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ORIGINAL PAPER

Stress chaperone mortalin regulates human melanogenesis

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Abstract In order to identify the cellular factors involved in human melanogenesis, we carried out shRNA-mediated loss-of-function screening in conjunction with induction of melanogenesis by 1-oleoyl-2-acetyl-glycerol (OAG) in human melanoma cells using biochemical and visual assays. Gene targets of the shRNAs (that caused loss of OAG-induced melanogenesis) and their pathways, as determined by bioinformatics, revealed involvement of proteins that regulate cell stress response, mitochondrial functions, proliferation, and apoptosis. We demonstrate, for the first time, that the mitochondrial stress chaperone mortalin is crucial for melanogenesis. Upregulation of mortalin was closely associated with melanogenesis in in vitro cell-based assays and clinical samples of keloids with hyperpigmentation. Furthermore, its knockdown resulted in compromised melanogenesis. The data proposed mortalin as an important protein that may be targeted to manipulate pigmentation for cosmetic and related disease therapeutics.

Keywords shRNA screening · mtHsp70/mortalin · Hsp60 · Regulation · Melanogenesis · Upregulation · Keloids

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Introduction

Skin, the largest organ of the body, is the most important tissue that interfaces and interacts with environment and regulates signaling pathways evoking adaptation/ defense to a variety of stresses involving chemicals, heat, radiations, and microbes (Jablonski and Chaplin, 2000). Skin color, although a heritable trait, is highly influenced by environmental and endocrine factors that cause its modulation either temporarily or permanently (Jablonski 2012; Jablonski and Chaplin 2010; Dadzie et al. 2014). It is determined by the amount, type, and distribution of melanin pigment, synthesized by a complex process of melanogenesis in subcellular organelles (melanosomes), in specialized cells (melanocytes) and involves melanogenic enzyme, tyrosinase (Jablonski and Chaplin 2000). Melanin synthesized in melanocytes is transferred to keratinocytes and determines skin color by virtue of its characteristics including type and proportion of small molecule constituents. Eumelanin (EM) that gives black and brown coloration is the most abundant melanin in humans. Pheomelanin (PM) imparts pink and red color and is concentrated in the lips, nipples, and glands (Jablonski and Chaplin, 2010; Nasti and Timares 2015; Hu et al. 2009). Besides its role in skin coloration, melanin has been assigned functions including stress protection and its susceptibility to disorders ranging from minor itching to metastatic cancers. For example, neuromelanin (NM) is the darker pigment produced in specific populations of catecholaminergic neurons in human brain and has been shown to play crucial role in protection from

metal toxicity and regulation of apoptosis in brain (Double 2006; Gerlach et al. 2006; Zucca et al. 2014).

Induction of photo-damage and oxidative stress are established as the initiating steps of skin carcinogenesis including melanomas, the most aggressive form of skin cancer (Marrot and Meunier 2008; Miller and Tsao 2010; Abdel-Malek et al. 2010). Melanin pigment provides protection against UV-induced DNA damage. Several population studies have shown that the regular use of sunscreens causes reduction in the lifetime incidence of ultraviolet-induced skin cancers (Marrot and Meunier 2008; Guercio-Hauer et al. 1994). Light complexion has been linked to high incidence of dysplastic nevi (DN, atypical moles) (Pavel et al. 2004). X-ray microanalysis of melanosomes from DN and melanomas revealed high sulfur (an indicator of pheomelanin), iron, and calcium (involved in oxidative stress) as compared to the normal skin melanocytes from the same individual suggesting that the phaeomelanogenesis is associated with oxidative imbalance (Pavel et al. 2004). DN cells also showed significantly high reactive oxygen species (ROS) and DNA fragmentation than the normal melanocytes from the same donor (Smit et al. 2008). Sulfur-containing substrate (tyrosine) analogs, *N*-acetyl-4-*S*-cysteaminylphenol (NAcCAP) and *N*-propionyl-4-*S*-cysteaminylphenol (NPrCAP), have been shown to possess selective cytotoxicity towards melanoma cell lines in vitro and in vivo (Thomas et al. 1999). In oxidative-stressed melanoma cells, tyrosinase and microphthalmia transcription factor (MITF), the two main regulators of melanogenesis, were downregulated suggesting that the oxidative stress may lead to hypopigmentation (Jimenez-Cervantes et al. 2001). Vitiligo skin, a patchy loss of inherited skin color, is also characterized by high level of oxidative stress (Denat et al. 2014; Kostyuk et al. 2010; Schallreuter et al. 2003). However, it has not been clinically associated with either increased photo-damage/skin aging or cancer due to high levels of wild-type functional dermal p53 protein, implicated in control of DNA damage (Salem et al. 2009; Schallreuter et al. 2003).

Melanogenesis is regulated by a variety of signal transduction pathways. In mammals, more than 100 genes have been shown to be involved in the process of melanogenesis. Several of these have also been implicated in oxidative stress response. The transcription factor, MITF, has been implicated to play a key role not only in the process of melanocyte development and melanogenesis but also in proliferation and survival (Vachtenheim and Borovansky 2010). The expression level of catalase, a main enzyme responsible for degrading H_2O_2 in melanocytes, was correlated with melanin content (Maresca et al. 2008). Darkly pigmented melanocytes therefore possess two protective mechanisms (high melanin and catalase activity) that may act synergistically for protection against UV. In contrast, light skin possesses lower level of melanin as well as catalase activity and therefore it is more susceptible to accumulate damage after UV exposure (Maresca et al. 2008). The KIT protein, a receptor tyrosine kinase that regulates hematopoiesis and melanogenesis, has been shown to suppress p53-mediated apoptosis by preventing depolarization of the mitochondrial membrane potential and generation of ROS (Lee 1998). Induction of p53 expression, a hallmark for genotoxic stress, was shown to cause induction of pigmentation in human melanocytes (Marrot et al. 1998). Cellular thiols were shown to regulate the activities of tyrosinase and glutathione peroxidase in a dose-dependent manner (Benathan 1997). p38 MAPK signaling was found to be involved in stress (alpha-melanocyte-stimulating hormone and ultraviolet irradiation)-induced melanogenesis (Stepien 2010; Bellei et al. 2010). Several synthetic and natural antioxidants have been shown to modulate skin color and its characteristics to protect against UV damage and cancer (Bagchi et al. 2004). Molecular mechanisms of regulation of melanin synthesis and its photoprotective action, in context to its melanogenic, antioxidant, and cell survival activities, have not been fully elucidated. Understanding these mechanisms is of high significance in cosmetic (skin whitening from beauty perspectives and skin tanning products for reducing the potential risk from skin cancer) as well as pharmaceutical (therapy for pigmentary diseases) applications. With these in view, we undertook a loss-of-function screening using human shRNA library in conjunction with 1-oleoyl-2-acetyl-glycerol (OAG, an established inducer of pigmentation; Gordon and Gilchrest 1989; Friedmann et al. 1990)-induced melanogenesis in human melanoma cells. Pathway and molecular analyses of the identified gene targets revealed that the proliferation, stress, and apoptotic signaling mediated by heat shock 70 family chaperone mtHsp70/mortalin (Wadhwa et al. 1993) are critically involved in the process of melanogenesis.

Materials and methods

Cell culture, transfections, and drug treatments

Human skin melanoma (G361) cells obtained from the Japanese Collection of Research Bioresources (JCRB, Japan) were cultured in McCoy's 5A medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum. Primary human melanocyte cells were obtained from Kurabo Industries Ltd (Osaka) and cultured in DermaLife Basal Medium (Life Line Cell Technology, Carlsbad, CA). OAG (Sigma, Japan), dissolved in dimethylsulfoxide (DMSO), was added to the sub-confluent (60–70 % confluence) cells at a concentration of 15 μg/ml for primary human melanocytes and 30 μg/ml for G361 melanoma. Repeated freezing and thawing of OAG was avoided by making small aliquots of the stock solution and storage at −20 °C. Transfections of shRNA were performed using X-tremeGENE 9 (Roche Applied Science, Indianapolis, USA). Conditions for the best transfection efficiency were

determined by using GFP expression plasmid. Typically, 100 ng and 2 μg of plasmid DNA were used per 96-well and 6-cm dish of cells, respectively, at 60–70 % confluency. Cells were selected in puromycin (2 μg/ml)-supplemented medium and then used for assays as described below. Epolactaene (ETB, an heat shock protein 60 (Hsp60) inhibitor) (3 μM), creatine (inducer of mitochondrial fragmentation) (10 μM), and carbonilcyanide *p*-triflouromethoxyphenylhydrazone (FCCP, an uncoupler of mitochondrial oxidative phosphorylation) (10 μM) were used to inhibit mitochondrial functions. MKT-077 at a subtoxic dose (100 nM) was used as an inhibitor of mortalin.

Preparation of shRNAs and screening of shRNA library

shRNAs were cloned in a U6-driven expression vector as described earlier (Widodo et al. 2007). Cells were plated in 96-well plates and transfected at about 70 % confluency with 100 ng of the plasmid DNA. Twenty-four hours post-transfection, cells were selected in puromycin (2 μg/ml)-supplemented medium for 48–72 h, expanded to 70 % confluency, and then treated with OAG. shRNAs that resulted in abrogation of OAG-induced increase in melanin content and tyrosinase activity were selected. Cells were transfected with these selected shRNAs, and OAG-induced melanogenesis was determined by quantitative assays for melanin and tyrosinase once again. Similar cycle of selection was repeated four times.

Melanin content

To estimate the melanin concentration, cells (5×10^3/well for primary human melanocytes and 2×10^3/well for G361 melanoma) plated in 96-well dish were treated with shRNA and/or OAG for 24 h as described. The cells were incubated with 0.85 N KOH (100 μl) overnight with slow shaking at room temperature (RT). Melanin content was estimated by reading absorbance at 405 nm using a spectrophotometer (Tecan, Switzerland). Relative amount of melanin was calculated by using synthetic melanin (Sigma) as a standard in similar assays and normalized against protein content.

Tyrosinase ELISA

Cells were plated in 96-well plates (NUNC-IMMUNO, Maxisorp) and cultured until (24 or 48 h) they attached well to the surface. The cells were treated with OAG and shRNA, as indicated, for 24 h followed by two washing with cold PBS (shaking for 5 min each time). Cells were lysed with RIPA buffer (Thermo Fisher Scientific Inc., IL) and stored in −80 °C until further processing. Protein concentration was estimated using Pierce BCA Protein Assay Kit (Thermo Scientific, IL, USA). Equal amounts of the protein from control and treated cells were diluted in coating buffer (0.1 M sodium bicarbonate pH 9.6 with 0.02 % sodium azide) and incubated in plates for either 3 h at RT or overnight at 4 °C. The uncoated proteins were removed by aspirating the protein lysate softly. Plates were washed with washing buffer (PBS–0.5 % Tween, pH 7.4) by shaking for 10 min (twice). Cells were blocked in blocking buffer (1 % bovine serum albumin and 0.02 % NaN_3, pH 7.4) by incubation at either RT (for 3 h) or 4 °C (overnight). Blocking buffer was then discarded, and plates were washed with washing buffer (twice, 5 min shaking each time). Cells were then incubated with anti-tyrosinase polyclonal antibody (M-19)-R (1:5000 dilution in blocking buffer) for 1 h at RT. Cells were washed thrice with washing buffer and then incubated with secondary antibody (alkaline phosphatase-goat anti-rabbit IgG) (1:1000 dilution in blocking buffer) followed by washing in washing buffer (with shaking for 5 min × 5 times). Cells were then incubated with AP substrate *p*-nitrophenyl phosphate (pNPP; 1 mg/ml) (PIERCE) in substrate buffer (50 mM $NaHCO_3$ and 10 mM $MgCl_2 \cdot 6H_2O$, pH 9.8) at RT for 30 min, followed by measurement of absorbance at 405 nm. In order to avoid evaporation, plates were kept sealed during the process.

Pathway analysis

Gene network and pathway analyses of the 40 selected candidate genes were performed using STRING database v9.1 and Ingenuity Pathway Analysis software (Franceschini et al. 2013; Szklarczyk et al. 2014). Outcome of these analyses was also compared with the analysis performed using STRING database v9.1 and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. The top ten pathways with high probability scores were selected and investigated for their interaction with the melanogenesis regulatory genes.

Western blotting

Cells were harvested upon completion of treatment (as indicated). They were washed with PBS and lysed in RIPA buffer containing complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Whole cell lysate (20 μg) was resolved using SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane using semidry transfer apparatus (ATTO Corporation, Japan). The membrane was blocked with 1 % BSA/TBS, and the expression level of indicated proteins was determined by incubating the membrane with specific primary antibodies (tyrosinase (M-19)-R, mortalin (Wadhwa et al. 1993), p53 (DO-1) Santa Cruz Biotechnology, Santa Cruz, CA, Hsp60 (N-20) followed by incubation with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and detection by ECL PLUS (GE Healthcare, UK)). The membranes were

probed by anti β-actin antibody (Abcam, Cambridge, UK) as an internal loading control.

Immunostaining

Cells were plated on coverslips placed in a 12-well culture plate. After the indicated treatments, cells were fixed with pre-chilled methanol/acetone (1:1) for 5–10 min. After washing with PBS and PBS with 0.2 % Triton X-100 (PBS-T) for 10 min each, cells were incubated with primary antibodies, anti-p53 (DO-1; Santa Cruz Biotechnology), anti-myc tag (Cell Signaling), anti-melanosome (HMB45- recognizes 10-kD segment of a sialylated glycoconjugate) (Dako), and anti-mortalin (Wadhwa et al. 1993) at 4 °C overnight. Cells were washed extensively with PBS-T (four times, 10 min each) followed by incubation with fluorochrome-conjugated secondary antibodies (Alexa-488-conjugated goat anti-rabbit or anti-mouse or Alexa-594-conjugated goat anti-rabbit or anti-mouse) (Molecular Probes). Cells were processed for imaging after extensive washing with PBS-T. Mitochondrial membrane potential was determined in control and treated cells by using JC-1 Assay Kit (Cell Technology Inc., USA) that uses a unique cationic dye (5,5,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) to signal the loss of mitochondrial membrane potential. While healthy mitochondria are stained bright red, the collapse of mitochondrial membrane potential is seen as green fluorescence as described earlier (Divi et al. 2007). ROS were detected by fluorescent staining using the Image-iT^{TM} LIVE Green ROS Detection Kit (Molecular Probes, Eugene, OR). Images, in all cases, were captured on a Zeiss Axiovert 200M microscope and analyzed by AxioVision 4.6 software (Carl Zeiss Microimaging, Thornwood, NY).

Immunohistochemistry

Keloid tissues were obtained from the Institute for Human Tissue Restoration, Department of Plastic & Reconstructive Surgery, College of Medicine, Yonsei University. Keloid and normal abdominal skin tissues were obtained for fibroblast culture, histology, and immunohistochemical analyses with excision. Keloid fibroblasts were obtained from both the central dermal layer of keloids. All the experiments involving humans were performed in adherence to the Helsinki Guidelines. Keloid and normal abdominal skin tissue sections were incubated at 4 °C overnight with an in-house anti-mortalin monoclonal antibody (Clone C1-3), followed by incubation with secondary antibody (Dako Envision™ Kit, Dako, Glostrup, Denmark) at RT for 20 min. Diaminobenzidine/hydrogen peroxidase (DAKO, Carpinteria, CA) was used as the chromogen substrate. All the slides were counterstained with Mayer's hematoxylin. The expression level of mortalin was semi-quantitatively analyzed using MetaMorph® image analysis software (Universal Image Corp., Buckinghamshire, UK). Results were expressed as the mean optical density for six different digital images.

Statistical analysis

All the experiments were performed, at least, three times. Quantitation of data was performed using ImageJ software (NIH, MA). Statistical significance of the data was calculated by QuickCals *t* test calculator (GraphPad Software, Inc., CA). Significance values were $*p<0.05$, $**p<0.01$, and $***p<0.001$.

Results and discussion

In order to identify the cellular factors involved in human melanogenesis, we used human melanoma (G361) and primary melanocytes from Caucasian skin (PM-C). The cells were subjected to OAG at a dose predetermined to cause induction of melanogenesis but not apoptosis, in independent experiments including cell morphology and protein assays. From these experiments, we determined that 15 and 30 μg/ml OAG were non-toxic to G361 and PM-C cells, respectively. As shown in Fig. 1, OAG-treated G361 cells showed an increase in melanosome staining. Quantitative assay on melanin content revealed nearly twofold increase, normalized by treatment with vitamin C (a positive control for depigmentation) (Choi et al. 2010; Panich et al. 2011) (Fig. 1b). In consistent with the melanin assays, tyrosinase expression (as determined by immunostaining, Western blotting, and ELISA) increased in OAG-treated cells (Fig. 1c–e) and decreased in the presence of vitamin C (Fig. 1e). Furthermore, whereas OAG-treated PM-C cells showed increase in melanosome staining (Fig. 1f), its expression level remained unchanged in human osteosarcoma (U2OS), breast carcinoma (MCF7), and amelanotic melanoma (A357) suggesting that the OAG-induced melanogenesis is a reliable and sensitive assay to screen cellular factors involved in this process.

We next recruited shRNA-induced loss-of-function screening using G361 cells and OAG-induced melanogenesis. Schematic diagram of the screening assay is presented in Supplementary Fig. 1. Cell transfection conditions were determined by microscopic observations of GFP protein in pEGFPC1-transfected G361 cells. In order to further rule out the differences in the transfection efficacy of the shRNA plasmids, the transfected cells were selected in puromycin-supplemented medium for 24–48 h and then treated with OAG. Increase in melanin was parallely examined by melanosome staining, melanin content, and tyrosinase activity assays. The shRNAs that caused abrogation of OAG-induced increase in melanosome, melanin content, and tyrosinase activity were selected, and the process was repeated for next

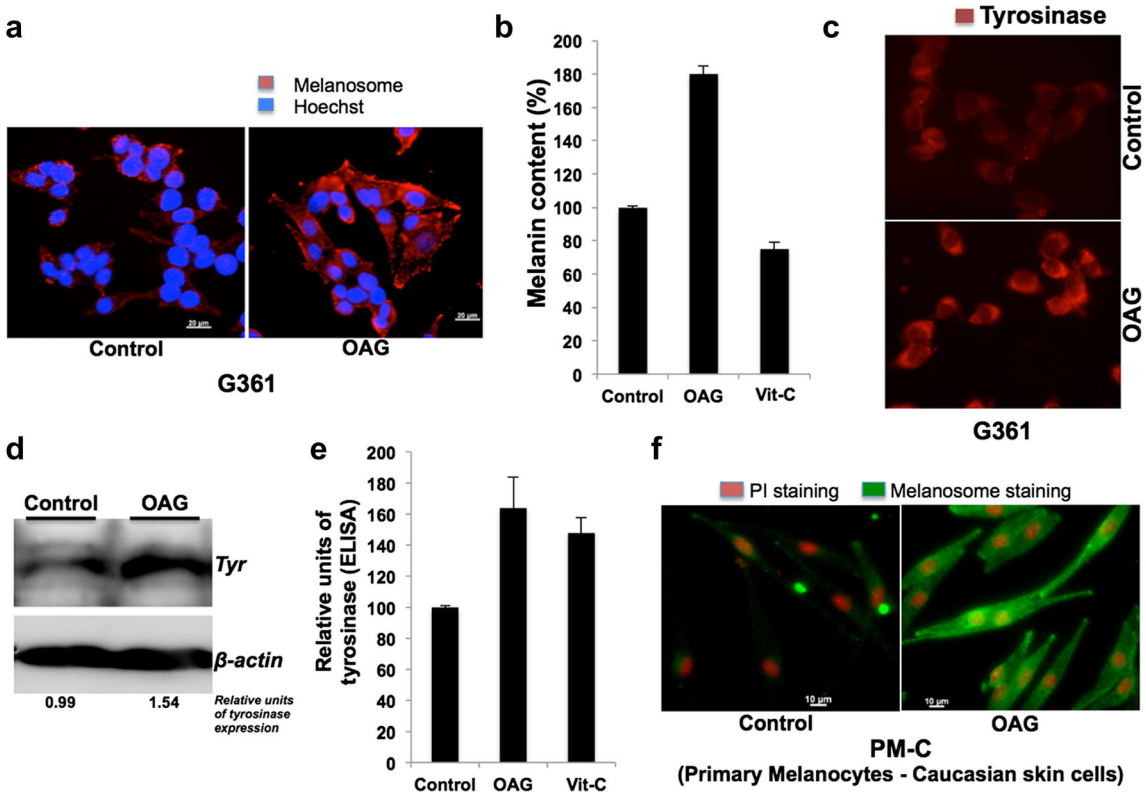


Fig. 1 Induction of melanogenesis with OAG. G361 cells were treated with OAG (30 μg/ml) for 24–48 h. Upregulation of melanin was examined by immunostaining (**a**) and biochemical quantitative assays (**b**). Tyrosinase was detected by immunostaining (**c**), Western blotting (**d**), and tyrosinase ELISA (**e**). All the assays revealed an increase in melanin as well as tyrosinase in OAG-treated cells. Vitamin C (Vit-C), used as a positive control for depigmentation, caused downregulation of melanin as shown in (**b**). (**f**) Primary melanocytes (PM-C), when treated with OAG (10 μg/ml for 24 h), showed an increase in melanosome staining

round of screening. The effect of 2044 shRNAs was investigated by conducting four rounds of screenings, and 40 shRNAs that caused reduction in all the three parameters were finally selected (Fig. 2a). We next investigated the effect of these genes on tyrosinase expression (Western blotting) during OAG-induced melanogenesis. We found that the knockdown of several, but not all, selected genes abrogated OAG-induced increase in tyrosinase (Fig. 2b) suggesting the possibility of identification of new gene/cellular factors that may regulate melanogenesis, independent to that of tyrosinase signaling. Involvement of selected gene targets in melanogenesis was assessed by gene interaction and pathway analyses using bioinformatic tools (Fig. 2c, 1–4). The analysis revealed the involvement of tyrosinase-mediated melanin pathway suggesting the reliability of the screening assay. Furthermore, involvements of NF-κB, TGF-beta, and p53 stress signaling pathways were identified (Fig. 2c) suggesting that the oxidative stress and melanogenesis are closely linked. Interestingly, MITF (a key regulator of melanocyte development and pigment production), an upstream regulator of genome instability and induction of cellular senescence mediated by ROS, p53, p21, and p16 activities in melanoma cells (Giuliano et al. 2011), was identified in our screening. MITF is connected with stress and cell cycle regulatory factors. Nishiura et al. (2012) identified bystander effect of UVA-induced melanogenesis in B16 murine melanoma cells and demonstrated it to be mediated by increased levels of intracellular oxidation and mitochondrial membrane potential. Upregulation of Nrf2, a key factor in protection of cells against oxidative stress and xenobiotic damage, caused a decrease in melanin synthesis in normal human melanocytes (Shin et al. 2014). It was associated with a decrease in expression of tyrosinase and tyrosinase-related protein 1 and activation of PI3K/Akt signaling. The inhibitory effect of Nrf2 on melanogenesis was reversed by overexpression of Keap1, an intracellular regulator of Nrf2. Similarly, wortmannin inhibitor of PI3K activity reversed the depigmentary effects of Nrf2 suggesting that Nrf2 negatively regulates melanogenesis by modulating the PI3K/Akt signaling pathway (Shin et al. 2014). In view of these reports, it was suggestive that the genes identified in our screening could be functionally relevant to the process of melanogenesis. We selected seven genes (p53, p21, p14, Hsp60, Bcl-2, Bcl-xL, and mortalin) involved in control of cell proliferation, stress signaling, and mitochondrial functions for further analyses.

In order to investigate the role of selected genes in melanogenesis, we first examined the expression in control and OAG-treated G361 cells. As shown in Fig. 3a, induction of

a

	Gene Name
1	Cyclin-dependent kinase 5 (CDK5)
2	Fibroblast growth factor (FGFG3)
3	Retinoic acid receptor, alpha (RARA)
4	Mitogen-activated protein kinase 7 (MAP2K7)
5	Interleukin-1 receptor-associated kinase 3 (IRAK3)
6	Mitogen-activated protein kinase 7 (MAPK7)
7	p21(CDKN1A)-activated kinase 7 (PAK7)
8	p16 cyclin dependent kinase inhibitor (p16INK4A)
9	Heat shock protein 60 (HSP60)
10	Mitochondrial heat shock protein 70 (HSP9A)
11	Pyruvate dehydrogenase kinase, isoenzyme 2 (PDK2)
12	Polymerase-gamma 22 (POLG-22)
13	Transcription factor 1 (ATF1)
14	Tyrosine phosphatase receptor type C (PTPRC)
15	Tyrosine phosphatase non-receptor type 3 (PTPN3)
16	Phosphatase 2 regulatory subunit A (PR 65)
17	Aurora kinase C (AURKC)
18	Tyrosine kinase (TXK)
19	Mixed-lineage leukemia translocated to 6 (MLLT6)
20	Lymphotoxin alpha TNF superfamily member 1 (LTA)
21	TNFR-associated factor 6 (TRAF)
22	G kinase anchoring protein 1 (FKSG2)
23	DNA binding Ku antigen protein (G22P1)
24	Synovial apoptosis inhibitor 1, synoviolin (HRD1)
25	Mitogen-activated protein kinase 8 interacting protein 1 (MAPK8IP1)
26	Phosphatase 11 (RNA/RNP complex 1-interacting) (DUSP11)
27	Protein phosphatase 1 regulatory (inhibitor) subunit 3A (PPP1R3A)
28	Serine/threonine kinase 38 like (STK38L)
29	Adenylate kinase 5 (AK5)
30	Protein kinase C and casein kinase substrate in neurons 3 (PACSIN3)
31	Liver-specific bHLH-Zip transcription factor (LISCH7)
32	T-cell leukemia, homeobox 2 (TLX2)
33	Engrailed homolog 2 (EN2), mRNA.
34	CCAAT/enhancer binding protein alpha (CEBPA)
35	Activating transcription factor 7 (ATF7)
36	ETS oncogene family member (ELK1)
37	TGFB-induced factor (TALE family homeobox) (TGIF)
38	Nijmegen breakage syndrome 1 (nibrin) (NBS1)
39	Germ cell specific Y-box binding protein (YBX2)
40	Bagpipe homeobox homolog 1 (Drosophila) (BAPX1)

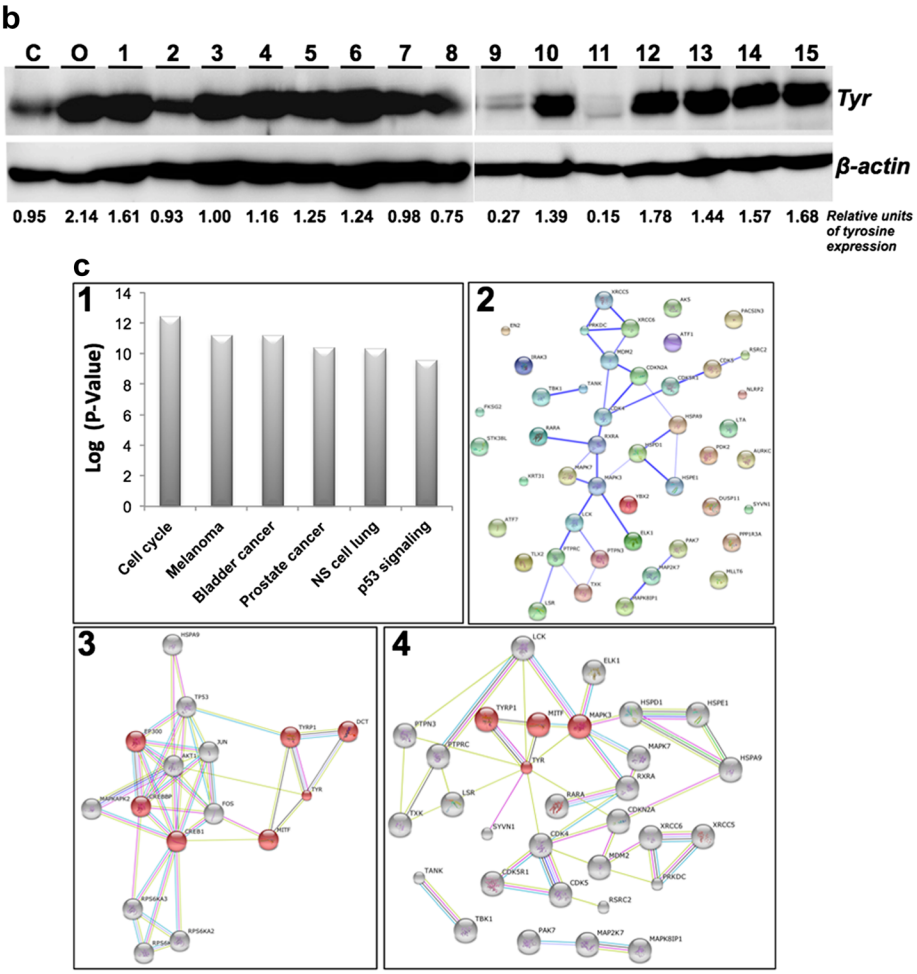
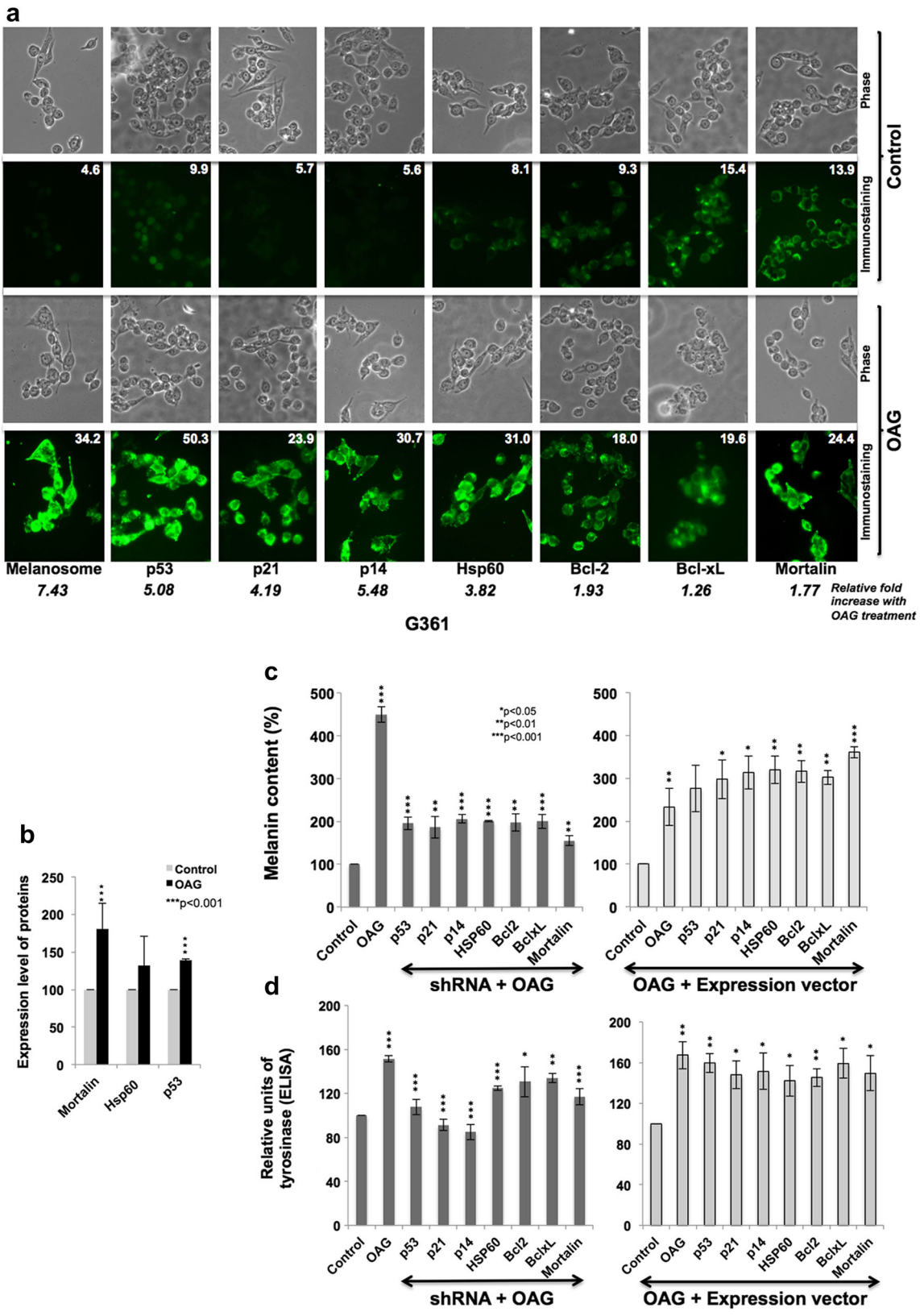


Fig. 2 Identification and validation of genes involved in melanogenesis pathway. **a** Gene targets of the 40 selected shRNAs that caused loss of OAG-induced upregulation of melanogenesis, from four rounds of screening, are listed. **b** Representative Western blot of tyrosinase in shRNA (corresponding to the gene targets shown in table)-transfected cells showing a decrease in its expression with some but not all. **c** The network analyses of target genes and their association with melanogenesis. The target genes showed their connection with cellular mechanisms involving cell cycle, cancer, and p53 signaling (*1–2*). Melanogenesis genes, i.e., MIFT, TYR, TYRP1, DCT, and CREB (*red circle*), showed connections with HspA9/mortalin, p53 (*3*) and HspD1, HspE1, CDKN2A, and CDK4 (*4*). Analyses were performed using STRING and Ingenuity Pathway/Kyoto Encyclopedia of Genes and Genomes (KEGG)

melanogenesis was associated with an increase in the expression of p53, p21, p14, Hsp60, Bcl2, Bcl-xL, and mortalin. Of note, cell cycle regulatory proteins (p53, p21, p14) showed higher increase as compared to the stress regulatory and anti-apoptotic proteins (Hsp60, mortalin, Bcl-2, and Bcl-xL). Increase in expression of mortalin, Hsp60, and p53 was also confirmed by Western blot analysis (Fig. 3b). Melanin assays in cells compromised for the target gene expression by specific shRNA caused a decrease in OAG-induced increase in melanin (Fig. 3c). Tyrosinase assay in control, OAG-treated, and shRNA-transfected cells also exhibited decrease, although to a variable level, in shRNA-treated cells suggesting that these genes are involved in melanogenesis (Fig. 3d). In order to further validate their role in melanogenesis, we generated G361 cells with overexpression of these proteins. Melanin assay in these cells revealed small increase in OAG-induced melanin (Fig. 3c). However, it was not associated with an increase in tyrosinase activity (Fig. 3d) suggesting that these genes may regulate melanogenesis by tyrosinase-independent

Fig. 3 Expression analyses of the selected genes in control and OAG (10 μM)-treated cells. **a** Immunostaining of control and OAG-treated cells for indicated proteins showed OAG-induced upregulation of their expression. Images were scanned and quantitated with ImageJ software. Expression level in control and OAG-treated cells and fold increase in the latter are indicated on the images (*white*) and bottom of the panel, respectively. **b** Expression level of proteins in control and OAG-treated cells, as determined by Western blotting. Quantitation from three independent experiments is shown. **c** Quantitative estimation of melanin and tyrosinase activity in cells transfected with either shRNAs (*left*) or expression plasmids (*right*) in the presence of OAG. Cells compromised for the indicated genes showed significant attenuation of OAG-induced increase in melanin content (**c**, *left*). Decrease in tyrosinase activity in all cases, although to a variable extent, was also observed (**d**, *left*). Overexpression of proteins caused small enhancement of OAG-induced increase in melanin content (**c**, *right*) and was not associated with increase in tyrosinase activity (**d**, *right*)



pathways. Together with the data shown in Figs. 2b and 3d (left panel), it was suggestive that these genes are involved in melanogenesis; their knockdown compromised OAG-induced increase in melanin in a tyrosinase-dependent manner. However, an increase in expression of these genes that resulted in small increase in OAG-induced melanin occurred

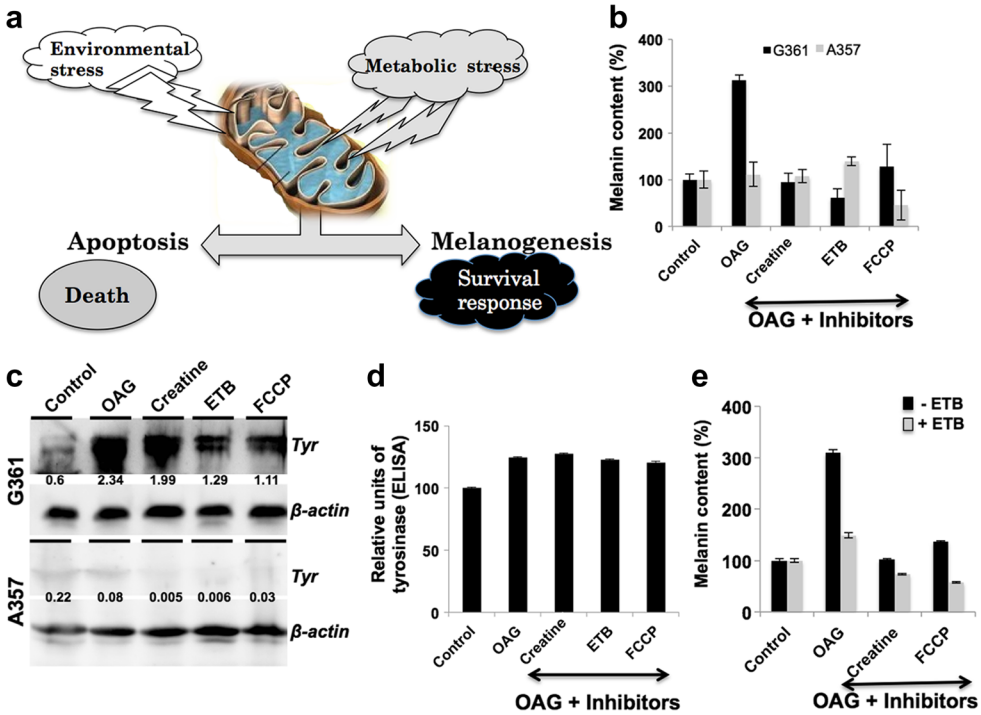
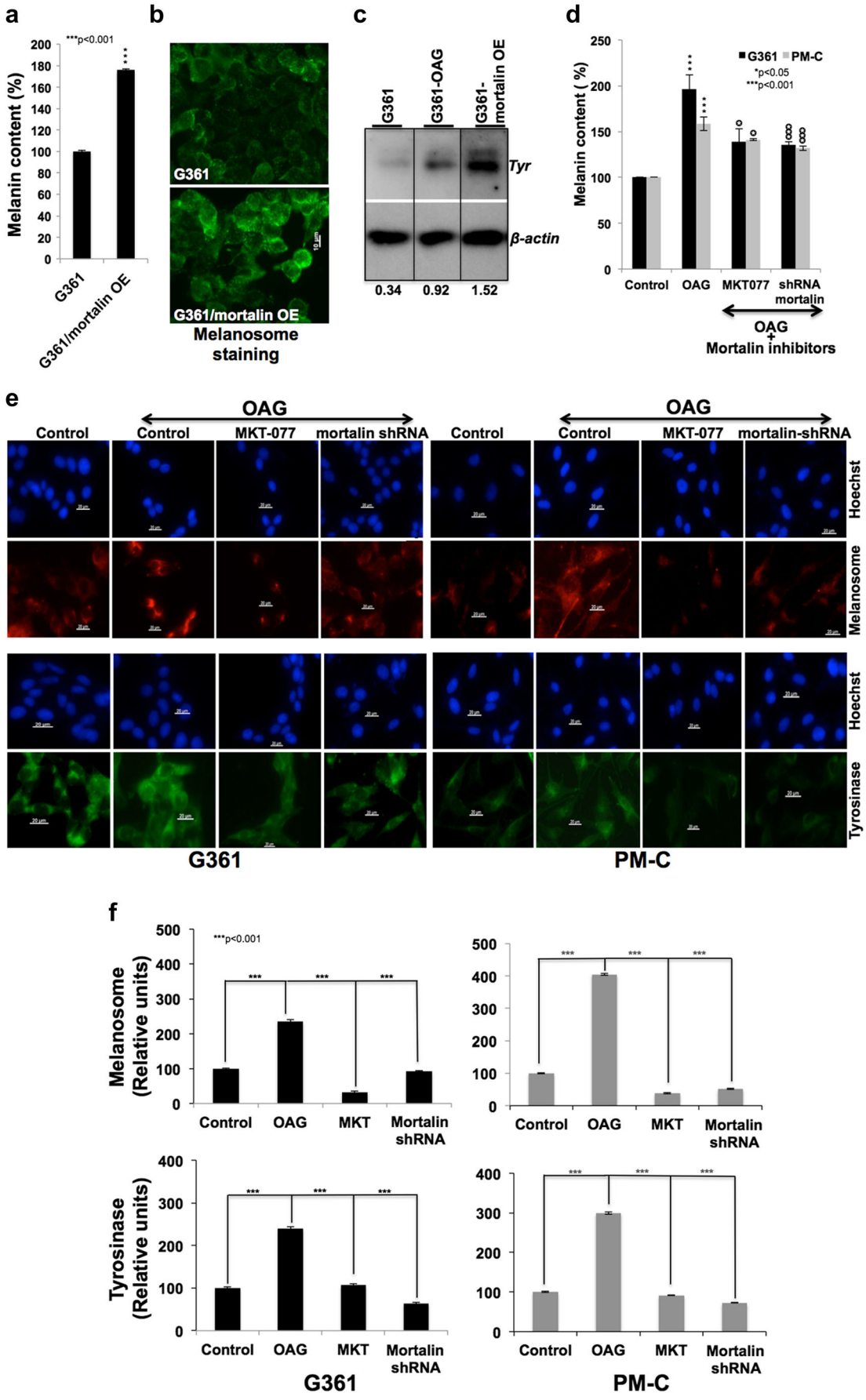


Fig. 4 Effect of mitochondrial inhibitors on melanogenesis. A drawing showing melanogenesis as a survival response triggered in mitochondria in response to internal/external stresses (**a**). G361 cells were treated with mitochondrial inhibitors (Creatine, 10 μM; FCCP, 10 μM) and Hsp60 inhibitor (ETB, 3 μM) for 24–48 h with/without OAG (20 μg/ml). As shown, the inhibitors caused abrogation of OAG-induced upregulation of melanin content in G361 but not in amelanotic melanoma A357 cells (**b**). Under the similar conditions, OAG-induced increase in tyrosinase activity was not affected by mitochondrial inhibitor creatine (**c**, **d**). ETB- and FCCP-mediated abrogation of OAG-induced increase in melanin content was accompanied by a decrease in tyrosinase (**a**, **c**, and **d**). ETB potentiated the inhibition of OAG-induced melanogenesis by mitochondrial inhibitors (Creatine and FCCP) (**e**)

through tyrosinase-independent pathway. Terrein, a fungal metabolite derived from *Aspergillus terreus*, has been shown to have a variety of biological activities including anti-inflammatory, antioxidant, anticancer, and inhibition of melanogenesis. It was shown to affect melanin synthesis via downregulation of MITF, mitochondria integrity leading to increase in p53, p21, ERK, and Bax expression levels suggesting a link between proliferation control and melanogenesis signaling (Park et al. 2004; Porameesanaporn et al. 2013). Snyder et al. (2005) screened for pigment enhancing reagents by cell-based assays and identified a small molecule melanogenin that targeted mitochondrial protein, prohibitin. It was shown that prohibitin is required for melanogenin-induced pigmentation and a potential target for treatment of pigmentary disorders. Based on these results, the role of mitochondria in pigmentation signaling has been highlighted (Rosania 2005). Kim and Lee (2013) reported that the hydrogen peroxide-induced melanogenesis caused upregulation of PAH, TYR, MITF, phosphorylated CREB, ATP5B, and mitochondrial F1 complex. It was also associated with increased intracellular ATP levels suggesting the involvement of mitochondria. Interestingly, electron tomography study by Daniele et al. (2014) showed that the mitochondria physically contact melanosomes through fibrillar bridges involving mitofusin 2, also connects mitochondria to ER. Such mitochondria-melanosome contacts were shown to be associated with melanogenesis process. Knockdown of mitofusin 2 resulted in reduction of contacts and was associated with aberrant melanogenesis. Furthermore, pharmacological inhibition of mitochondrial ATP synthesis severely reduced contacts between mitochondria and melanosomes and impaired melanosome biogenesis. Williams et al. (2004) reported the identification of compounds for correction of albinism. The selected compounds were shown to target mitochondrial F1F0-ATP synthase and correct mistrafficked tyrosinase enzyme.

In light of these reports and our data (described above) on the selection of stress and mitochondrial proteins involved in melanogenesis, we hypothesized that the melanogenesis may represent a stress-survival response mediated through mitochondria (Fig. 4a). We, therefore, investigated the role of

Fig. 5 Upregulation of melanogenesis in mortalin-overexpressing cells. ▶ Mortalin overexpressing G361 derivatives showed increase in melanin content (**a**, **b**) and tyrosinase expression (**c**). Treatment of cells with mortalin inhibitor, MKT-077 (50–100 nM for 24 h), caused a decrease in OAG-induced melanin (**d**–**f**) and tyrosinase (**e**, **f**), similar to the effect caused by anti-mortalin shRNA. Quantitation from three independent experiments is presented


a
b
c
d
***p<0.001
Melanin content (%)
G361
G361/mortalin OE
G361
G361/mortalin OE
Melanosome staining
G361
G361-OAG
G361-mortalin OE
Tyr
β-actin
0.34
0.92
1.52
G361
PM-C
*p<0.05
***p<0.001
Melanin content (%)
Control
OAG
MKT077
shRNA mortalin
OAG + Mortalin inhibitors
e
OAG
OAG
Control
Control
MKT-077
mortalin shRNA
Control
Control
MKT-077
mortalin-shRNA
Hoechst
Melanosome
Hoechst
Tyrosinase
G361
PM-C
f
***p<0.001
Melanosome (Relative units)
Control
OAG
MKT
Mortalin shRNA
Tyrosinase (Relative units)
G361
PM-C

mitochondria and mitochondrial stress proteins, Hsp60, and mtHsp70/mortalin in OAG-induced melanogenesis. We first used mitochondrial inhibitors and found that the OAG-induced increase in melanin was compromised in cells treated with mitochondrial inhibitors (creatine induces mitochondrial fragmentation and FCCP, an uncoupler of oxidative phosphorylation) (Fig. 4b). Amelanotic melanoma (A357), used as a negative control, did not show the effect of inhibitors on melanin content (Fig. 4b). Of note, there was no significant decrease in tyrosinase in cells treated with creatine (Fig. 4c, d). These data suggested that the functional mitochondria is involved in the process of melanogenesis and may not have direct effect on tyrosinase. We also examined the mitochondrial membrane potential and ROS levels in control and OAG-treated cells. As shown in Supplementary Fig. 2, induction of ROS and collapse of mitochondrial membrane potential was observed in cells treated with H_2O_2 and creatine (positive controls). OAG-treated cells showed mild induction of ROS and collapse of mitochondrial membrane potential, suggesting an induction of oxidative stress signaling.

We next investigated the functional significance of mitochondrial stress proteins, Hsp60, and mtHsp70/mortalin in melanogenesis. Both Hsp60 and mortalin are essential proteins that localize in multiple subcellular sites and shown to be involved in protein assembly and folding, oxidative, and anti-apoptotic stress signaling (Singh and Gupta 2009; Kaul et al. 2007). They are upregulated in several kinds of tumors and contribute to carcinogenesis by multiple pathways (Deocaris et al. 2009, 2013). Both Hsp60 and mortalin have been shown to complex with Bax in the cytoplasm and deregulate apoptosis in cancer cells (Gupta and Knowlton 2002; Lu et al. 2011). Interestingly, in case of birds, coloration is acquired from carotenoids consumed from the diet. While the healthy birds express dense carotenoid-based colors, the parasite-infected birds exhibit pale coloration and high level of stress chaperone Hsp60 (del Cerro et al. 2010). In case of fish, melanophore index, a primary stress indicator, was inversely correlated with increase in the expression of heat shock protein 70 (Hsp70)/Hsp60 heat shock proteins and melanin concentrating hormone (MCH) in response to a variety of stressed aquatic conditions (Daiwile et al. 2015). In light of these reports and our data, we investigated the role of Hsp60 in OAG-induced melanogenesis. G361 cells treated with ETB, an inhibitor of Hsp60, showed a decrease in OAG-induced increase in melanin (Fig. 4b, e). Furthermore, co-treatment with ETB and mitochondrial inhibitors showed stronger effect (Fig. 4e). These data supported the

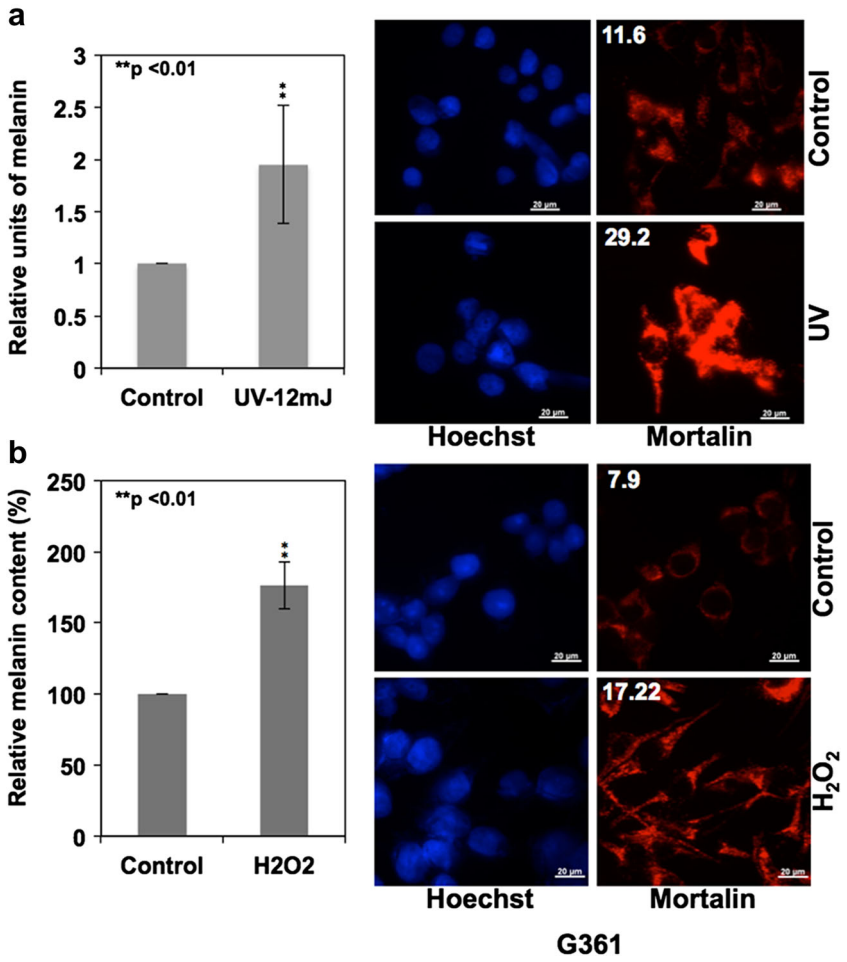


Fig. 6 Upregulation of mortalin in oxidatively stressed cells. G361 cells with oxidative stress by exposure to either UV (12 mJ/cm^2) or H_2O_2 (40 μM) for 24–48 h showed an increase in melanin (*left*, quantitation from three independent experiments is shown) as well as mortalin (*right*, images; scanned and quantitated by ImageJ software) revealed two- to threefold increase in mortalin

role of Hsp60 in regulation of OAG-induced pigmentation in human cells.

Mortalin, similar to Hsp60, influenced OAG-induced melanogenesis (Figs. 2 and 3). To the best of our knowledge, there is no report so far on the involvement of mortalin in the process of melanogenesis. We, therefore, extended this finding to further analyses. We examined melanin content in G361 cells and their mortalin-overexpressing derivatives and found that the latter possess higher level of melanin (Fig. 5a–c) that was also associated with increase in tyrosinase expression. Anti-mortalin molecule, MKT-077 (100 nM), caused reduction in OAG-induced melanin, similar to the one caused by anti-mortalin shRNA (Fig. 5d), not only in G361 cells but also in normal primary melanocytes, as examined by immunostaining assays (Fig. 5e, f). Furthermore, the inhibitor-treated cells showed a decrease in the expression of tyrosinase suggesting that mortalin is an important factor for melanogenesis (Fig. 5e, f). In view of the established relationship of oxidative stress and melanogenesis (Denat et al. 2014; Shin et al. 2014), we next recruited H_2O_2 and UV-induced oxidative stress models and examined if mortalin was upregulated in oxidative stress-induced melanogenesis. As shown in Fig. 6a, b, both UV and H_2O_2-induced upregulation of melanin was associated with increase in mortalin. In order to investigate further the physiological relevance of role of mortalin in melanogenesis, we examined its expression in the clinical samples of keloids that possess hyperpigmentation. As shown in Fig. 7, we found that the hyperpigmentation of skin in keloids was tightly associated with the higher (~3-fold) expression of mortalin.

Mortalin, a stress chaperone, predominantly localizes in the mitochondria. Its functions have been classified into two major classes based upon subcellular location: (i) functions in mitochondria including import of nuclear-encoded proteins into the mitochondrion, nascent protein folding, protein degradation, and interaction with sub-mitochondrial constituents to maintain its integrity and function and (ii) extra-mitochondrial function including its interaction and functional regulation of several cytoplasmic, endoplasmic reticulum and nuclear proteins, centrosomes, growth factors, immune system constituents, and metabolic constituents (Deocaris et al. 2009, 2013). Besides its functionality in human carcinogenesis (Deocaris et al. 2013), mortalin has been documented to play a significant role in neuronal functions including

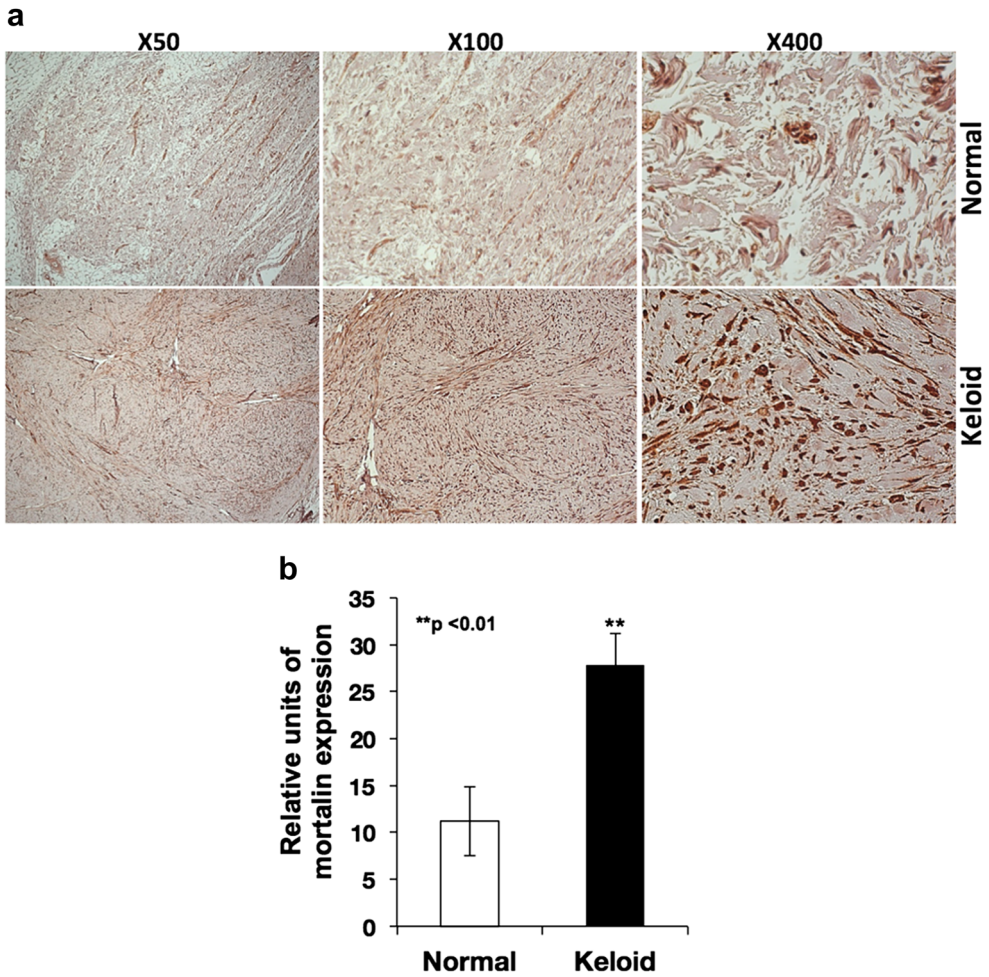


Fig. 7 Upregulation of mortalin in hyperpigmented keloid skin. Immunohistochemical staining of mortalin in keloid skin sections (**a**). Quantitation was performed using MetaMorph® image analysis software and the statistical significance of the data were calculated by Statistical Package for the Social Sciences (SPSS) software (**b**)

neuronal differentiation (Shih et al. 2011). Consistent with the concept that the mitochondrial dysfunction is involved in the pathogenesis of several major neurological diseases, including neurodegenerative disorders (Alzheimer's and Parkinson's diseases) and cerebral ischemia, mortalin dysfunction was shown to be involved in these diseases (Park et al. 2014; Qu et al. 2011; 2012).

Several epidemiological studies have reported high co-occurrence of Parkinson's disease (PD) and melanoma. It has been shown that the UV-induced increase in neuromelanin, the melanin synthesized in dopaminergic neurons, enhances the susceptibility to oxidative stress and induced neuronal injury relevant to PD. Furthermore, alpha-synuclein (PD-regulatory protein) was shown to interact with tyrosinase and inhibit tyrosine hydroxylase and attenuate melanin synthesis in melanoma cells. However, an overexpression of alpha-synuclein in dopaminergic neurons resulted in an increase in their melanin content suggesting positive association between PD and melanoma (Pan et al. 2012). Mortalin has been shown to interact with alpha-synuclein and contributes to PD pathogenesis (Gaweda-Walerych and Zekanowski 2013; Chiasserini et al. 2011; Jin et al. 2007; Xu et al. 2008; Zhu et al. 2013). Most recently, it was shown that there is a physical contact between the mitochondria and melanosomes through fibrillar bridges. Mitofusin (Mfn) 2, a protein that bridges ER to mitochondria and localizes in melanosome-mitochondrion contacts, was shown to be involved in the process of melanogenesis. Mfn2 knockdown abrogated OA1-induced melanogenesis, and the pharmacological inhibition of mitochondrial ATP synthesis caused reduction in contact formation and impaired melanosome biogenesis (Daniele et al. 2014). Oxidative stress-induced melanogenesis was regulated by upregulation of PAH and intracellular cAMP levels. Kim et al. (2013) also predicted the role of mortalin in these processes.

In contrast to the above-described role of mortalin in melanogenesis, constitutive Hsp70 was shown to inhibit melanin production (Usui et al. 2015; Yamashita et al. 2010; 2012) and has been used as a marker to isolate hypopigmenting reagents. Exposure to UV and other stresses leading to induction of melanogenic proteins and darkening of skin has been firmly established. Kim et al. (2003) reported that the expression of MITF and tyrosinase activity were upregulated during induction of melanogenesis by IBMX and not suppressed in Hsp70-overexpressing cells suggesting the involvement of additional factors that upregulate melanogenesis during stressed conditions. Our results suggest that mtHsp70/mortalin, an essential mitochondrial protein involved in redox signaling and protection against oxidative stresses (Qu et al. 2011, 2012), is involved in melanogenesis and skin pigmentation.

Mitochondria biogenesis requires import of several precursor proteins synthesized in the cytosol. Translocation of proteins from cytosol across the mitochondrial inner membrane is driven by the action of an essential mitochondrial inner membrane translocase motor protein complex, Tim44-complex. The latter interacts with two major mitochondrial chaperones (mortalin and Hsp60) (Schiller et al. 2008) that also interact with each other (Wadhwa et al. 2005; Bottinger et al. 2015). It has been established that Hsp60 together with its co-factor Hsp10 catalyzes folding of a subset of mortalin-client proteins. On the other hand, mortalin interacts with Hsp60 co-chaperone (Hsp10) and promotes the formation of functional heptameric Hsp60 rings required for folding of client proteins (Wadhwa et al. 2005; Bottinger et al. 2015). Mortalin and mtHsp40 dysfunction has also been associated with mitochondrial fragmentation in which molecular ratio of mtHsp40 to mtHsp70 was shown to determine their chaperone function and mitochondrial morphology (Lee et al. 2015). It has been established that these chaperones collectively ensure normal structure and function of mitochondria, also required for melanogenesis (Daniele et al. 2014). Their dysfunction has been associated with several pathologies, also called chaperonopathies (Cappello et al. 2014). Identification of both Hsp60 and mortalin in our screening is suggestive that cooperative function of these proteins along with their co-chaperones and client proteins may be involved in regulation of the process of melanogenesis. Molecular insights to these mechanisms warrant further studies.

We provide evidence that (i) the hyperpigmented keloid skin possesses significantly higher level of mortalin expression, (ii) mortalin is increased during oxidative stress and drug-induced melanogenesis, and (iii) overexpression of mortalin causes an increase in pigmentation and its compromise leads to reduction in melanin content. Further studies are warranted to (i) elucidate the molecular mechanism of the role of tyrosinase in mortalin-mediated melanogenesis and (ii) validate mortalin as a target and drug discovery tool for manipulation of skin pigmentation for therapeutic and cosmetic purposes.

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Compliance with ethical standards

Conflict of Interest The authors declare no conflict of interest.

Author Contributions RW and SCK conceived and coordinated the study and wrote the paper. RW, DP, RG, NW, NN, LL, and HA performed the experiments. RW, COY, NA, CM, and SCK analyzed the data. All authors reviewed the results and approved the final version of the manuscript.

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