See related commentary on pg 645

Impact of NAD(P)H:Quinone Oxidoreductase-1 on Pigmentation

Tae-Young Choi[1], Kyung-Cheol Sohn[1], Jin-Hwa Kim[2], Seong-Min Kim[2], Cheol-Hee Kim[3], Jae-Sung Hwang[4], Jeung-Hoon Lee[1], Chang Deok Kim[1] and Tae-Jin Yoon[2]

We obtained metastasized melanoma tissue from a primary acral lentiginous melanoma (ALM) patient and established a melanoma cell line named primary culture of melanoma cell derived from lymph node (PML)-1. PML-1 cells had a light brown color and decreased the expression of melanogenesis markers, including tyrosinase (TYR), microphthalmia-associated transcription factor, and tyrosinase-related protein-1. To identify genes differentially regulated in PML-1 melanoma cells, we performed DNA microarray and two-dimensional matrix-assisted laser desorption ionization-time of flight mass spectrometry analyses. Among the candidate genes identified, we chose NAD(P)H:quinone oxidoreductase-1 (NQO1) for further study. Reverse transcription–PCR and western blot analyses showed that NQO1 was markedly decreased in PML-1 cells and in several amelanotic melanoma cell lines. To investigate whether NQO1 affects the melanogenesis, we treated the cultured normal human melanocytes (NHMC) and zebrafish with NQO1 inhibitors, ES936 and dicoumarol. Interestingly, melanogenesis was significantly decreased by the addition of NQO1 inhibitors in both NHMC and zebrafish models. In contrast, overexpression of NQO1 using a recombinant adenovirus clearly induced melanogenesis, concomitantly with an increase of TYR protein level. These results suggest that NQO1 is a positive regulator of the pigmentation process.

Journal of Investigative Dermatology (2010) **130,** 784–792; doi:10.1038/jid.2009.280; published online 17 September 2009

INTRODUCTION

Melanocytes are specialized cells that produce melanin pigments, thereby contributing to the appearance of skin color. Melanins in the skin provide a primary defense system against noxious environmental insults, including UV radiation and visible light (Costin and Hearing, 2007). Melanins are a complex group of heterogeneous biopolymers, which can be classified into black/brown eumelanins and red/yellow pheomelanins. Melanins are synthesized from tyrosine through a well-characterized biochemical pathway in which tyrosinase (TYR) acts as the rate-limiting enzyme (Land *et al.*, 2004; Wang and Hebert, 2006). Although the biochemical pathway involved in melanin synthesis has been well established, many of the factors regulating the pigmentation process remain to be elucidated.

Melanoma is a type of skin cancer that originates from melanocytes. The incidence of melanoma is relatively lower than other types of skin cancers; however, it accounts for 80% of the deaths from all skin cancers (Miller and Mihm, 2006). Acral lentiginous melanoma (ALM) is one type of malignant melanoma that is prevalent in Asian and African compared with Caucasian populations (Phan *et al.*, 2006). In this study, we established a melanoma cell line from the metastatic lymphoid tissue of a primary ALM patient, and named it PML-1 (primary culture of melanoma cell derived from lymph node). Interestingly, PML-1 cells had a light brown color, whereas normal melanocytes cultured from the same patient had an intense black color. We thought that PML-1 cells would be a good model for studying the regulation of pigmentation and attempted to identify differentially expressed genes using oligonucleotide microarrays, two-dimensional (2D) gel electrophoresis, and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOFMS). Among the several candidate genes identified, we chose NAD(P)H:quinone oxidoreductase-1 (NQO1) for further study, as it was remarkably decreased in PML-1 cells.

NQO1 (EC 1.6.5.2) is a ubiquitous flavoenzyme that catalyzes the two-electron reduction of quinones to hydroquinones using

[1]*Department of Dermatology, School of Medicine, Research Institute for Medical Sciences, Chungnam National University, Daejeon, Korea;* [2]*Department of Dermatology, School of Medicine, Institute of Health Sciences, Gyeongsang National University, Jinju, Korea;* [3]*Department of Biology and GRAST, Chungnam National University, Daejeon, Korea and* [4]*Department of Genetic Engineering, College of Life Sciences, Kyung Hee University, Suwon, Korea*

Correspondence: Dr Chang Deok Kim, Department of Dermatology, School of Medicine, Research Institute for Medical Sciences, Chungnam National University, 55 Munhwa-ro, Daejeon 301-747, Korea.
E-mail: cdkimd@cnu.ac.kr
or Dr Tae-Jin Yoon, Department of Dermatology, School of Medicine, Institute of Health Sciences, Gyeongsang National University, 49 Gangnam-ro, Jinju 660-702, Korea. E-mail: yoontj@gnu.ac.kr

Abbreviations: ALM, acral lentiginous melanoma; DCT, dopachrome tautomerase; MALDI-TOFMS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; MITF, microphthalmia-associated transcription factor; NQO1, NAD(P)H:quinone oxidoreductase 1; PML-1, primary culture of melanoma cell derived from lymph node; TYR, tyrosinase; TYRP1, tyrosinase-related protein-1

Received 21 April 2009; revised 19 July 2009; accepted 20 July 2009; published online 17 September 2009

NAD(P)H as an electron donor (IUBMB page: http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/6/5/2.html; Lind *et al.*, 1990; Riley and Workman, 1992; Asher *et al.*, 2006). NQO1 provides cells with multiple layers of protection against oxidative stress, such as the direct detoxification of highly reactive quinones, the maintenance of lipid-soluble antioxidants in reduced forms, and stabilization of the tumor suppressor p53 (Nioi and Hayes, 2004). In addition, a recent study showed that the nuclear factor erythroid 2-related factor 2 pathway and downstream NQO1 are involved in the adaptation of skin keratinocytes and melanocytes to environmental stress (Marrot *et al.*, 2008). Although its protective role has been well recognized, the impact of NQO1 on melanogenesis has not been studied earlier. Interestingly, evidence indicates that oxidative stress and H_2O_2 are major players in the regulation of melanogenesis in melanocytes, whereas NQO1 is an important enzymatic antioxidant (Schallreuter *et al.*, 2008; Wood and Schallreuter, 2008). We speculate that NQO1 may have an impact on pigmentation, and show for the first time that NQO1 affects melanogenesis positively through the regulation of TYR.

RESULTS

Establishment of the PML-1 melanoma cell line

We obtained metastasized melanoma tissue from a lymph node in the right inguinal area of a Korean ALM patient (Supplementary Figure 1), and then established the melanoma cell line named PML-1. PML-1 melanoma cells showed quite a different morphology compared with normal human melanocytes (NHMC) cultured from the same patient and from a melanotic melanoma cell line MNT-1; i.e., enlarged cell bodies and multiple dendrites (Figure 1a). Although PML-1 cells were established from metastasized melanoma tissue, they showed a remarkably slow rate of cell growth, with an estimated doubling time of more than 3 weeks (data not shown). The expression of tumor suppressors and cell cycle regulators, including retinoblastoma, p53, p21, and cyclin D1, was significantly reduced in PML-1 cells (Figure 1b). Interestingly, PML-1 cells had a light brown color, whereas NHMC and MNT-1 cells had intense black colors. Thus, we next examined the protein levels of various pigmentation markers, including microphthalmia-associated transcription factor (MITF), TYR, tyrosinase-related protein-1 (TYRP1), and dopachrome tautomerase (DCT/TRP2). Protein levels of MITF, TYR, and TYRP1 were markedly decreased in PML-1 cells, whereas the DCT level was not changed (Figure 1c). These results indicate that the pigment-producing potential is significantly reduced in PML-1 cells, and is at least part of the reason why PML-1 cells have a light brown color.

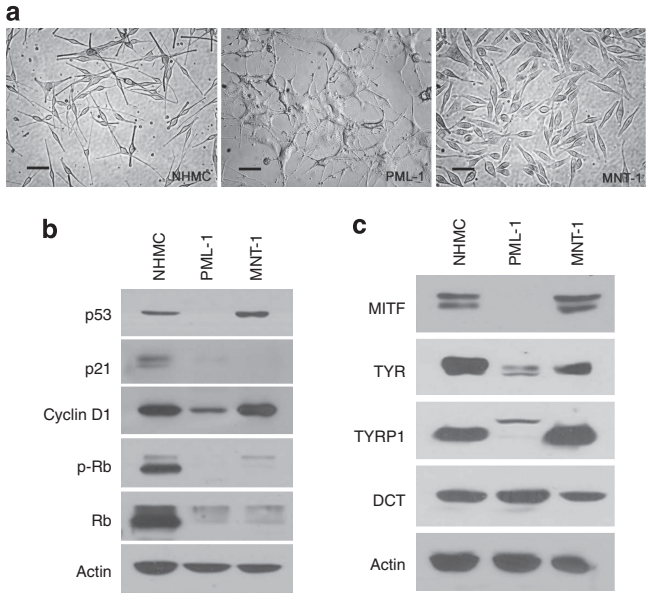

Figure 1. Characteristics of the PML-1 melanoma cells. (**a**) Morphology of PML-1 melanoma cells derived from the metastatic lymphoid tissue of a primary acral lentiginous melanoma (ALM) patient. Compared with normal human melanocytes (NHMC) derived from the same patient and melanotic MNT-1 melanoma cells, PML-1 cells show enlarged cell bodies and multiple dendrites. (**b**) Western blot analysis of cell cycle-related genes in PML-1 cells. (**c**) Western blot analysis of pigmentation markers; MITF, microphthalmia-associated transcription factor; TYR, tyrosinase; TYRP1, tyrosinase-related protein-1; DCT, dopachrome tautomerase. Actin was included as a loading control. Scale bar = 25 μm.

Identification of differentially expressed genes in PML-1 cells

To identify genes differentially expressed in PML-1 cells, we performed oligonucleotide microarray, 2D gel electrophoresis, and MALDI-TOFMS analyses. We obtained 604 differentially expressed genes by oligonucleotide microarray (Supplementary Table 1) and 36 candidate proteins by MALDI-TOFMS (Supplementary Table 2). Comparing the results obtained by those two analytical methodologies, we identified nine candidate genes whose expression was markedly increased and/or decreased in PML-1 cells (Figure 2a and b). We chose the downregulated gene NQO1 for further study, reasoning that the reduced expression of pigmentation-related proteins such as MITF, TYR, and TYRP1 could result from the downregulation of melanogenic regulators. To confirm the MALDI-TOFMS data (Figure 3a), we first examined the expression level of NQO1 using reverse transcription (RT)–PCR and western blot analyses, which confirmed that the mRNA and protein levels for NQO1 (isoform 1) were markedly decreased in PML-1 cells (Figure 3b and c). As 3 splicing variants exist for the NQO1 transcript (Jaiswal *et al.*, 1988; Gasdaska *et al.*, 1995), we also examined the transcript levels for the other NQO1 isoforms (2 and 3) using RT–PCR. The results showed that NQO1 isoforms 2 and 3 were also decreased, suggesting that NQO1 expression is regulated at the transcriptional level in PML-1 cells (Figure 3b). Immunohistochemical analysis revealed that the expression of NQO1 was not obvious in the metastatic tissue of the ALM patient, whereas its expression was easily detectable in the epidermis of a normal skin region (Figure 3d).

Impact of NQO1 on pigmentation

On the basis of expression data, we hypothesized that NQO1 may affect the pigmentation process directly and/or

a

Accession number	Gene	GEV
NM_001398	Enoyl coenzyme A hydratase 1	3.61±0.41
NM_002073	Guanine nucleotide-binding protein	3.96±0.61
NM_017827	Seryl-tRNA synthetase 2	3.16±0.42
NM_004341	Carbamoyl-phosphate synthetase 2	2.70±0.19
NM_001909	Cathepsin D	2.34±0.31
XM_377109	40S ribisomal protein SA	2.28±0.34
XM_496430	Heat-shock 10 kDa protein 1	2.13±0.05
NM_000436	3-oxoacid CoA transferase 1	2.05±0.01
NM_000903	NAD(P)H:quinone oxidoreductase 1	0.18±0.01

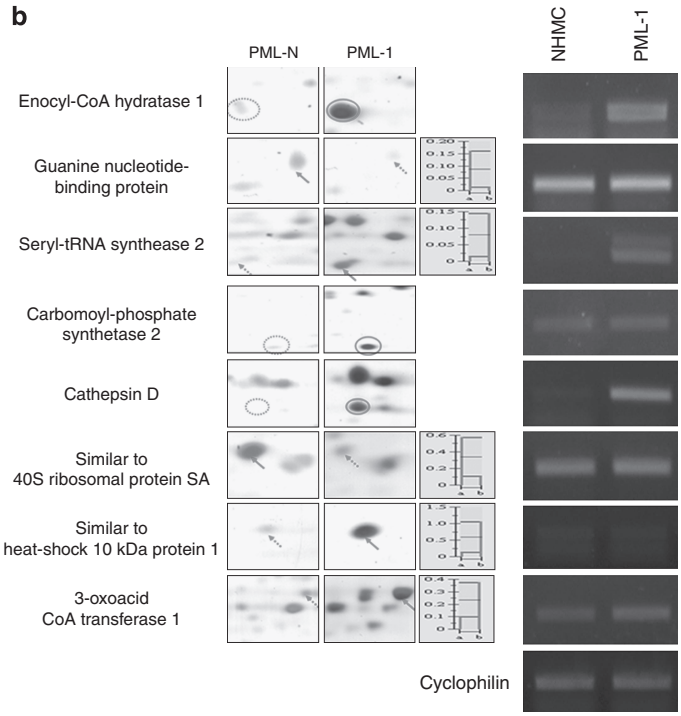

Figure 2. Identification of differentially expressed genes in PML-1 melanoma cells. (**a**) List of differentially expressed genes, which were selected by comparison of the results between the oligonucleotide microarray and the two-dimensional (2D) MALDI-TOFMS. Microarray analysis was performed with Cy5- and Cy3-labeled probes, and then fluorescence intensity was assigned as the gene expression value (GEV) for determination of the relative gene expression level. The results are shown as the mean values ± SD of duplicate measurements. (**b**) Expression of candidate genes in 2D PAGE and reverse transcription–PCR analysis.

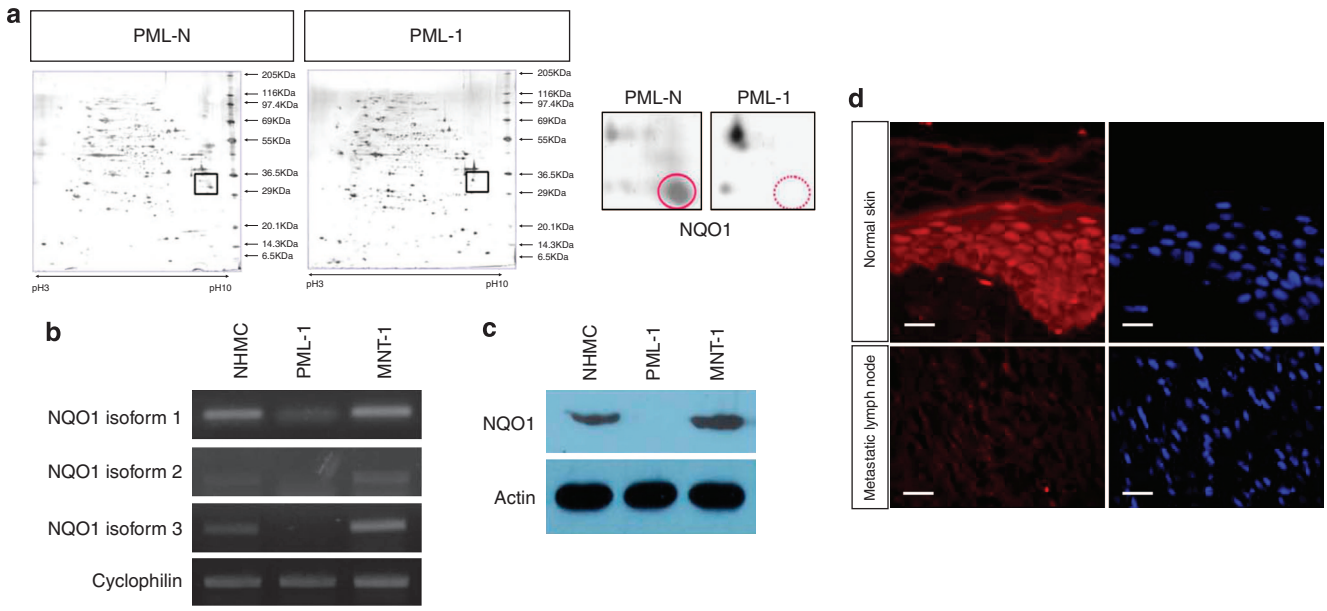

Figure 3. Expression of NAD(P)H:quinone oxidoreductase-1 (NQO1) in PML-1 melanoma cells. (**a**) Two-dimensional gel analysis for comparison of protein levels between normal human melanocytes (NHMC) (PML-N) and PML-1 cells. After MALDI-TOFMS analysis, NAD(P)H:quinone oxidoreductase-1 (NQO1) was selected as a differentially expressed protein in PML-1 cells (red circle). Right panels show magnified images of the boxes in the left panels. PML-N, normal melanocytes isolated from the acral lentiginous melanoma (ALM) patient; PML-1, melanoma cells established from the metastatic lymphoid tissue of the same patient. (**b**) mRNA levels of NQO1 isoforms were determined by reverse transcription–PCR. Cyclophilin was included as a loading control. (**c**) Protein level of NQO1 determined by western blot. Actin was used as a loading control. (**d**) Immunohistochemistry. The normal skin specimen and the metastatic lymph node were obtained from the ALM patient, and then were fixed and paraffin-embedded. Sections were stained with the anti-NQO1 antibody. Right panels show the DAPI staining. Scale bar = 10 μm.

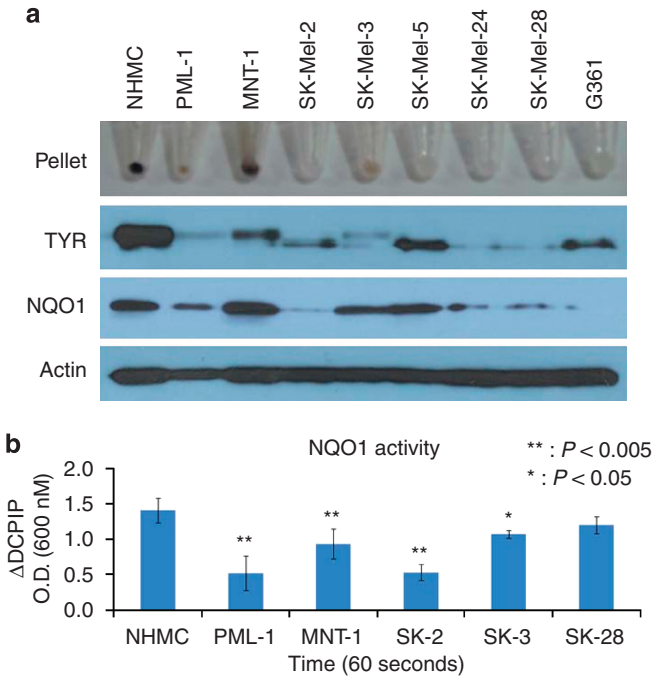

Figure 4. Expression and activity of NAD(P)H:quinone oxidoreductase-1 (NQO1) in melanoma cell lines. (**a**) Upper panels show the pellet color of various melanoma cells cultured *in vitro*. Protein levels of tyrosinase (TYR) and NAD(P)H:quinone oxidoreductase-1 (NQO1) were determined by western blot; actin is included as a loading control. Normal human melanocytes (NHMC) and MNT-1 cells show an intense black color; PML-1 and SK-MEL-3 melanoma cells show a yellow-brown color; SK-MEL-2, SK-MEL-5, SK-MEL-24, SK-MEL-28, and G361 cells were amelanotic. (**b**) NQO1 activity in various melanoma cells cultured *in vitro*. Cell extracts were incubated with reaction mixture, and then absorbances at 600 nm were chased for 60 seconds. Enzyme activity is represented by the change of absorbance. Data are averages and SEM of triplicate measurements.

indirectly. We first examined the protein level of NQO1 in various melanoma cell lines that showed different melanogenic potentials. NHMC and MNT-1 cells were highly melanogenic and produced melanin of black color, PML-1, and SK-MEL-3 melanoma cells produced melanin of brown color, whereas SK-MEL-2, SK-MEL-5, SK-MEL-24, SK-MEL-28, and G361 cells were amelanotic (Figure 4a, pellet). Interestingly, the melanogenic potential of various, if not all, melanoma cell lines seemed to correspond with the NQO1 level (Figure 4a). Total cellular NQO1 activities matched well with the protein levels in melanoma cell lines (Figure 4b). These results suggest that NQO1 may exert its role as a positive regulator of melanogenesis. Thus, we next investigated the effect of NQO1 inhibitors on pigmentation using an *in vitro* cell culture system and an *in vivo* zebrafish model (Choi *et al.*, 2007). We used two NQO1 inhibitors in this study. The first one was ES936 (5-methoxy-1,2-dimethyl-3-[(4-nitrophenoxy)methyl]indole-4,7-dione), which is a mechanism-based inhibitor with a Ki of 450 nM. The inhibition of NQO1 by ES936 is NADH-dependent and involves the generation of reactive iminium species that alkylate a Y residue of NQO1 (Winski *et al.*, 2001; Dehn *et al.*, 2003). The second NQO1 inhibitor used was dicoumarol [3,3′-methylenebis(4-hydroxycoumarin)], which is the most potent competitive known inhibitor of NQO1 with a Ki of 1–10 nM. Dicoumarol competes with NAD(P)H for binding with NQO1 and prevents the electron transfer to FAD (Cullen *et al.*, 2003). Treatment of NHMC with ES936 led to a decrease in expression of melanogenic proteins such as MITF, TYR, and TYRP1 in a dose-dependent manner (Figure 5a). The inhibition of NQO1 by dicoumarol elicited similar results, regardless of additional supplements to the growth medium such as PMA or endothelin-1 (Figure 5b). Interestingly, those two NQO1 inhibitors did not significantly affect the DCT level, which was constant in PML-1 cells compared with NHMC and MNT-1 cells (Figure 1c). These results suggest that NQO1 may be an important regulator of melanin production. In the zebrafish model, treatment with NQO1 inhibitors reduced the pigmentation dramatically, although severe side effects were observed in the dicoumarol-treated zebrafish embryos (Figure 5c).

The human NQO1 gene comprises six exons and five introns, and produces three transcript variants by alternative splicing (Figure 6a). In NHMC and in melanoma cells, all three isoforms of NQO1 transcripts were identified (Figure 3b). To further investigate the effects of NQO1 on pigmentation, we generated recombinant adenoviruses expressing NQO1 isoforms. After transduction into PML-1 cells, overexpressed NQO1 isoforms were detected by western blot analysis (Figure 6c). Overexpression of NQO1 isoforms did not influence cell morphology (Figure 6b). Interestingly however, secretion of melanin was significantly increased by NQO1 isoform 1, but not by isoforms 2 or 3 (data not shown). We speculated that NQO1 may enhance the melanogenic potential in PML-1 cells, and then examined the protein levels for MITF, TYR, and TYRP1. As shown in Figure 6c, overexpression of NQO1 isoform 1 increased the TYR protein level, whereas the levels of MITF and TYRP1 were slightly decreased. Again, no effect was noted in response to NQO1 isoforms 2 or 3, suggesting that NQO1 isoform 1 is the one involved in the regulation of pigmentation. When NQO1 isoform 1 was overexpressed in PML-1 cells, the TYR enzymatic activity was also increased in a dose-dependent manner (Figure 6d), which supports the notion that NQO1 is an authentic regulator of pigmentation. To further confirm the role of NQO1, we transduced NHMC and amelanotic SK-Mel-2 melanoma cells with an adenovirus expressing NQO1 isoform 1, which showed that overexpression of NQO1 also led to an increase of TYR protein level in both types of cells (Figure 6e). To investigate whether NQO1 regulates TYR at the transcriptional level, we performed RT–PCR analysis. After the adenovirus-driven expression of NQO1 in PML-1 cells, the mRNA levels for MITF, TYR, TYRP1, and DCT were not changed significantly (Figure 6f). These results suggest that NQO1 may affect the stability of TYR rather than the expression of its gene.

DISCUSSION

Although many genes involved in the regulation of pigmentation have been discovered, they are not sufficient to understand the sophisticated molecular mechanisms underlying

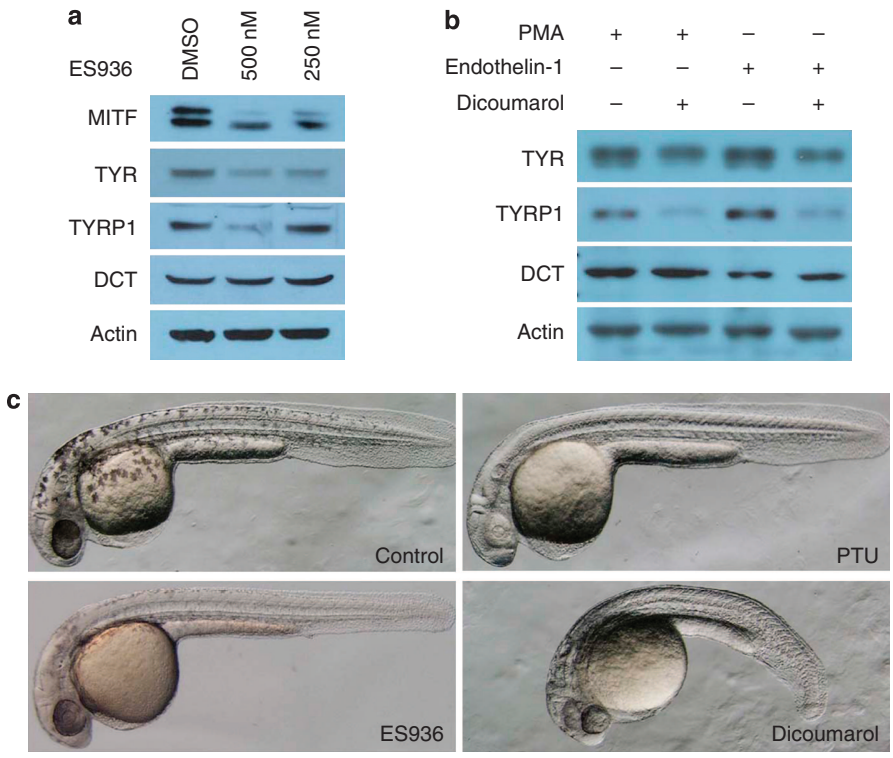

Figure 5. Effect of NAD(P)H:quinone oxidoreductase-1 (NQO1) inhibitors on pigmentation. Normal human melanocytes (NHMC) were treated with the NQO1 inhibitors ES936 (**a**) or dicoumarol (**b**) at the indicated concentrations for 24 hours. Protein levels for microphthalmia-associated transcription factor (MITF), tyrosinase (TYR), tyrosinase-related protein-1 (TYRP1), and dopachrome tautomerase (DCT) were determined by western blot. (**c**) Synchronized zebrafish embryos were treated with NQO1 inhibitors (ES936, 5 μM; dicoumarol, 50 nM at 9 hpf). The effects on the pigmentation of zebrafish embryos were observed under a stereomicroscope at 30 hpf. As a positive control, 0.2 mM PTU (1-phenyl-2-thiourea), which is a tyrosinase inhibitor used routinely to inhibit pigment production in zebrafish (29), was included.

this process. In this study, we identified a previously unidentified melanogenic regulator, NQO1, using oligonucleotide microarray and 2D MALDI-TOFMS methods. The results highlight the previously unidentified role of NQO1 in melanogenesis. Specifically, we found that the melanogenic potential is relatively well matched with NQO1 levels in various melanoma cell lines, that the inhibition of NQO1 activity decreases melanogenesis, and that overexpression of NQO1 enhances pigmentation through an increase of TYR.

NQO1 is a flavoenzyme that exerts its role in the cellular defense mechanism against oxidative stress. NQO1 knockout mice are more susceptible to oxidative stress, and show a higher frequency of skin tumor development when carcinogens such as benzo(a)pyrene and 7,12-dimethylbenz(a)-anthracene are applied topically (Long *et al.*, 2000, 2001). Furthermore, individuals with a lack of NQO1 owing to a genetic polymorphism show an increased susceptibility to certain cancers (Ross *et al.*, 2000). In this study, we showed that NQO1 protein level and enzymatic activity are reduced in many melanoma cell lines, including PML-1, which was derived from the metastatic lymphoid tissue of a primary ALM patient. Considering the physiological function of NQO1 to detoxify potentially mutagenic compounds, it could be suggested that decreased NQO1 activity predisposes melanocytes to a cancerous stage. A correlation between NQO1 and melanoma development will be an interesting further study.

In this study, we showed that the inhibition of NQO1 activity decreases melanogenesis, whereas the overexpression of NQO1 enhances pigmentation, likely through the regulation of the TYR level. Although the precise mechanism remains to be elucidated, our data suggest that NQO1 may affect the stability of TYR rather than its gene expression, because the overexpression of NQO1 did not increase the mRNA level for TYR in PML-1 cells. As it has been shown that NQO1 interacts with a broad range of substrates including p53 and ornithine decarboxylase (Anwar *et al.*, 2003; Asher *et al.*, 2005), we hypothesized that TYR could be a direct substrate for NQO1, thereby acquiring the more stable configuration. However, in a preliminary immunoprecipitation assay, we were unable to show a direct interaction between NQO1 and TYR (data not shown), which suggests that the stabilization of TYR by NQO1 is mediated through another regulatory mechanism. One possible candidate is the proteasomal degradation machinery. Evidence shows that NQO1 inhibits the ubiquitin-independent degradation of ornithine decarboxylase by the 20S proteasome (Asher *et al.*, 2005). Furthermore, NQO1 protects the tumor suppressor p53 against 20S proteasomal degradation, leading to the stabilization and

activation of p53 (Gong *et al.*, 2007). A similar phenomenon has also been shown for the tumor suppressor p33 (ING1b) (Garate *et al.*, 2008). Together, these results suggest that NQO1 may inhibit the activity of the 20S proteasome, thereby stabilizing a broad range of target proteins that are destined for proteasomal degradation. Elucidation of whether NQO1 regulates the TYR level in a similar manner will be an interesting further study.

In summary, we identified a previously unidentified melanogenic regulator, NQO1, and showed its positive regulatory role in melanogenesis. Our results provide a previously unidentified insight on which to base further investigations of the molecular events that occur during the process of pigmentation.

MATERIALS AND METHODS

Cell culture

Metastatic lymphoid and normal tissue of primary ALM patients and normal foreskin samples were obtained under the written informed consent of the donors, in accordance with the ethics committee approval process of the Gyeongsang National University Hospital. The study was conducted according to the Declaration of Helsinki Principles. Skin samples were briefly sterilized in 70% ethanol, minced, and then treated with dispase overnight at 4 °C. The epidermis was separated and placed in a solution containing 0.05% trypsin and 0.02% EDTA (Gibco BRL, Rockville, MD) for 30 minutes at 37 °C. After vigorous pipetting, the cells were pelleted and resuspended in a growth medium composed of Medium 154 and human melanocyte growth supplement (Cascade Biologics,

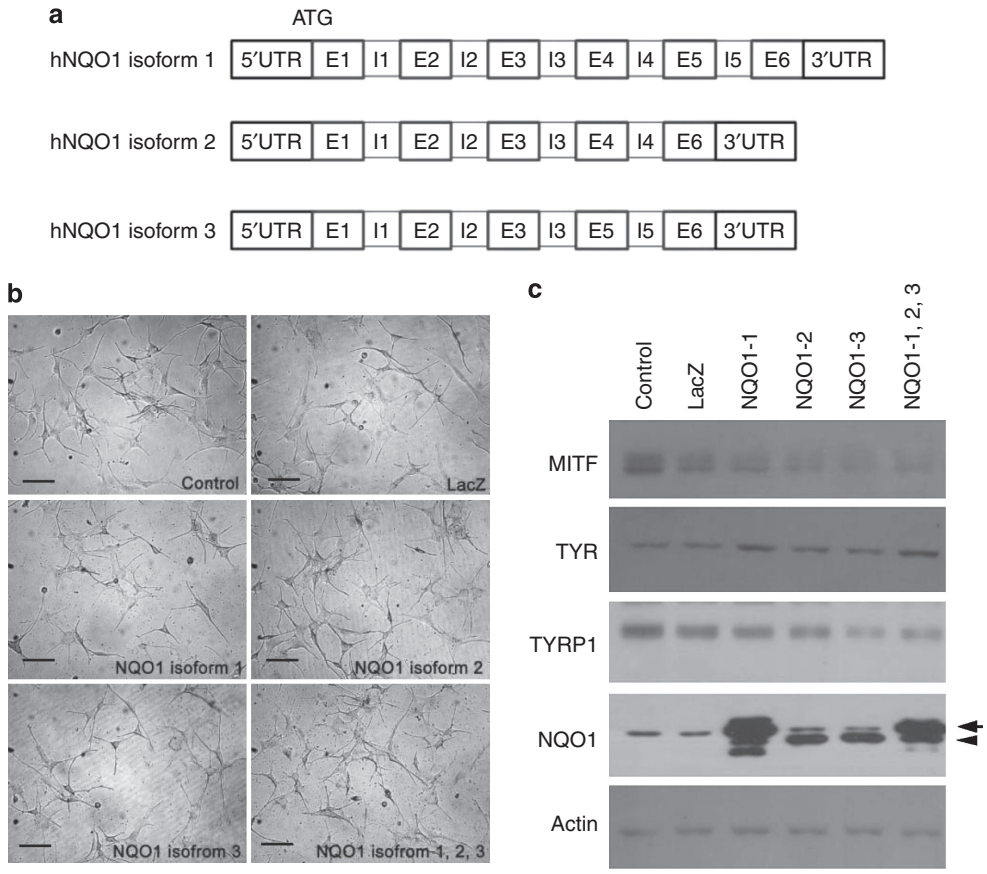

Figure 6. Impact of NAD(P)H:quinone oxidoreductase-1 (NQO1) on pigmentation. (**a**) Gene structure of human NAD(P)H:quinone oxidoreductase-1 (NQO1) and splicing variants. The NQO1 isoform 1 has all exons in its mRNA transcript, whereas isoform 2 lacks exon 5 and isoform 3 lacks exon 4 in their mRNA transcripts. (**b**) Morphology of primary culture of melanoma cell derived from lymph node (PML-1) cells after transduction of adenoviruses expressing NQO1 isoforms. Cells were incubated with the recombinant adenoviruses (10 MOI, multiplicity of infection) for 18 hours, and then were replenished with fresh medium and incubated for 3 days. Overexpression of NQO1 isoforms did not affect cell morphology significantly. (**c**) PML-1 cells were transduced with adenoviruses (10 MOI), and then protein levels for microphthalmia-associated transcription factor (MITF), tyrosinase (TYR), tyrosinase-related protein-1 (TYRP1), and NQO1 were determined by Western blot. Arrow indicates the NQO1 isoform 1. Arrowhead indicates the NQO1 isoforms 2 and 3. Actin was used as a loading control. (**d**) Effect of NQO1 (isoform 1) on TYR activity in PML-1 cells. After adenoviral transduction at the indicated MOI, cell extracts were prepared. For measurement of TYR activity, 250 μg total protein was incubated with L-DOPA (0.5 mM) (upper panel), and then quantified using a spectrometer (middle panel). The results are shown as percentage of control. All experiments were repeated three times. The lower panel shows protein levels for TYR and NQO1 after adenoviral transduction. (**e**) Effect of NQO1 (isoform 1) on tyrosinase activity in normal human melanocytes (NHMC) and in amelanotic melanoma SK-Mel-2 cells. Cells were transduced with 10 MOI adenovirus expressing NQO1, and then protein levels for TYR and NQO1 were determined by Western blot. (**f**) Effect of NQO1 (isoform 1) on the transcription of pigmentation markers in PML-1 cells. Cells were transduced with 10 MOI adenovirus, and then mRNA levels for MITF, TYR, TYRP1, DCT, and NQO-1 were determined by reverse transcription–PCR. Cyclophilin was used as a loading control. Scale bar = 25 μm.

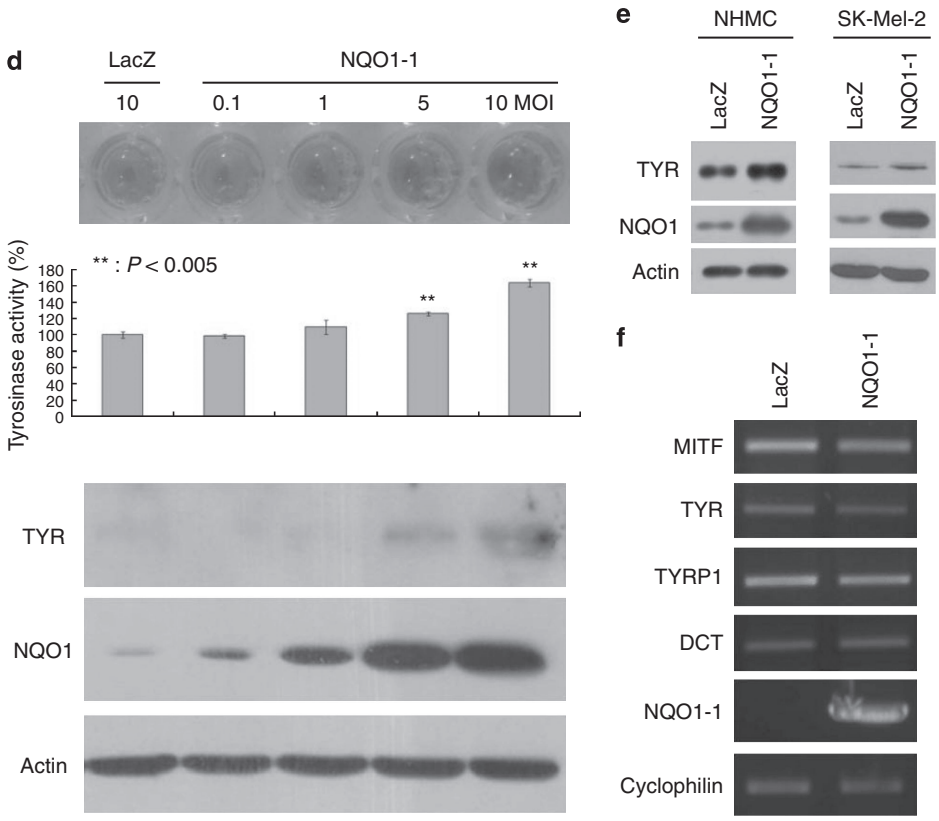

Figure 6. Continued.

Portland, OR). During the primary culture of human melanocytes, 200 μg ml^{-1} G418 (geneticin sulfate, Duchefa, Haarlem, The Netherlands) was added to the growth medium to suppress the proliferation of fibroblasts. PML-1 melanoma cells were established in a similar manner. Other melanoma cells were maintained in DMEM supplemented with 10–20% fetal bovine serum (Gibco BRL).

Oligonucleotide microarray and 2D MALDI-TOFMS

To identify genes differentially expressed in PML-1 cells, we used two different methodologies: oligonucleotide microarray at the mRNA level and 2D MALDI-TOFMS at the protein level. Comparisons were performed between melanoma cells (PML-1) and normal melanocytes (PML-N), both of which were established from the same patient.

For oligonucleotide microarray, total RNAs were isolated using a Tri reagent (Sigma, St Louis, MO). Fluorescent-labeled probes were prepared and applied to the 27 K human oligonucleotide microarray slides (GenomicTree, Daejeon, Korea) as reported earlier (Seo *et al.*, 2005). Microarray slides were analyzed using the GenePix Pro 4.0 software (Axon Instruments, Union City, CA) and GeneSpring 7.2 software (Agilent Technologies, Redwood City, CA). Oligonucleotide microarray was repeated twice with the total RNAs isolated from different batches of culture.

For 2D MALDI-TOFMS, cellular proteins were solubilized in urea buffer (Yang *et al.*, 2006). Two hundred μg cellular proteins were first separated using an immobiline DryStrip (pH 3–10, GE Healthcare Bio-Sciences, Piscataway, NJ), and then SDS–PAGE was performed on 8–16% gradient gels. After SDS–PAGE, gels were stained with silver nitrate and scanned using the Proteome Weaver software (Definiens, Munchen, Germany). Selected spots were in-gel digested by trypsin and analyzed by MALDI-TOFMS (Voyager-DE STR, Applied Biosystems, Foster City, CA).

Production of an adenovirus expressing NQO1 isoforms

A replication-incompetent adenovirus was created using the Virapower adenovirus expression system (Invitrogen, Carlsbad, CA) as reported earlier (Yoon *et al.*, 2008). Briefly, the full-length NQO1 and its isoforms were obtained using the primers 5′-ATGGTCG GCAGAAGAGCACTGA and 5′-CAGATCAAAGCTAGAAAATGA, subcloned into the pENT/CMV-GFP entry vector. Site-specific recombination between the entry vector and the adenoviral destination vector was achieved by LR clonase II (Invitrogen). The resulting adenoviral expression vector was transfected into 293A cells using Lipofectamine 2000 (Invitrogen). Cells were grown until 80% cytopathic effect was seen, and then were harvested for preparation of stock recombinant adenovirus. The adenovirus was purified with cesium chloride according to the method reported earlier (Tollefson *et al.*, 2007).

Tyrosinase and NQO1 activity

TYR activity was determined as described earlier with a slight modification (Choi *et al.*, 2007). Briefly, cells were sonicated in protein extraction solution, after which lysates were clarified by centrifugation. After quantification, 250 μg total proteins in 100 μl lysis buffer were transferred into 96-well plates, and 100 μl of 1 mM L-DOPA was added. After incubation for 60 minutes at 37 °C, absorbances were measured at 475 nm. TYR activity is expressed as a percentage of

the control. All experiments were performed at least three times with similar results.

For measurement of NQO1 activity, cells were homogenized in a solution containing 0.25 M sucrose, 50 mM Tris-Cl, pH 7.4, and proteinase inhibitors. Lysates were clarified by centrifugation at 50,000 r.p.m. for 1 hour, incubated in the reaction mixture (25 mM Tris-Cl, pH 7.4, 200 μM NADH, 200 μg ml^{-1} BSA, and 50 μM DCPIP), and absorbances were measured at 600 nm.

Western blot analysis

Cellular proteins were separated by SDS–PAGE, transferred onto nitrocellulose membranes, and then incubated with appropriate antibodies overnight at 4 °C with gentle agitation. Blots were then incubated with peroxidase-conjugated secondary antibodies for 30 minutes at room temperature, and visualized by enhanced chemiluminescence (Intron, Daejeon, Korea). The following primary antibodies were used in this study: p53, p21, cyclin D1, phosphor-retinoblastoma, total-retinoblastoma, MITF, and NQO1 (Santa Cruz Biotechnologies, Santa Cruz, CA), TYR, TYRP1, and DCT (kindly provided by Dr Vincent J. Hearing, NIH, Bethesda, MD), and actin (Sigma).

Zebrafish test

Adult zebrafish were kept in a 5 l acrylic tank at 28.5 °C with a 14/10 hours light/dark cycle. Embryos were obtained from natural spawning that was induced in the morning by turning on the light. Collection of embryos was completed within 30 minutes. Synchronized embryos were placed in 35-mm culture dishes containing a 2 ml embryo medium. Test compounds were dissolved in 0.1% DMSO, and then added to the embryo medium from 9 hours postfertilization (hpf) to 30 hpf. To observe the effect on pigmentation, embryos were dechorionated by forceps, anesthetized in tricaine methanesulfonate solution (Sigma), mounted in 3% methyl cellulose on a depression slide (Aquatic Eco-Systems, Apopka, FL), and photographed under the stereomicroscope MZ16 (Leica Microsystems, Ernst-Leitz-Strasse, Germany).

Statistical analysis

Data were evaluated statistically using Student's *t*-test. Statistical significance was set at $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Dr Vincent J. Hearing for providing antibodies. This work was supported by the Korea Research Foundation Grant funded by a Korean Government (KRF-2008-313-E00395) and by a grant of the National R&D Program for Cancer Control, Ministry for Health, Welfare and Family Affairs, Republic of Korea (0820050), and in part by a clinical research fund from the Gyeongsang National University Hospital.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Anwar A, Dehn D, Siegel D, Kepa JK, Tang LJ, Pietenpol JA *et al.* (2003) Interaction of human NAD(P)H:quinone oxidoreductase 1 (NQO1) with the tumor suppressor protein p53 in cells and cell-free systems. *J Biol Chem* 278:10368–73

Asher G, Bercovich Z, Tsvetkov P, Shaul Y, Kahana C (2005) 20S proteasomal degradation of ornithine decarboxylase is regulated by NQO1. *Mol Cell* 17:645–55

Asher G, Dym O, Tsvetkov P, Adler J, Shaul Y (2006) The crystal structure of NAD(P)H quinone oxidoreductase 1 in complex with its potent inhibitor dicoumarol. *Biochemistry* 45:6372–8

Choi TY, Kim JH, Ko DH, Kim CH, Hwang JS, Ahn S *et al.* (2007) Zebrafish as a new model for phenotype-based screening of melanogenic regulatory compounds. *Pigment Cell Res* 20:120–7

Costin GE, Hearing VJ (2007) Human skin pigmentation: melanocytes modulate skin color in response to stress. *FASEB J* 21:976–94

Cullen JJ, Hinkhouse MM, Grady M, Gaut AW, Liu J, Zhang YP *et al.* (2003) Dicumarol inhibition of NADPH:quinone oxidoreductase induces growth inhibition of pancreatic cancer via a superoxide-mediated mechanism. *Cancer Res* 63:5513–20

Dehn DL, Siegel D, Swann E, Moody CJ, Ross D (2003) Biochemical, cytotoxic, and genotoxic effects of ES936, a mechanism-based inhibitor of NAD(P)H:quinone oxidoreductase 1, in cellular systems. *Mol Pharmacol* 64:714–20

Gasdaska PY, Fisher H, Powis G (1995) An alternatively spliced form of NQO1 (DT-diaphorase) messenger RNA lacking the putative quinone substrate binding site is present in human normal and tumor tissues. *Cancer Res* 55:2542–7

Gong X, Kole L, Iskander K, Jaiswal AK (2007) NRH:quinone oxidoreductase 2 and NAD(P)H:quinone oxidoreductase 1 protect tumor suppressor p53 against 20 seconds proteasomal degradation leading to stabilization and activation of p53. *Cancer Res* 67:5380–8

Garate M, Wong RP, Campos EI, Wang Y, Li G (2008) NAD(P)H quinone oxidoreductase 1 inhibits the proteasomal degradation of the tumour suppressor p33(ING1b). *EMBO Rep* 9:576–81

Jaiswal AK, McBride OW, Adesnik M, Nebert DW (1988) Human dioxin-inducible cytosolic NAD(P)H:menadione oxidoreductase. cDNA sequence and localization of gene to chromosome 16. *J Biol Chem* 263:13572–8

Land EJ, Ramsden CA, Riley PA (2004) Quinone chemistry and melanogenesis. *Methods Enzymol* 378:88–109

Lind C, Cadenas E, Hochstein P, Ernster L (1990) DT-diaphorase: purification, properties, and function. *Methods Enzymol* 186:287–301

Long DJ II, Waikel RL, Wang XJ, Roop DR, Jaiswal AK (2001) NAD(P)H:quinone oxidoreductase 1 deficiency and increased susceptibility to 7, 12-dimethylbenz[a]-anthracene-induced carcinogenesis in mouse skin. *J Natl Cancer Inst* 93:1166–70

Long DJ II, Waikel RL, Wang XJ, Perlaky L, Roop DR, Jaiswal AK (2000) NAD(P)H:quinone oxidoreductase 1 deficiency increases susceptibility to benzo(a)pyrene-induced mouse skin carcinogenesis. *Cancer Res* 60:5913–5

Marrot L, Jones C, Perez P, Meunier JR (2008) The significance of Nrf2 pathway in (photo)-oxidative stress response in melanocytes and keratinocytes of the human epidermis. *Pigment Cell Melanoma Res* 21:79–88

Miller AJ, Mihm MC Jr (2006) Melanoma. *N Engl J Med* 355:51–65

Nioi P, Hayes JD (2004) Contribution of NAD(P)H:quinone oxidoreductase 1 to protection against carcinogenesis, and regulation of its gene by the Nrf2 basic-region leucine zipper and the arylhydrocarbon receptor basic helix-loop-helix transcription factors. *Mutat Res* 555:149–71

Phan A, Touzet S, Dalle S, Ronger-Savle S, Balme B, Thomas L (2006) Acral lentiginous melanoma: a clinicoprognostic study of 126 cases. *Br J Dermatol* 155:561–9

Riley RJ, Workman P (1992) DT-diaphorase and cancer chemotherapy. *Biochem Pharmacol* 43:1657–69

Ross D, Kepa JK, Winski SL, Beall HD, Anwar A, Siegel D (2000) NAD(P)H:quinone oxidoreductase 1 (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms. *Chem Biol Interact* 129:77–97

Schallreuter KU, Kothari S, Chavan B, Spencer JD (2008) Regulation of melanogenesis – controversies and new concepts. *Exp Dermatol* 17:395–404

Seo EY, Namkung JH, Lee KM, Lee WH, Im M, Kee SH *et al.* (2005) Analysis of calcium-inducible genes in keratinocytes using suppression subtractive hybridization and cDNA microarray. *Genomics* 86:528–38

Tollefson AE, Kuppuswamy M, Shashkova EV, Doronin K, Wold WS (2007) Preparation and titration of CsCl-banded adenovirus stocks. *Methods Mol Med* 130:223–35

Wang N, Hebert DN (2006) Tyrosinase maturation through the mammalian secretory pathway: bringing color to life. *Pigment Cell Res* 19:3–18

Winski SL, Swann E, Hargreaves RH, Dehn DL, Butler J, Moody CJ *et al.* (2001) Relationship between NAD(P)H:quinone oxidoreductase 1 (NQO1) levels in a series of stably transfected cell lines and susceptibility to antitumor quinones. *Biochem Pharmacol* 61:1509–16

Wood JM, Schallreuter KU (2008) A plaidoyer for cutaneous enzymology: our view of some important unanswered questions on the contributions of selected key enzymes to epidermal homeostasis. *Exp Dermatol* 17:569–78

Yang WH, Kim JE, Nam HW, Ju JW, Kim HS, Kim YS *et al.* (2006) Modification of p53 with *O*-linked *N*-acetylglucosamine regulates p53 activity and stability. *Nat Cell Biol* 8:1074–83

Yoon HK, Sohn KC, Lee JS, Kim YJ, Bhak J, Yang JM *et al.* (2008) Prediction and evaluation of protein–protein interaction in keratinocyte differentiation. *Biochem Biophys Res Commun* 377:662–7