The Essential Role of p53 in Hyperpigmentation of the Skin via Regulation of Paracrine Melanogenic Cytokine Receptor Signaling*

Daiki Murase, Akira Hachiya¹, Yasuko Amano, Atsushi Ohuchi, Takashi Kitahara, and Yoshinori Takema
From the Kao Biological Science Laboratories, Haga, Tochigi 321-3497, Japan

Hyperpigmentation of the skin is characterized by increases in melanin synthesis and deposition. Although considered a significant psychosocial distress, little is known about the detailed mechanisms of hyperpigmentation. Recently, the tumor suppressor protein p53 has been demonstrated to promote ultraviolet B-induced skin pigmentation by stimulating the transcription of a melanogenic cytokine, POMC (pro-opiomelanocortin), in keratinocytes. Given that p53 can be activated by various kinds of diverse stresses, including sun exposure, inflammation, and aging, this finding led us to examine the involvement of p53 in cytokine receptor signaling, which might result in skin hyperpigmentation. Immunohistochemical and reverse transcription-PCR analyses revealed the increased expression and phosphorylation of p53 in the epidermis of hyperpigmented spots, accompanied by the higher expression of melanogenic cytokines, including stem cell factor, endothelin-1, and POMC. The involvement of p53 in hyperpigmentation was also indicated by the significantly higher expression of p53 transcriptional targets in the epidermis of hyperpigmented spots. Treatment of human keratinocytes and melanocytes with known p53 activators or inhibitors, including pifithrin-α (PFT), demonstrated significant increases or decreases, respectively, in the expression of melanogenic factors, including cytokines and their receptors. Additionally, PFT administration abolished stem cell factor-induced phosphorylation of mitogen-activated protein kinase in human melanocytes. Furthermore, when organ-cultured hyperpigmented spots, in vitro human skin substitutes, and mouse skin were treated with PFT or p53 small interfering RNA, the expression of melanogenic cytokines and their receptors was significantly decreased, as were levels of tyrosinase and melanogenesis. Taken together, these data reveal the essential role of p53 in hyperpigmentation of the skin via the regulation of paracrine-cytokine signaling, both in keratinocytes and in melanocytes.

There are various types of hyperpigmentations (pigmented spots) of the skin characterized by increases in melanin synthesis and deposition, such as freckles, postinflammatory hyperpigmentation, UV-induced pigmentation (UV-melanoses), senile lentigines (age spots), pigmentation petaloids actinica, and melasma. These hyperpigmentations are well known to cause significant psychosocial distress, but little is known about their detailed mechanisms.

Hyperpigmentation generally results from three major steps in the epidermis: the proliferation of melanocytes, the synthesis and activation of tyrosinase to produce melanin, and the transfer of melanosomes to keratinocytes (1–4). During the first two steps, a complicated network composed of paracrine and autocrine cytokines secreted by keratinocytes and by melanocytes, respectively, plays an important role in regulating melanogenesis in collaboration with their corresponding receptors, whose expression is also regulated by various cytokines (5–23). In addition, several types of cross-talk among cytokine receptor signaling pathways are involved in the enhanced proliferation and melanogenic activities of melanocytes. These paracrine and/or autocrine cytokine receptor signalings include basic fibroblast growth factor (5), ET-1 (endothelin-1) (6–12, 21), α-melanocyte-stimulating hormone (13–15, 17, 19, 20), SCF (stem cell factor) (9, 11, 22, 23), nitric oxide (18), and their specific receptors. During the process of melanosome transfer from melanocytes to keratinocytes, the expression of a melanosome phagocytic protein, PAR2 (protease-activated receptor 2), is induced by UV irradiation, and inhibition of PAR2 activation prevents UVB-induced pigmentation in Yucatan swine skin (24, 25).

In UVB-induced pigmentation, which disappears a few months after the initial exposure, we previously demonstrated that the expression of SCF and ET-1 is remarkably enhanced at the mRNA transcript and protein levels in the earlier and later phases of UVB-induced pigmentation, respectively (23). SCF and ET-1 are both potent mitogens and melanogens, and they synergistically activate the phosphorylation of mitogen-activated protein kinase (MAPK)² in human melanocytes (9, 11). SCF also stimulates the expression of ET₄R (endothelin B receptor) so that its enhanced expression is harmonized with that of ET-1 in human epidermis (23). Taken together, these findings suggest that the biphasic expression of SCF and ET-1 intrinsically plays a pivotal role in the proliferation and melanogenesis of human melanocytes in vivo during UVB-induced hyperpigmentation.

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² The abbreviations used are: MAPK, mitogen-activated protein kinase; UVB, ultraviolet B; 5-FU, 5-fluorouracil; PFT, pifithrin-α; NHEK, normal human epidermal keratinocyte; NHEM, normal human epidermal melanocyte; HSS, human skin substitute; ELISA, enzyme-linked immunosorbent assay; siRNA, small interfering RNA; RT, reverse transcription.
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In addition to UVB-induced pigmentation, the contribution of two receptor-mediated signaling cascades (ET-1/ETαR and SCF/KIT) has also been reported to play a role in the formation of one type of persistent hyperpigmentation, senile lentigines, in which both long term sun exposure and chronological aging are thought to be involved (26, 27). The higher expression of both ET-1 and SCF has been confirmed in the lesional skin of senile lentigines compared with control perilesional skin. These cytokine receptor networks have also been reported to be involved in other human hyperpigmentation disorders, such as dermatofibromas, café-au-lait macules, and seborrheic keratoses (28–30). Such evidence indicates that the mechanisms of several types of hyperpigmentation, such as UV-induced pigmentation (suntanning) and senile lentigines, commonly share increased SCF/KIT and ET-1/ETBR signaling and reveals their coordinated roles in regulating epidermal melanogenesis as a part of skin homeostasis consistent with a previous study (31). Additionally, Motokawa et al. (32) reported that expression of the POMC (pro-opiomelanocortin) gene, which encodes a precursor of α-melanocyte-stimulating hormone, is higher in the epidermis of senile lentigines than in peripheral epidermal controls.

Recently, Cui et al. (33) focused on the role of p53, a tumor suppressor protein, in the induction of UV-induced epidermal hyperpigmentation by its direct activation of POMC transcription in keratinocytes, which results in the enhanced melanogenesis of melanocytes in a paracrine manner. They demonstrated multiple instances of in vivo hyperpigmentation due to activation of the p53 cascade, which mimics the pathway of UV-induced pigmentation. Given that p53 can be activated by various diverse stresses, including sun exposure, inflammation, and aging, their findings led us to assess the potential involvement of p53 in melanogenic paracrine cytokine receptor signaling that causes hyperpigmentation disorders, such as senile lentigines, which are strongly related to photoaging and chronological aging.

Therefore, we investigated the involvement and the role of p53 in the regulation of melanogenic paracrine cytokine receptor networks in hyperpigmentation of the skin. We report for the first time that p53 plays a pivotal role in the formation of hyperpigmentation via the stimulation of paracrine cytokine networks in human epidermis.

EXPERIMENTAL PROCEDURES

Materials—Normal human epidermal keratinocytes (NHEKs), melanocytes (NHEMs), and three-dimensional human skin substitutes (HSSs; MEL-300A) were purchased from Kurabo Co. (Osaka, Japan). C57BL/6 female mice 5–7 weeks old were supplied by CLEA Japan, Inc. (Tokyo, Japan). Human recombinant SCF, polyclonal antibodies specific for SCF and KIT, and the ET-1 ELISA kit were provided by Immuno-Biological Laboratories Co. (Gunma, Japan). Human recombinant ET-1 and anti-β-actin-specific antibody were supplied by Sigma. Antibodies specific for p53 (DO-7 and DO-1) and phospho-p53 were purchased from Dako Inc. (Carpinteria, CA), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and Cell Signaling Technology, Inc. (Danvers, MA), respectively. Specific antibodies for p21, MDM2, and Gadd45a were supplied by Santa Cruz Biotechnology. The specific siRNA directed against human or mouse p53 and the control siRNA were provided by Invitrogen. Other chemicals were of reagent grade.

Cell Culture—NHEKs were preliminarily incubated in EpiLife medium (Kurabo) supplemented with 10 μg/ml insulin, 0.1 μg/ml human recombinant epidermal growth factor, 0.5 μg/ml hydrocortisone, 50 μg/ml gentamycin, 50 ng/ml amphotericin B, and 0.4% (v/v) bovine pituitary extract at 37 °C in an atmosphere of 5% (v/v) CO2. NHEMs were maintained in Medium 254 (Kurabo) supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 3 ng/ml human recombinant fibroblast growth factor, 0.18 μg/ml hydrocortisone, 3 μg/ml heparin, 10 ng/ml phorbol 12-myristate 13-acetate, 0.2% (v/v) bovine pituitary extract, and 0.5% (v/v) fetal bovine serum at 37 °C with 5% CO2, as previously described (6). Cells were seeded in 6-well plates (BD Biosciences) at a density of 1.0 × 10^6 cells/well and then were incubated for 24 h, followed by incubation in the medium without bovine pituitary extract and human epidermal growth factor for NHEK and in the medium without phorbol 12-myristate 13-acetate for NHEM. Following another 24-h incubation, cells were treated with each reagent and incubated according to each experimental design.

siRNA Transfection—NHEKs and NHEMs were transfected with 40 nM specific siRNA directed against p53 or a control siRNA, using Lipofectamine 2000 (Invitrogen) or TransIT-TKO Transfection Reagent (Mirus Bio Co., Madison, WI) according to the manufacturer’s instructions.

Suction Blister Method—Human epidermal sheets were obtained from the upper arms of five healthy 43–52-year-old Japanese subjects using the suction blister technique (34) with approval by the Ethical Committee of Kao Biological Science Laboratories. Following informed consent from the volunteers, samples were collected from each subject at three sites: a hyperpigmented spot in a sun-exposed area (site 1), a sun-irradiated control skin in the peripheral area of site 1 (site 2), and a non-exposed control skin from the ventral side of the upper arm (site 3). Blisters were induced with a 1- or 2.5-mm diameter syringe (Terumo, Tokyo, Japan) at each site for ~1 to 2 h. The blister roof was removed with sterile scissors.

Organ Culture of Human Pigmented Spots—Punch biopsy skins, with or without hyperpigmentation spots on the upper arm or shoulder from healthy 50–59-year-old Caucasian females with Type II skin (Stephens and Associates, Carrollton, TX) were used in this study. Collection of the skins was approved by the Institutional Review Board of IntegReview Ltd. (Austin, TX), and informed consent was obtained from the volunteers prior to the procedure. Each punch biopsy skin with hyperpigmentation was divided into two equal pieces, put onto collagen sponges (MedChem Products Inc., Woburn, MA), and incubated in Dulbecco’s modified Eagle’s medium or in a mixture of 50% Epilife, 50% Medium 254 without phorbol 12-myristate 13-acetate (Cascade Biologies, Portland, OR). One half was then treated with 10 μM PFT for 72 h, and the other half was incubated in medium without PFT. Media were changed every day, and finally the epidermal sheets were peeled off and used for gene expression analysis.
Injection of siRNA into Mouse Ears Followed by UVB Irradiation—C57BL/6 female mice, 8 weeks old at the beginning of the study, were used. The animals had free access to food and chlorinated water and were housed in cages. Mouse ears were injected subepidermally with 5 μg of mouse p53-specific siRNA or control siRNA with a cationic polymer transfection reagent, in vivo-jetPEI™ (Polyeus-transfection SA, Illkirch, France). Immediately after the injection, they were irradiated with UVB irradiation with a dose of 50 mJ/cm² of UVB light, at which dose most of the energy of the emitted is a peak of emission near 306 nm, using UVB lamps (Sanyko Denki Co., Kanagawa, Japan). In total, they were administered with siRNA injection and UVB irradiation three times at 24-h intervals.

Measurement of Skin Color—The intensity of UVB-induced pigmentation was measured by a color difference meter (Nippon Denshoku Industries, Tokyo, Japan) 6 days after the first siRNA injection and UVB exposure and was expressed as the L* value.

Immunohistochemistry—The specimens, obtained from hyperpigmented (spots) and from peripheral areas, were fixed in 10% buffered formalin and then embedded in paraffin. Following antigen retrieval by heating in a target retrieval solution (10 mM Tris-HCl buffer, including 1 mM EDTA (pH 9.0) or 10 mM sodium citrate buffer (pH 6.0)) at 95 °C for 45 min, the sections were treated with 0.3% H₂O₂ solution at room temperature for 30 min. Immunoreactivity was assessed using antibodies specific for p53 or p53 phosphorylated at serine 392 as primary antibodies and peroxidase-labeled anti-mouse or anti-rabbit IgG polyclonal antibodies (Dako Inc.) as secondary antibodies after blockage of nonspecific binding using 3% bovine serum albumin and 3% goat normal serum (Vector Laboratories, Burlingame, CA). Normal rabbit or mouse IgG (Vector Laboratories) in place of the primary antibody was used as a negative control. The sections were developed using the Histomark® TrueBlue™ peroxidase system (Kirkegaard & Perry Laboratories, Gaithersburg, MD), rinsed in distilled water, and counterstained with NUCLEAR FAST RED (Vector Laboratories), followed by washing in running water.

Quantitative Real Time RT-PCR—Total RNA extracted from human epidermal sheets and from cultured cells using an RNaseasy microkit (Qiagen, Valencia, CA) and TRIzol reagent (Invitrogen), respectively, were used for the single-stranded cDNA synthesis. Real time quantitative RT-PCR was performed with the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA), and its on-demand probes for genes of interest were normalized against RPLP0 (ribosomal protein large P0) using an ABI PRISM 7500 Sequence Detector System (Applied Biosystems).

Western Blotting Analysis—Whole skin of mouse ears injected with p53-specific siRNA or control siRNA with or without UVB irradiation and cultured cells treated with 5 μM nutlin-3 (EMD Biosciences, Inc., San Diego, CA), 10 μg/ml 5-fluourouracil (5-FU; EMD Biosciences, Inc.), 10 μg/ml pifithrin-α (PFT; EMD Biosciences, Inc.) or 40 nm siRNA specific for p53 together with Lipofectamine 2000 or TransIT-TKO, in the presence or absence of 10 nm SCF and/or 10 nm ET-1, were washed with phosphate-buffered saline, solubilized in 0.1 ml of radioimmune precipitation buffer (Sigma) supplemented with a protease inhibitor mixture (Roche Applied Science) and then homogenized using ultrasonication and/or a glass homogenizer. The resulting supernatants were collected and recovered as whole cell lysates, and their protein concentrations were determined using the BCA protein assay reagent (Pierce). The whole cell lysates were separated using 7.5, 10, or 15% SDS-polyacrylamide gel electrophoresis and transferred to Sequi-Blot® polyvinylidene difluoride membranes (Bio-Rad). The membranes were then incubated with the primary antibodies specific for p53 (DO-7 (1:1000 dilution) or DO-1 (1:400 dilution)), for p53 phosphorylated at serine 15 (1:1000 dilution), for SCF (1:100 dilution), for p21 (1:200 dilution), for MDM2 (1:200 dilution), for Gadd45α (1:200 dilution) for ERK1 and -2 (MAPK) (Thr(P)185/Tyr(P)187) (1:1000 dilution; Biomol, Plymouth Meeting, PA), for ERK1 and -2 (MAPK) (1:1000; Biomol dilution), for KIT (1:1000 dilution), or for tyrosinase (1.0 μg/ml; Upstate Biotechnology, Inc., Lake Placid, NY). The blots were then washed and incubated with appropriate secondary antibodies (anti-mouse IgG peroxidase-linked F(ab′)2, fragment (1:2500 dilution; GE Healthcare) or anti-rabbit IgG peroxidase-linked F(ab′)2 fragment (1:5000 dilution; GE Healthcare)). Immunoreactive protein bands were visualized with ECL Western blotting detection reagents (GE Healthcare). The amounts of protein loaded were normalized against a control protein, β-actin, using a monoclonal antibody specific for β-actin (1:5000 dilution; Sigma) as an internal standard.

ELISA—NHEKs were seeded in 6-well plates at a density of 1 × 10⁴ to 2 × 10⁵ cells/ml and were treated with 5 μM nutlin-3, 10 μg/ml 5-fluourouracil, or siRNA specific for p53 together with Lipofectamine 2000. The conditioned media were then collected and quantified at 100 μl/well for the levels of ET-1 using an ET-1 ELISA kit (Immuno-Biological Laboratories Co.) according to the manufacturer’s instructions. The standard curve was linear from 78.1 to 5000 pg/ml.

Measurement of Melanin Contents in Three-dimensional HSSs and NHEMs—Three-dimensional HSSs were maintained in EPI-100NM-113 medium (Kurabo Co.) at 37 °C with 5% CO₂, according to the manufacturer’s instructions. To investigate the effects of p53 on paracrine cytokine receptor signaling and melanin synthesis, HSSs were incubated with 10 μM PFT in the presence of 10 nm SCF and ET-1 for 14 days. Media were changed every other day. Photographs of HSSs were taken when cells were harvested. Cells were washed three times with phosphate-buffered saline, 5% (v/v) trichloroacetic acid, and diethyl ether mixed with three volumes of ethanol. Cells were then washed one time with diethyl ether and incubated at 50 °C for 2 h until dry. Cells were solubilized in 200 μl of 2 M NaOH, and melanin contents were measured using an absorbance meter (Microplate Reader model 550; Bio-Rad) at 405 nm and melanin standard (Sigma). To measure the amount of melanin content in human melanocytes, 5 × 10⁴ NHEMs were cultured in the conditioned medium in 6-well plates and transfected with specific siRNA directed against p53 or a control siRNA. After the transfection, the wells were washed three times with phosphate-buffered saline, and cells were solubilized in 200 μl of 2 M NaOH on day 10. Melanin content in NHEMs was measured as described above.
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**RESULTS**

The Expression and Phosphorylation of p53 Is Stimulated in Hyperpigmented Skin Accompanied by Significant Increases in the Expression of Paracrine Melanogenic Cytokines—In order to investigate the potential involvement of p53 in the increased expression of paracrine cytokines from keratinocytes, which might result in the formation of hyperpigmentation, mRNA transcripts or protein levels (and phosphorylations) were examined in pigmented spots, which are frequently observed as benign brownish patches on sun-exposed skin (Fig. 1A). Histologically, those tissues were clearly found to contain melanin granules in the basal and spinous layers of the epidermis accompanied by acanthosis (Fig. 1B). Quantitative real time RT-PCR analysis demonstrated significant increases in mRNA transcript levels of well known melanogenic paracrine cytokines (i.e. SCF, ET-1, and POMC, in the pigmented spots compared with peripheral control areas (Fig. 1C), which is consistent with previous studies (26, 27, 32). In addition, significantly greater expression of p53 (represented by the blue color) was observed in the nuclei of epidermal keratinocytes in pigmented spots compared with peripheral control areas using immunohistochemical analysis (Fig. 2, A and B, and Table 1). Interestingly, higher expression of p53 was also observed in melanocytes in the pigmented spots (Fig. 2A, arrow in the magnified image). Consistent with the higher levels of p53 expression, greater levels were also observed of p53 phosphorylated at serine 15 (data not shown) and at serine 392 (Fig. 2C and D) in the epidermis of hyperpigmented areas.

Additionally, the involvement of p53 in the formation of hyperpigmentation was also evident from the greater expres-
Inhibition of p53 Decreases in the Expression of Genes Involved in Paracrine Cytokine Receptor Signaling, Resulting in a Significant Reduction of Melanin Synthesis in Organ-cultured Pigmented Spots, Three-dimensional HSSs, and/or Mouse Skin—To further examine the role of p53 in the formation of hyperpigmented spots, the expression of melanogenic paracrine cytokines and their receptors and the amounts of melanin present were examined in organ-cultured pigmented spots or in three-dimensional HSSs in the presence of 10 μM PFT. PFT treatment of pigmented skin spots for 72 h significantly inhibited the mRNA expression of SCF and ET-1 compared with untreated controls of the same samples (Fig. 7, A and B). On the other hand, POMC expression was not changed by treatment with PFT in our organ-cultured pigmented spots (data not shown), which contrasts with a previous report that suggested the involvement of p53 in the production of POMC by keratinocytes following UV irradiation (33). In addition to the decreased expression of paracrine cytokines, the mRNA transcript levels of KIT, ET_{A,R}, MC1R (melanocortin-1 receptor), and tyrosinase, which are expressed by melanocytes, were also significantly suppressed by treatment with PFT (Fig. 7, C–F). To investigate the possible involvement of p53 in melanosome transfer to keratinocytes, PAR2 expression was also examined but was not detected (data not shown).

Consistent with the suppressed expression of genes involved in the paracrine cytokine receptor signaling by p53 inhibition in organ-cultured pigmented spots, the melanin contents in three-dimensional HSSs demonstrated a significant inhibition of melanogenesis following treatment with PFT for 14 days compared with the untreated controls (Fig. 8, A and B). Subsequently, we investigated whether inhibition of p53 decreases cytokine-induced melanogenesis, especially in the presence of SCF and ET-1. Interestingly, the remarkable induction of melanin synthesis induced by SCF and ET-1 was abolished by the addition of PFT, indicating the pivotal role of p53 in maintaining hyperpigmentation by regulating paracrine cytokine receptor signaling in melanocytes.

In order to further clarify the pivotal role of p53 in cutaneous hyperpigmentation in vivo via the cytokine receptor signaling, the knockdown experiment with p53-specific siRNA was performed in mouse skin after UVB exposure, since the expression of SCF and ET-1 has been reported to be remarkably enhanced at the mRNA transcript and protein levels in the earlier and later phases of UVB-induced pigmentation, respectively (23). Intradermal injection of p53-specific siRNA was found to significantly inhibit UVB-induced hyperpigmentation compared with control siRNA injection (Fig. 9A and B). Additionally, Western blotting analyses clearly illustrated that UV-induced tyrosinase expression was concomitantly suppressed by the administration of p53-specific siRNA as well as a p53-specific siRNA-induced dramatic reduction in p53 and SCF protein expression (Fig. 9C).
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The Significant Role of p53 in the Transmission of Melanogenic Paracrine Cytokine Signaling in NHEMs—The findings that PFT or p53-specific siRNA significantly decrease the mRNA transcript or protein levels of tyrosinase and KIT and melanin synthesis in organ-cultured pigmented spots, mouse skin, and three-dimensional HSSs in the presence of SCF and ET-1 prompted us to investigate the involvement of p53 in melanocytes to maintain enhanced melanogenesis in hyperpigmented skin. Following the confirmation of the phosphorylation of p53 at serine 15 in NHEMs, which peaked 30 min after the addition of SCF and ET-1 (Fig. 10A), Western blotting analysis also indicated that SCF and ET-1 synergistically phosphorylated MAPK in NHEMs, which is consistent with a previous study (11), and only the SCF-induced phosphorylation of MAPK was abolished by p53 deactivation (Fig. 10B). Furthermore, transfection of NHEMs with a p53-specific siRNA was found to abolish the protein expression of p53 as well as tyrosinase and KIT, resulting in a significant decrease in cellular melanin content. (Fig. 10, C and D). Additionally, PFT treatment of NHEMs for 24 h also inhibited the mRNA or protein levels of tyrosinase and MITF (microphthalmia-associated transcription factor) (Fig. 10, E and F).

DISCUSSION

It has been reported that melanogenesis is stimulated in the basal layer of human epidermis after UV irradiation. Melanin granules are transferred mostly to the upper portion of the cytoplasm of basal and suprabasal keratinocytes in the epidermis, which results in the formation of supranuclear caps that protect the skin against dangerous UV and visible light radiation by directly shielding them and absorbing free radicals generated in the cytoplasm (45–48). Although hyperpigmentation with excess amounts of melanin has been considered a significant psychosocial distress due to its unfavorable appearance, little is known about the detailed mechanisms underlying its formation. Recently, Cui et al. demonstrated the role of p53 in the induction of cutaneous pigmentation after UVB irradiation due to the up-regulation of POMC transcript expression in keratinocytes (33). Given that p53 can be activated by various types of stress, such as sun exposure, inflammation, and aging, and that several cytokines (including α-melanocyte-stimulating hormone derived from POMC) play roles in the induction and maintenance of hyperpigmentation, the potential involvement of p53 activation in paracrine cytokine receptor signaling in those processes, especially in senile lentigines, deserved clarification. There are many similarities between the clinical and histological features, such as a generally high localization in sun-exposed areas, the appearance of epidermal hyperpigmentation, a thicker epidermis, etc., between UVB-melanosis and suprabasal keratinocytes in the epidermis, which results in the formation of supranuclear caps that protect the skin against dangerous UV and visible light radiation by directly shielding them and absorbing free radicals generated in the cytoplasm (45–48). Although hyperpigmentation with excess amounts of melanin has been considered a significant psychosocial distress due to its unfavorable appearance, little is known about the detailed mechanisms underlying its formation. Recently, Cui et al. demonstrated the role of p53 in the induction of cutaneous pigmentation after UVB irradiation due to the up-regulation of POMC transcript expression in keratinocytes (33). Given that p53 can be activated by various types of stress, such as sun exposure, inflammation, and aging, and that several cytokines (including α-melanocyte-stimulating hormone derived from POMC) play roles in the induction and maintenance of hyperpigmentation, the potential involvement of p53 activation in paracrine cytokine receptor signaling in those processes, especially in senile lentigines, deserved clarification. There are many similarities between the clinical and histological features, such as a generally high localization in sun-exposed areas, the appearance of epidermal hyperpigmentation, a thicker epidermis, etc., between UVB-melanosis and senile lentigines (27). In this study, we show for the first time that administration of a p53 inhibitor, PFT, or p53-specific siRNA significantly suppresses the expression of mRNAs and proteins encoding paracrine cytokines (including SCF and ET-1) as well as melanogenic factors (such as KIT, ETBR, MC1R, MITF, and tyrosinase) and results in the inhibition of hyperpigmentation.
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in organ-cultured skin, three-dimensional HSSs, and mouse skin. These data reveal that p53 plays an essential role in the formation of hyperpigmentation by regulating melanogenic paracrine cytokine networks both in keratinocytes and in melanocytes.

One of the most important issues addressed in this study refers to how the p53-mediated pathway acts on keratinocytes to induce and maintain melanogenesis in hyperpigmentation in addition to the well known role of cutaneous keratinocytes in modulating UV-induced apoptosis (49). As expected, quantitative real time RT-PCR, Western blotting, and immunohistological analyses demonstrated that the greater expression and phosphorylation of p53 (at least at serine 15 and serine 392) are present in epidermal cells, including keratinocytes and melanocytes, in pigmented spot areas. They further showed that the inhibition of p53 results in decreased melanin synthesis accompanied by the suppressed expression of the melanogenic paracrine cytokines, SCF and ET-1, in organ-cultured pigmented spots, three-dimensional HSSs, and/or mouse skin. However, the increased expression of POMC in hyperpigmented skin was not affected by the inhibition of p53 by PFT in our pigmented spot organ culture system, whereas the essential role of POMC and its dependence on p53 function in UVB-induced pigmentation have been well documented by the UV sensitivity of subjects who have mutations either in POMC or in its receptor MC1R (33, 50, 51). Recently, Saito et al. (52) demonstrated that UV irradiation induced a more robust and prolonged p53 phosphorylation at various sites (including serine 15 and serine 392), which reached a maximum within 24 h and lasted for about 96 h after UV exposure in human lung carcinoma cells. Consistent

FIGURE 5. Increased expression of p53 promotes the expression of melanogenic paracrine cytokines in NHEKs. A, NHEKs were treated with or without 5 μM nutlin-3 for the indicated times. Total RNAs were extracted from the cells, and gene expression was analyzed by quantitative RT-PCR using the TaqMan gene expression assay. The mRNA expression of genes of interest was normalized against the expression of RPLP0 mRNA. The relative amount of mRNA expression in the treated cells is expressed as a ratio against that of the control without any treatment. Values represent means ± S.D. from three samples. **, p < 0.01; *, p < 0.05 (ANOVA, Dunnett). B, NHEKs were treated with or without 10 μg/ml 5-FU for the indicated times. Total RNAs extracted from the cells were used for gene expression analysis as described above. Values represent means ± S.D. from three samples. **, p < 0.01; *, p < 0.05 (ANOVA, Dunnett). C, NHEKs were treated with or without 5 μM nutlin-3. After treatment for the times indicated, cells were harvested for Western blotting analysis using SCF-specific, p21-specific, or MDM2-specific antibodies. Blots were reprobed with a β-actin-specific antibody as a loading control.

FIGURE 6. Inhibition of p53 expression decreases the expression of melanogenic paracrine cytokines in NHEKs in a dose-dependent manner. A, NHEKs were transfected with p53-specific or nonspecific siRNAs. Seventy-two hours after the transfection, cells were harvested for Western blotting analyses using p53-specific (DO-7), SCF-specific, p21-specific, or MDM2-specific antibodies. Blots were reprobed with a β-actin-specific antibody as a loading control. B, NHEKs were transfected as described under "Experimental Procedures." Conditioned media were collected and quantified for the levels of ET-1 by ELISA as detailed under "Experimental Procedures." Values represent means ± S.D. from three samples. **, p < 0.01; *, p < 0.05 (ANOVA, Dunnett). C, NHEKs were transfected as described under "Experimental Procedures." Forty-eight hours after the transfection, total RNA was extracted from the cells, and gene expression was analyzed by quantitative RT-PCR using the TaqMan Gene Expression Assay. The mRNA expression of SCF was normalized against the expression of RPLP0 mRNA. The amount of SCF mRNA expression in cells treated with the p53-specific siRNA is expressed relative to that of control cells treated with the nonspecific siRNA. Values represent means ± S.D. from three samples. **, p < 0.01; *, p < 0.05 (ANOVA, Bonferroni).
with this, our immunohistologic analysis of pigmented spots found greater phosphorylation of p53 at serine 15 and serine 392, which have been reported to be essential to stabilize p53 (53, 54) and to regulate the oligomerization state of p53 (55) and its ability to bind DNA in a sequence-specific manner (56, 57). Although it has been reported that the expression of POMC is stimulated following UVB irradiation both in keratinocytes and in melanocytes in the skin to induce pigmentation and that POMC expression is also increased in pigmented spots (senile lentigo) (13, 15, 32, 58–63), this does not necessarily involve p53 in the stimulation of POMC production. This is consistent with a previous report that the induction of POMC expression following UV exposure is mediated by p38 stress-activated kinase signaling to the transcription factor USF-1 in melanocytes (64). On the other hand, the increased expression of SCF and ET-1 in pigmented spots was found to be dependent on the activation of p53 in this study. We clearly demonstrated that the induction or suppression of p53 expression by nutlin-3 or 5-FU or by a p53 siRNA or PFT, respectively, significantly increased or decreased the expression of SCF and ET-1 in keratinocytes. In addition, the conspicuously enhanced melanogenesis in three-dimensional HSSs in the presence of SCF and ET-1 was abolished by p53 deactivation. Consistently, UVB-induced pigmentation where the up-regulated expression of SCF and ET-1 contributed was significantly inhibited by the administration of p53-specific siRNA in mouse skin. These findings led us to investigate the impact of p53 on the transmission of paracrine cytokine signals as well as the expression regulation of melanogenic factors in melanocytes. We first investigated whether the action of paracrine cytokines, especially SCF and/or ET-1, whose greater expression in hyperpigmented skin was regulated by p53, was transmitted by p53 in NHEMs. Western blotting analysis clearly showed that they triggered the phosphorylation of p53 and MAPK and that SCF-induced phosphorylation of MAPK was abolished by p53 inhibition in NHEMs, which is consistent with a previous study showing the activation of ERK by p53 in carboplatin-induced apoptosis in cervical carcinoma (67). Following this finding, Western blotting and quantitative real time RT-PCR analyses revealed that treatment of NHEMs with p53-specific siRNA or PFT significantly decreased the mRNA and/or protein expression of the melanocytic master transcriptional regulator MITF, KIT, and tyrosinase, resulting in a significant decrease in melanin synthesis. Consistent with this, previous studies have demonstrated that MITF regulated by SCF/KIT signaling through the MAPK pathway stimulates transcription of the tyrosinase gene in melanocytic cells (68, 69) and that KIT expression is closely linked to MITF by the existence of an E-box in the KIT promoter (70, 71). In addition, it has been reported that the physiologic mechanism underlying the stimulated SCF expression by p53 stabilization causing epidermal melanocytosis (66). Further analysis remains to be done to clarify whether p53 promotes the mRNA transcript expression of ET-1 directly or indirectly in human keratinocytes, because there is no information about whether there is a consensus p53-binding region in the preproendothelin-1 (ET-1 precursor) promoter region. Taken together, these results suggest for the first time that increased expression and phosphorylation of p53 is involved in the enhanced production of SCF and ET-1, which results in hyperpigmentation of the skin.

It would also be very interesting to consider the role of p53 to induce melanin synthesis in melanocytes. In the present study, we found that treatment of organ-cultured pigmented spots with PFT elicited a significant decrease in the mRNA expression of KIT, ETαR, MC1R, and tyrosinase in melanocytes apart from the expression of SCF and ET-1 in keratinocytes. In addition, the conspicuously enhanced melanogenesis in three-dimensional HSSs in the presence of SCF and ET-1 was abolished by p53 deactivation. Consistently, UVB-induced pigmentation where the up-regulated expression of SCF and ET-1 contributed was significantly inhibited by the administration of p53-specific siRNA in mouse skin. These findings led us to investigate the impact of p53 on the transmission of paracrine cytokine signals as well as the expression regulation of melanogenic factors in melanocytes. We first investigated whether the action of paracrine cytokines, especially SCF and/or ET-1, whose greater expression in hyperpigmented skin was regulated by p53, was transmitted by p53 in NHEMs. Western blotting analysis clearly showed that they triggered the phosphorylation of p53 and MAPK and that SCF-induced phosphorylation of MAPK was abolished by p53 inhibition in NHEMs, which is consistent with a previous study showing the activation of ERK by p53 in carboplatin-induced apoptosis in cervical carcinoma (67). Following this finding, Western blotting and quantitative real time RT-PCR analyses revealed that treatment of NHEMs with p53-specific siRNA or PFT significantly decreased the mRNA and/or protein expression of the melanocytic master transcriptional regulator MITF, KIT, and tyrosinase, resulting in a significant decrease in melanin synthesis. Consistent with this, previous studies have demonstrated that MITF regulated by SCF/KIT signaling through the MAPK pathway stimulates transcription of the tyrosinase gene in melanocytic cells (68, 69) and that KIT expression is closely linked to MITF by the existence of an E-box in the KIT promoter (70, 71). In addition, it has been reported that the
activation of p53 by DNA damage is accompanied by stimulation of the melanogenic rate-limiting enzyme tyrosinase gene, although no consensus p53-binding motif has been detected in the tyrosinase promoter (72–75). It remains to be determined how p53 plays a role in the induction and the persistence of enhanced expression of melanogenic factors, including paracrine cytokine receptors, in melanocytes that causes cutaneous hyperpigmentation. However, the mechanisms underlying depigmentation by inhibition of p53 may be explained by the cascade triggered by that inhibition, followed by the suppression of tyrosinase expression due to the decrease in MITF activity accompanied by the inhibition of KIT expression that results in insufficient paracrine cytokine receptor signaling. Apart from its role as a transcription factor, p53 has been documented to directly activate the proapoptotic Bcl-2 protein Bax to permeabilize mitochondria and engage the apoptotic program in a transcription-independent manner (76, 77), which shows the multiple ways that p53 can induce and prolong the hyperpigmentation in human melanocytes as well as in keratinocytes.

In this study, we demonstrated how the p53-mediated pathway acts on paracrine cytokine receptor signaling, which results in the hyperpigmentation of the skin. Increased levels and activation of p53 expression were found in hyperpigmented skin, in concert with significant increases in the expression of paracrine cytokines. More importantly, abrogation of the p53-mediated pathway using the p53 inhibitor, PFT, and/or p53-specific siRNA suppressed their expression in keratinocytes as well as the expression of their receptors and melanogenic factors, including MITF and tyrosinase, in melanocytes, resulting in the depigmentation of organ-cultured hyperpigmented skin, HSSs, and hyperpigmented mouse skin. Considering the harmonized suppressed expression of paracrine cytokines and their receptors in keratinocytes and melanocytes, respectively, by the inhibition of p53, p53 is considered to be a master regulator triggering and maintaining greater activities of paracrine cytokine receptor signaling that causes hyperpigmentation of the skin.
Role of p53 in Hyperpigmentation

FIGURE 10. Inhibition of SCF-induced MAPK phosphorylation significantly decreased the expression of melanogenic factors due to the inhibition of p53 in NHEMs. NHEMs were treated with or without 10 μM PFT for 2 h before the treatment with 10 nM SCF and/or ET-1. Cells were harvested 4352 JOURNAL OF BIOLOGICAL CHEMISTRY

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These findings provide new insights for the fundamental understanding of regulatory mechanisms underlying hyperpigmentation as well as providing a basis to develop an efficient strategy for the treatment of pigmentary skin disorders.

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