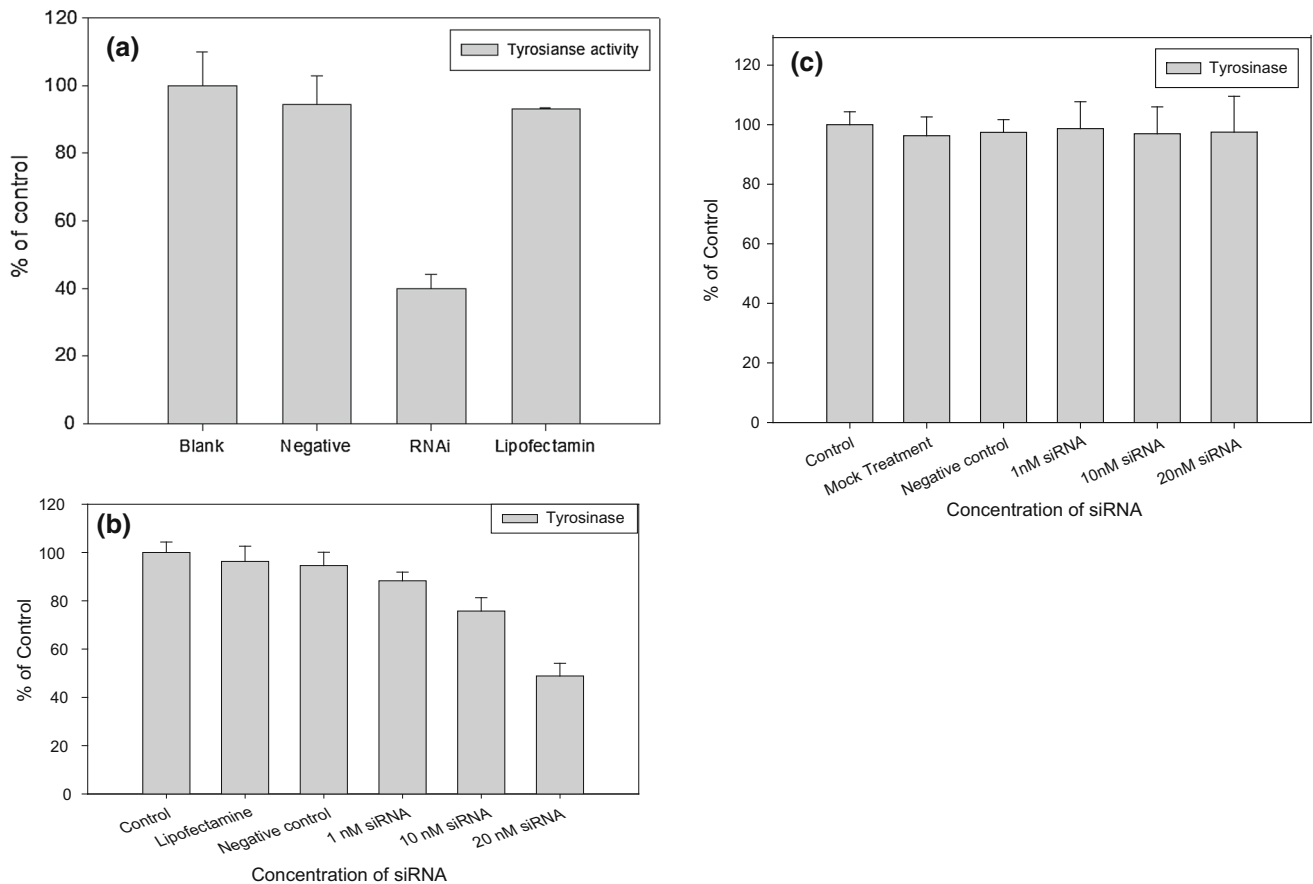
Fig. 7 Effect of P-protein-siRNA on cellular tyrosinase activity **a** Melan-a cell, (SiRNA 10 nM) **b** B16F10 cells siRNA treatment at different concentration and **c** melan-p1 cells siRNA treatment at different concentration
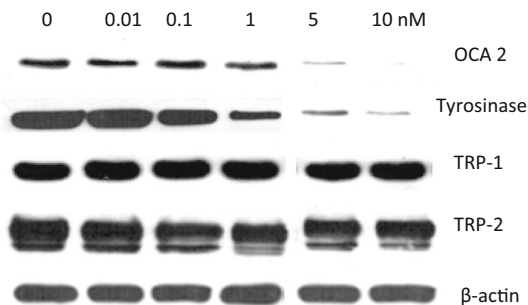

Fig. 8 Dose-dependent down regulation of P-protein by P-protein-siRNA and their effect on the expression of tyrosinase, TRP-1, and TRP-2 in melan-a melanocyte cells

proportion of the tyrosinase into media [16]. In this study, the cellular tyrosinase activity was down regulated in a dose-dependent manner (Fig. 7). However, the siRNA was not affected the cellular tyrosinase activity as well as the expression in melan-p1 cells (Figs. 7c, 9c). Previous

studies on p-mutant also reported the diminution in tyrosinase expression [25–27]. The siRNA had no effect on expression of TRP-1 and TRP-2 in melan-a, B16F10 and melan-p1 cells. This showed that the other proteins were not affected by the P-protein down regulation. These results were similar to the previously reported studies on melan-p1 cells in compare to melan-a and melan-c cells [14, 25]. These results supported the study which stated that P-protein regulated the post-translational processing of tyrosinase [16]. However, P-protein-siRNA did not show significantly the effect on the expression of TRP-1 and TRP-2 protein. This study demonstrated the selective inhibitory effect of P-protein siRNA on melanin synthesis and melanosomal biology in melan-a, B16F10 cells, and may provide a novel experimental tool for studying the skin cell pigmentation. This study concluded that the P-protein have the significant role in melanosomal biology, especially in terms of melanosome structure and type.
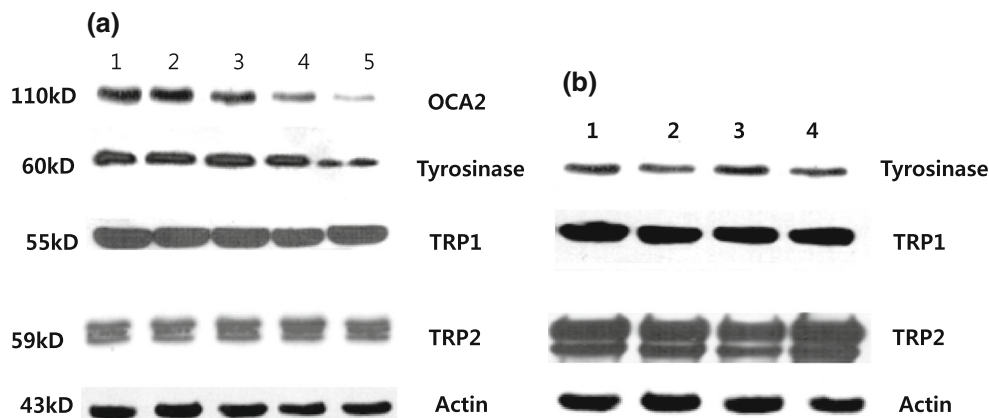
(a)

(b)

Fig. 9 Effect of P-protein-siRNA on the expression of melanosomal protein, **a** B16F10 Melanoma Cells (*Lane*, *1* Control, *2* Mock treatment with lipofectamine, *3* Negative control using scrambled RNA, *4* siRNA-treated cells (1nM), *5*) siRNA-treated cells (5 nM); **b** melan-p1, null at p-locus melanocyte cells (*Lane*, *1* Control, *2* Negative control using scrambled RNA, *3* siRNa-treated cells (1 nM), *4* siRNa-treated cells (5 nM))

Conclusion

Present study exhibits that the P-protein is one of the key melanosomal membrane proteins which have very important role in melanosome structure and delivers fruitful information for constructing a rational approach for controlling the synthesis of melanin as well as melanosome biology.

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