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Tyrosinase Gene Expression is Regulated by p53

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Tyrosinase, the rate-limiting enzyme for melanin synthesis, is induced after ultraviolet irradiation as part of the tanning response, the major recognized photoprotective response of human skin. Other DNA-damaging agents and DNA fragments such as thymidine dinucleotides also induce tyrosinase gene expression. Moreover, like ultraviolet light they also activate p53. To determine whether p53 activation is required for this increased tyrosinase expression, we employed two experimental systems: (i) a human melanoma line (WM35) known to express wild-type p53 *versus* WM35 cells engineered to express a transcriptionally inactive dominant-negative p53 (WM35-p53^{DN}) or the empty vector alone (WM35-pCMV7) and (ii) mice with wild-type p53 *versus* p53 knockout mice. In WM35-p53^{DN} cells, the baseline p53 protein level was higher than in WM35 or WM35-pCMV7 cells, and tyrosinase transcripts were lower. After ultraviolet irradiation, in all cell lines the p53 protein level increased within the first 24 h, as expected; and at 24 h tyrosinase mRNA levels were decreased. Consistent with the literature, these data in combination suggest that increased p53 protein level downregulates tyrosinase mRNA. In WM35 and WM35-pCMV7 cells at 48 and 72 h, however, whereas p53 levels remained elevated, tyrosinase mRNA levels compared to pre-irradiation levels tripled, whereas in WM35-p53^{DN} cells levels remained below baseline. In thymidine-dinucleotide-treated WM35 and WM35-pCMV7 cells there was a comparable upregulation of tyrosinase mRNA within 24 h that persisted through 72 h, but there was no upregulation of tyrosinase mRNA in WM35-p53^{DN} cells any time after ultraviolet irradiation or thymidine dinucleotide treatment. In ear skin of p53 wild-type mice, topical application of thymidine dinucleotide induced a 4–5-fold increase in epidermal melanin content after 3 wk, but in p53 knockout mice thymidine dinucleotide application caused no detectable increase in melanin. Together, these data demonstrate that p53 activation increases tyrosinase mRNA level and subsequently pigmentation. The data further suggest that tanning is part of a p53-mediated adaptive response of mammalian skin to DNA damage from ultraviolet irradiation.* *Key words: melanogenesis/photoprotection/pigmentation. J Invest Dermatol 118:126–132, 2002*

Tyrosinase is the rate-limiting enzyme for melanin biosynthesis (Hearing and Jiménez, 1989), the process that largely determines skin color. Tyrosinase gene transcription and enzyme activity are known to be regulated by a number of hormonal, environmental, and pharmacologic agents (Halaban *et al*, 1984; Fuller *et al*, 1987; Friedmann and Gilchrest, 1987; Ranson *et al*, 1988; Bolognia *et al*, 1989; Gordon and Gilchrest, 1989; Abdel-Malek *et al*, 1995; Eller *et al*, 1996; Gilchrest *et al*, 1998), and at least two second messenger systems are involved in melanin biosynthesis: protein kinase C (Gordon and Gilchrest, 1989; Park *et al*, 1993; 1999; Allan *et al*, 1995) and the cyclic adenosine monophosphate (cAMP)–protein kinase A pathway (Pawelek *et al*, 1975; Wong and Pawelek, 1975; Halaban *et al*, 1984).

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Abbreviation: pTpT, thymidine dinucleotide.

Tanning or ultraviolet (UV) induced melanogenesis is a familiar photoprotective response of human skin in which epidermal melanin content gradually increases over several days following a sufficient sun exposure, rendering the skin more resistant to subsequent UV damage (McGregor and Hawk, 1999). Although UV effects on skin are multiple and complex (McGregor and Hawk, 1999), considerable evidence implicates DNA damage or its repair in stimulating tanning (Eller *et al*, 1996; Gilchrest *et al*, 1998; Gilchrest and Eller, 1999). For example, treatment of UV-irradiated pigment cells with T4 endonuclease V, an enzyme known to mediate the first and rate limiting step in repairing thymine dimers and not known to have any other activity, enhances their tanning response to the UV irradiation (Gilchrest *et al*, 1993). Moreover, melanogenesis is enhanced by treatment of skin and skin-derived cells with small DNA oligonucleotides such as thymidine dinucleotides (pTpT), known to enter the nucleus (Hadshiew *et al*, 2001) and presumed to mimic UV-damaged DNA (Eller *et al*, 1994; 1996; Gilchrest and Eller, 1999). Melanogenesis is enhanced also by agents such as UV-mimetic chemotherapeutic drugs and DNA restriction enzymes known specifically to damage DNA (Eller *et al*, 1996). Interestingly, all these agents also upregulate and activate the p53 tumor suppresser protein (Hupp

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et al, 1992; Lane, 1992; Lu and Lane, 1993; Zhan *et al*, 1993; Eller *et al*, 1997; Goukassian *et al*, 1999; Maeda *et al*, 1999), leading to increased DNA binding and transcription of genes whose promoter contains a p53 consensus sequence.

Kichina *et al* (1996) demonstrated that stable transfection of wild-type p53 into a pigmented melanoma line leads to overexpression of wild-type p53 associated with a decrease in the level of tyrosinase mRNA and tyrosinase activity. By transient cotransfection of a p53-expressing plasmid and a chloramphenicol acetyl transferase (CAT) reporter gene linked to a tyrosinase promoter, these authors showed that increasing p53 protein levels specifically repressed CAT gene expression by the tyrosinase promoter, but in this study the p53 activity status was not addressed. Preliminary data from our laboratory[1] suggested a role for p53 activation in upregulating tyrosinase expression, however, and more recently Nylander *et al* (2000) reported activation of a tyrosinase promoter construct in the p53-null human osteosarcoma cell line Saos-2 transfected with a wild-type p53 expression vector, under conditions in which p53 activation was demonstrated using yet another promotor construct. Although they mapped a p53-regulated transcriptional control element near the translation start site of the tyrosinase gene, it was not determined whether p53 directly or indirectly interacts with this site to affect tyrosinase transcription. Moreover, using a cell line of nonmelanocytic origin leaves open the possibility that the observed upregulation of the transfected tyrosinase promoter construct resulted from p53 acting on one or more genes not expressed in melanocytes, thus creating an artifactual connection between p53 and tyrosinase expression.

p53 is a nuclear phosphoprotein that functions as a transcription factor (Farmer *et al*, 1992; Fields and Jang, 1992) and has been shown to directly activate transcription of several genes such as those encoding the growth-arrest and DNA-damage-inducible protein 45 (GADD45) (Kastan *et al*, 1992), the 21 kDa protein (p21/WAF1) (el-Deiry *et al*, 1993), and murine double-minute 2 protein (Wu *et al*, 1993). p53 has also been shown to negatively regulate the promoters of some genes including those encoding Fos (Kley *et al*, 1992), Myc (Moberg *et al*, 1992), interleukin-6 (Santhanam *et al*, 1991), proliferating cell nuclear antigen (Yamaguchi *et al*, 1994; Shivakumar *et al*, 1995; Morris *et al*, 1996), multiple drug resistance 1 (Li *et al*, 1997), retinoblastoma protein (Martin *et al*, 1993; Rolley *et al*, 1995), and heat-shock protein 70 (Agoff *et al*, 1993). These findings indicate that p53 can act as either a transcriptional activator or repressor.

Recent work from our laboratory has shown that the small DNA dinucleotide pTpT upregulates and activates p53 as shown by nuclear translocation and enhanced binding of p53 to its consensus sequence DNA (Eller *et al*, 1997; Goukassian *et al*, 1999) as well as by increased transcription of a human growth hormone gene under control of a p53-regulated promoter (Maeda *et al*, 1999). Also, the genes encoding GADD45, excision-repair cross-complementing 3 (ERCC3), and p21, known to be transcriptionally activated by p53, have been shown to be upregulated by pTpT (Eller *et al*, 1997; Goukassian *et al*, 1999). Moreover, northern blot analysis of p53-null *versus* p53-transfected cells demonstrated that p21 upregulation by pTpT requires p53 (Eller *et al*, 1997). As noted above, tyrosinase gene expression is also induced by treatment of melanocytic cells with pTpT (Eller *et al*, 1994; 1996).

To further elucidate the impact of p53 protein level and activity on tyrosinase expression, we first examined the expression of tyrosinase mRNA in a wild-type p53-expressing melanoma cell line derived from an early primary tumor (WM35), in a cell line derived from WM35 stably transfected to constitutively express a transcriptionally dominant-negative p53 (WM35-p53^{DN}) and in WM35 cells transfected with the plasmid vector alone (WM35-pCMV7) as a control. We present evidence that the tyrosinase gene is regulated by p53 in a dual manner, with p53 protein level negatively regulating tyrosinase transcription, as previously reported (Kichina *et al*, 1996), and activation of p53 increasing tyrosinase mRNA expression. To further confirm the requirement for p53 in pTpT-induced pigmentation in intact skin, p53 knockout mice were compared to p53 wild-type controls in their ability to respond to topical application of pTpT. Only animals with wild-type p53 increased their epidermal melanin content.

[1]Khlgatian M, Asawanonda P, Eller M, Yaar M, Fujita M, Norris DA, Gilchrest BA: Tyrosinase expression is regulated by p53. *J Invest Dermatol* 112:548, 1999 (abstr.)

MATERIALS AND METHODS

Cell lines and culture conditions WM35 cells were derived from an early, radial growth phase primary melanoma (Cornil *et al*, 1991; Florenes *et al*, 1996). These cells contain wild-type p53 and express the protein at a low but detectable level. Mutant p53 cDNA was generated by substitution of a single nucleotide (C to T) at codon 143 resulting in a change of valine to alanine, creating an inactive dominant-negative protein (Baker *et al*, 1990). This cDNA in the pCMV7 plasmid vector was used to permanently transfect WM35 cells (WM35-p53^{DN}) and cells transfected with the vector alone (WM35-pCMV7) were generated as control. All cells were maintained in RPMI 1640 medium supplemented with 5% fetal bovine serum (both from Gibco/BRL, Gaithersburg, MD) at 37°C in 7% CO_2. To maintain selective growth, transfected cell lines (WM35-p53^{DN} and WM35-pCMV7) were always supplemented with medium containing 400 µg per ml geneticin (G418) (Gibco/BRL).

UV irradiation Cells were irradiated in phosphate-buffered saline (PBS) through the plastic culture dish cover with a solar simulator (Spectral Energy Corporation, Westwood, NJ), a protocol that exposes cells to a spectrum of light closely resembling terrestrial sunlight (Werninghaus *et al*, 1991). The 1 kW xenon arc lamp (XMN 1000–21; Optical Radiation, Azuza, CA) irradiance was adjusted to 8×10^{-5} W per cm^2 and metered at 285 ± 5 nm with a research radiometer fitted with a UVB probe (model IL1700 A; International Light, Newburyport, MA) (McGregor and Hawk, 1999) to deliver a dose of 10 mJ per cm^2. Sham-irradiated cultures were handled identically, except that they were shielded with aluminum foil during the irradiation. Cultures were given fresh medium after irradiation.

Cell growth analysis In order to determine the growth properties of the melanoma cells, cultures of each melanoma line were plated in 35 mm dishes at a density of 2×10^4 cells per dish. One day later, duplicate cultures of each line were either irradiated as described above or sham irradiated. Cells were then given fresh medium and collected 24, 48, and 72 h later for cell number as determined by Coulter Counter.

PTpT treatment of cultured cells In previous experiments, 100 µM pTpT consistently elicited UV mimetic responses including melanogenesis (Eller *et al*, 1994), induction of tyrosinase mRNA (Eller *et al*, 1996), and p53 activation/upregulation (Eller *et al*, 1997; Goukassian *et al*, 1999). Melanoma cells were provided with 100 µM pTpT (Midland Certified Reagent, Midland, TX) diluted from a 2 mM stock solution in DME or an equal volume of diluent (DME) alone as control.

PTpT treatment of mouse skin For *in vivo* experiments, a 2 mM stock solution of pTpT in H_2O was prepared and this stock solution or an equal volume of diluent as control was added to propylene glycol (Sigma, St. Louis, MO) to produce a 100 µM solution. C57B1/6 J p53 proficient (+/+) as well as C57B1/6 J p53 knockout (–/–) mice, 10–12 wk of age, were obtained from The Jackson Laboratory (Bar Harbor, MA). pTpT or diluent control was applied to the right and left ear of each mouse, respectively, once a day, 5 d per wk, for 3 wk. Three mice of each genotype were treated. After 3 wk, the animals were euthanized by inhalation of CO_2 and the ears were removed. Four-micron sections were stained with Fontana Masson and evaluated by microscope. Image analysis was performed using an Olympus BH-2 light microscope and a videocamera/computer system (DAGE CCTV, Dage-MTI, Michigan City, IN, with software by Southern Micro Instruments, Atlanta, GA). Statistical analysis was performed using the Student's *t* test.

Western blot analysis Total cellular proteins were collected in a buffer consisting of 0.25 M Tris–HCl (pH 7.5), 0.375 M NaCl, 2.5% sodium deoxycholate, 1% Triton X-100, 25 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mg per ml aprotinin as described previously (Yaar *et al*, 1994). Protein concentration was determined by the Bradford method and 50 µg of protein per lane was processed for Western blot analysis as described previously (Yaar *et al*, 1994). Antibody reactions were performed with a 1:250 dilution of affinity purified mouse monoclonal anti-p53 DO-1 (Ab-6) (Oncogene Science,

Cambridge, MA). Horseradish-peroxidase-linked sheep antimouse IgG was obtained from Amersham Life Science, Arlington Heights, IL. The secondary antibodies were used at 1:2000 dilution. Antibody binding was detected with the ECL detection kit (Amersham), followed by autoradiography (Kodak X-Omatic AR film).

Northern blot analysis Total RNA was isolated from cells with Tri-Reagent (Gibco/BRL) following the protocol of the manufacturer. RNA concentrations were determined by absorbance at 260 nm. The ratio of 260/280 was always between 1.8 and 2.0. Fifteen micrograms of RNA from each sample were electrophoresed on 1% agarose gels containing 2.2 M formaldehyde as described previously (Yaar *et al*, 1991). RNA was transferred to nylon membranes (Hybond-N, Amersham), and immobilized by shortwave UV irradiation (UV-Stratalinker 1800, Stratagene, La Jolla, CA). Blots were hybridized with ^{32}P-labeled human tyrosinase cDNA (Pmel 34), a kind gift from Dr. B. Kwon (Guthrie Research Institute, Sayre, PA); p21 cDNA, a kind gift from Dr. J. Smith (Center on Aging, Baylor College of Medicine, Houston, TX); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (ATCC #57090) as a loading control.

Densitometric analysis Autoradiographs of Western and northern blots were scanned (Microtek Scan Maker II) into a computer (Massachusetts Engineering). Band intensity was determined after background subtraction using the densitometric program Sigma Gel (Jandel Scientific). Bands of interest were normalized using Coomassie Blue stained gels (Western blots) or GAPDH band intensity (northern blots).

Statistical analysis ANOVA with Fisher *post hoc* analysis was used for the data in **Figs 1** and **3**. Repeated measures univariate ANOVA (Huynh-Feldt) (SPSS version 9) was used for the data shown in **Fig 2**.

RESULTS

Tyrosinase mRNA and p53 protein in WM35 melanoma cells To determine the constitutive p53 protein level in these melanoma cell lines, total cellular proteins were analyzed by Western blotting, using an antibody that recognizes mutant as well as wild-type p53 protein (Cohen *et al*, 1998). Western blot analysis showed that the constitutive p53 level was approximately twice as high in cells transfected with the dominant-negative p53 cDNA than in the parental cell line or cells transfected with the empty vector (**Fig 1*A*, *B***). To determine the constitutive level of tyrosinase mRNA, parental and transfected cell lines were processed for northern blot analysis. Densitometric analysis revealed that the tyrosinase mRNA level was higher in the parental ($p < 0.04$, ANOVA *post hoc* analysis by Fisher) and vector-transfected control cell lines ($p < 0.012$) than in the WM35-p53^{DN} cell line that overexpresses p53 (**Fig 1*C*, *D***) by approximately 2–3-fold. Thus, in these cells, in agreement with the report of Kichina *et al* (1996), tyrosinase mRNA is inversely related to the level of p53 protein.

Regulation of p53 in WM35 melanoma cells by UV Parental and transfected melanoma cell lines were sham or UV irradiated and harvested at intervals up to 72 h after irradiation. Consistent with reports using other cell lines and different UV doses (Lu and Lane, 1993; Yamaizumi and Sugano, 1994; Medrano *et al*, 1995; Maki and Howley, 1997), Western blot analysis showed an increase in the level of p53 in all irradiated melanoma lines ($p < 0.001$) (**Fig 2*A*, *B***). Compared to baseline, maximal p53 increases of 300% to 400% were observed at 48–72 h after UV irradiation (**Fig 2*B***). The level of p53 in the sham-irradiated cultures did not change during this 72 h period.

Induction of tyrosinase mRNA by active p53 Under basal conditions in normal cells, p53-driven transcriptional activity is low (Rogel *et al*, 1995), consistent with the low level of p53 protein (Oren *et al*, 1981). Because p53 activation transcriptionally upregulates the p21 gene (Halaban *et al*, 1983; Kastan *et al*, 1992; el-Deiry *et al*, 1993; 1995), increased p21 mRNA after UV irradiation was used as an assessment of p53 activity. Melanoma cell lines were sham or UV irradiated and p21 gene expression was analyzed by northern blotting. WM35 and WM35-pCMV7 cells expressing functional p53 approximately tripled their p21 mRNA levels as early as 24 h after UV irradiation and the increases persisted through 72 h, but there was no increase in the low baseline level of p21 mRNA in the WM35-p53^{DN} cells, further demonstrating the p53^{DN} phenotype of these cells (**Fig 3*A*, *B***). These results are consistent with active p53 being necessary for p21 induction but not for constitutive expression, as previously shown in p53-null cells transfected with wild-type p53 or an empty vector control construct (Eller *et al*, 1997).

In order to determine the effect of p53 activation following UV irradiation on tyrosinase mRNA expression, northern blots of the melanoma lines were rehybridized with the tyrosinase probe. As previously reported for either single or multiple exposures in Cloudman S91 melanoma cells and human melanocytes (Aberdam *et al*, 1993; Eller *et al*, 1996), UV irradiation led to an initial decrease of 25%–50% in tyrosinase mRNA levels 24 h after irradiation (**Fig 3*C*, *D***). In melanoma lines expressing activated p53 as determined by the UV-induced transactivation of the p21 gene, however, thereafter there was a progressive increase in tyrosinase mRNA after irradiation, to approximately 250% to 350% of baseline levels at 48 and 72 h, respectively ($p < 0.05$ at both

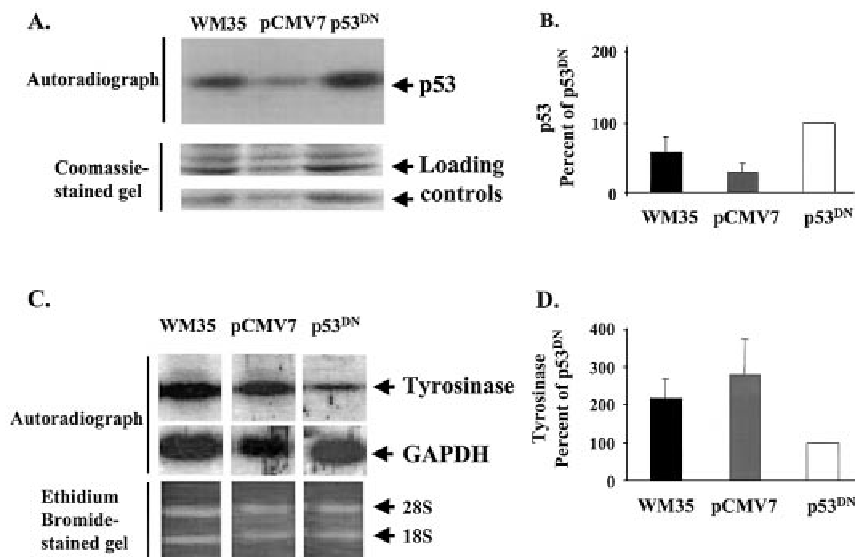

Figure 1. Relationship between p53 and tyrosinase mRNA in WM35 melanoma cells. Duplicate cultures of parental and transfected melanoma cells were plated at a density of 50×10^3 cells in 60 mm dishes and at near confluence (day 7) were harvested and processed for (*A*) Western and (*C*) northern blot analyses. The Western blot membrane was reacted with an anti-p53 antibody (DO-1), and the northern blot was sequentially hybridized with cDNAs for tyrosinase and GAPDH (as a loading control). The ethidium-bromide-stained gel is also shown verifying even loading. The Western and northern blot analyses derive from paired dishes of the same experiment so that relative p53 protein levels and tyrosinase mRNA levels can be directly compared among the different cell lines. Densitometric analyses are for two Western blots (*B*) and three northern blots (*D*), of which (*A*) and (*C*) are representative. For both Western and northern blots, the densitometric value for the p53^{DN} cell line was set at 100% and the other cell lines are represented as a percentage of that value. Measure of variance in (*B*) is standard deviation (SD) and in (*D*) is standard error (SE) of the mean.

times, ANOVA *post hoc* analysis by Fisher). In contrast, in the p53^{DN} cells, no delayed increase in tyrosinase mRNA was observed.

In normal cells, UV irradiation leads to growth arrest (el-Deiry *et al*, 1995), and decreased proliferative rate *per se* may be associated with a more differentiated phenotype. In order to exclude the possibility that increased tyrosinase expression in cells expressing wild-type p53 *versus* dominant-negative p53 was a nonspecific consequence of altered proliferative rate, growth curves were determined for the three cell lines. In all lines, sham-irradiated cultures grew more rapidly than paired UV-irradiated cultures, as expected. The p53^{DN}-transfected cells grew more slowly than controls, however, displaying a doubling time of approximately 36 h *versus* 24 h for controls, excluding more rapid proliferation as an explanation for their reduced tyrosinase expression.

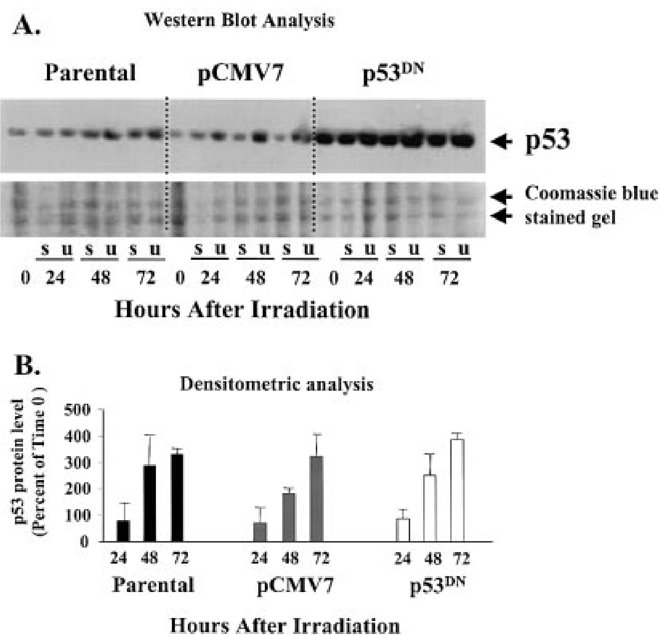

Figure 2. Effect of UV irradiation on p53 levels. Melanoma cell lines were irradiated with a physiologic dose of solar-simulated light or were sham irradiated, as described in *Materials and Methods*. (*A*) Total cellular proteins were collected at time 0 and at intervals up to 72 h after UV irradiation, and 50 μg of protein from each sample were processed for Western blot analysis with an anti-p53 antibody (DO-1). Three different blots are shown, so that quantitative comparison for p53 cannot be made among them. Protein loading was normalized using the mean densitometric intensity for two representative bands (shown) from the Coomassie blue-stained gel in which virtually all visible protein bands showed comparable variability among lanes. (*B*) Densitometric analysis showed that, after UV irradiation, p53 protein levels comparably increased in all melanoma cell lines through 72 h. Time 0 values for each cell line were set at 100% and values for all time points were expressed as a percentage of this value. All sham-irradiated values were ±10% of the time 0 values. In all histograms, SE is the measure of variance.

Induction of tyrosinase mRNA by pTpT in WM35 melanoma cells Previous work in our laboratory has shown that pTpT evokes a variety of photoprotective responses in large part, if not exclusively, by upregulation and activation of p53 (Eller *et al*, 1997; Goukassian *et al*, 1999; Maeda *et al*, 1999). pTpT treatment also leads to delayed increases in tyrosinase mRNA and pigmentation in human melanocytes and S91 Cloudman melanoma cells (Eller *et al*, 1994, 1996). To further confirm that activated p53 induces tyrosinase mRNA expression, and to avoid the complication of the membrane-associated pathways stimulated by UV irradiation that can affect transcription, the parental cells expressing wild-type p53 and p53^{DN}-transfected cells were stimulated with 100 μM pTpT and processed for northern blotting. At 24 and 72 h after pTpT supplementation, the tyrosinase mRNA level doubled in the WM35 melanoma cells that express wild-type activatable p53, whereas tyrosinase mRNA levels remained unchanged in WM35-p53^{DN} cells (**Fig 4*A*, *B***), confirming that activation of p53 is required for tyrosinase mRNA upregulation by pTpT.

Induction of melanogenesis by pTpT *in vivo* We have previously shown that pTpT induces melanogenesis in intact skin, at least in part through increasing tyrosinase mRNA and protein levels (Eller *et al*, 1994). In order to examine the role of p53 in this *in vivo* response, but avoiding the known effects of UV irradiation on cell membranes and on melanocyte proliferation (Gilchrest *et al*, 1998), p53 wild-type (+/+) and p53 knockout (–/–) mice were treated with pTpT and the pigmentation response was compared. Three weeks of five times per week application of pTpT to p53 proficient mice produced a 10-fold increase in epidermal melanin content as determined by confocal microscopy and image analysis (**Fig 5*A***). In contrast, no increase in epidermal melanin content was detected in the p53 (–/–) mice (**Fig 5*A*, *B***). As previously reported (Quevedo *et al*, 1966), there were very few or no melanocytes in murine epidermis, and none were detected by routine histology or by immunostaining for TRP-1 (Mel-5) in either pTpT-treated or vehicle-treated skin (data not shown). This fact, in combination with the growth inhibition rather than the

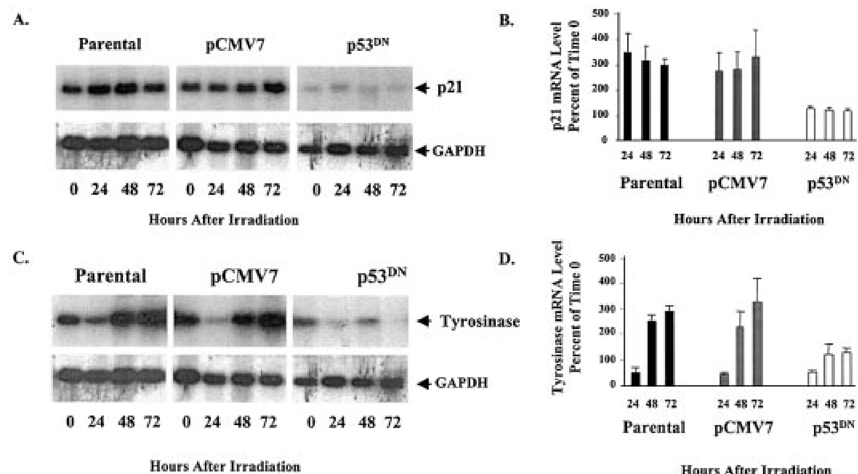

Figure 3. UV induction of p21 and tyrosinase mRNA by p53. Melanoma cell lines were UV irradiated as described in *Materials and Methods*. Cells were collected at time 0 and at intervals up to 72 h after irradiation and were processed for northern blot analysis with sequential hybridization using cDNA probes specific for p21 (*A*), tyrosinase (*C*), and GAPDH (loading control), followed by densitometric analysis. Note that samples from all melanoma lines were processed together in one gel, so that p21, tyrosinase, and GAPDH mRNA levels can be directly compared among them. Time 0 for each cell line was set at 100% and all time points were calculated as a percentage of this value for two separate experiments for p21 (*B*) and three separate experiments for tyrosinase (*D*). In the histogram, the measure of variance for p21 is SD and for tyrosinase is SE.

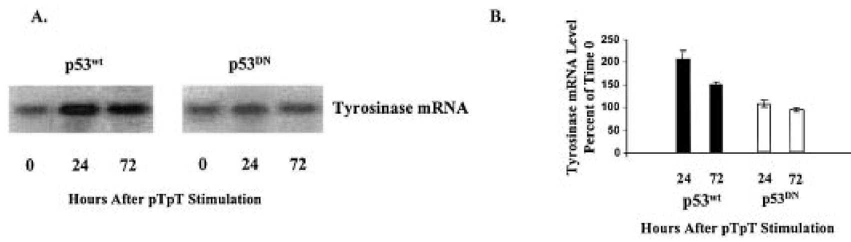

Figure 4. p53 requirement for pTpT-induced tyrosinase mRNA *in vitro*. Parental WM35 cells expressing wild-type p53 ($p53^{wt}$) and cells transfected with dominant-negative p53 ($p53^{DN}$) were supplemented with 100 μM pTpT. Cells were collected at time 0, 24, and 72 h after pTpT addition and total RNA was processed for northern blot analysis (*A*) as detailed in **Fig 1** legend. (*B*) Densitometric analysis was performed. Time 0 values for each cell line were set at 100% and other time points were calculated as a percentage of this value. In the histogram the measure of variance is SD.

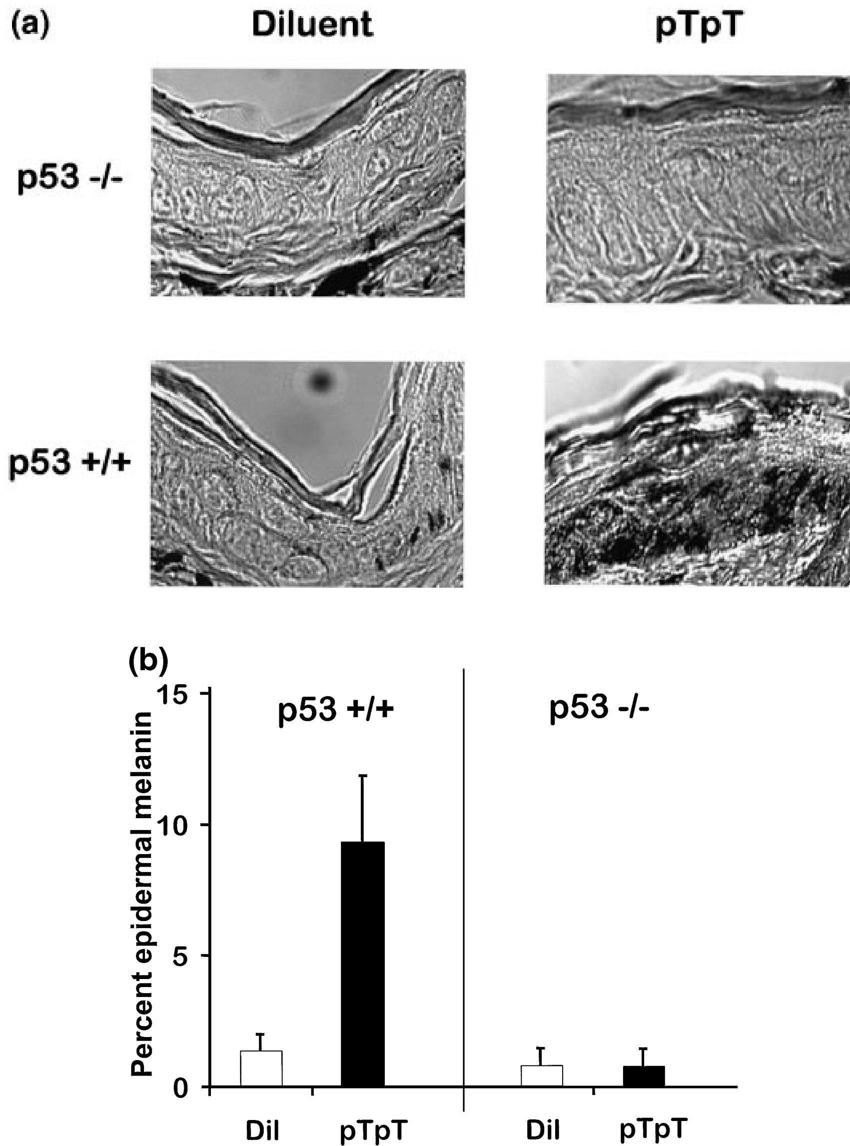

Figure 5. p53 requirement for pTpT-induced melanogenesis *in vivo*. Three p53 (+/+) and three knockout (–/–) mice were treated daily for 3 wk with 100 μM pTpT on one ear and propylene glycol as a diluent control on the other. Ear sections were stained with Fontana Masson to highlight melanin and then observed by confocal microscopy. Representative cross-sections of epidermis (*A*). The epidermal melanin content was determined by image analysis as described in *Materials and Methods* and graphed as mean ± SE (*B*).

stimulation observed after pTpT treatment of cultured cells (Eller *et al*, 1996), strongly suggests that the increased melanin content in pTpT-treated p53 +/+ murine skin is fully attributable to increased tyrosinase mRNA and protein levels, as *in vitro* (Eller *et al*, 1994), rather than to an increased number of melanocytes; and that the lack of melanin increase in pTpT-treated p53 –/– murine skin is due to lack of p53 upregulation of tyrosinase gene expression.

DISCUSSION

Tyrosinase is the rate-limiting enzyme for production of melanin (Hearing and Jiménez, 1989), a brown-black polymer that protects against UV-induced damage (Noda *et al*, 1993) by absorbing the UV photons and UV-induced free radicals before they can interact with cellular constituents (McGregor and Hawk, 1999). Both

tyrosinase activity and melanin content of skin increase 3–5 d after sufficient sun exposure (Kostanecki *et al*, 1976), a phenomenon termed delayed tanning. Of note, X-irradiation and certain DNA-damaging chemotherapeutic agents also induce tanning-like hyperpigmentation (Gilchrest and Eller, 1999).

Regulation of tyrosinase activity is complex but appears to occur in part at the level of gene transcription. The promoter of the human tyrosinase gene has been sequenced, and the potential regions regulating tyrosinase gene expression were determined by transient transfection with a CAT readout gene driven by tyrosinase promoters with various deletions (Ponnazhagan *et al*, 1994). Sequence analysis of the 2 kbp tyrosinase promoter revealed a TATA box 27 bp upstream from the transcription start site and a CAAT box 128 bp upstream. In addition, there were five putative AP-1 and two AP-2 sites, two glucocorticoid-responsive elements, three Oct-1 sites, and two UV-responsive elements. In addition, the tyrosinase promoter contains an ''M box'', a conserved element found in many melanocyte-specific genes and a known binding site for the microphthalmia-associated transcription factor (MITF), known to activate transcription of the tyrosinase gene (Bentley *et al*, 1994; Yasumoto *et al*, 1994). MITF can be induced by cAMP (Price *et al*, 1998), which can increase tyrosinase mRNA levels (Halaban *et al*, 1984). It is not known whether p53 regulates MITF expression but no p53 consensus sequence has been identified in the MITF promoter. Deletion mutations of the tyrosinase promoter region have shown that regions as small as 550 bp are active, albeit less than the full-length promoter (Ponnazhagan *et al*, 1994), suggesting that tyrosinase gene transcription is controlled by multiple regulatory elements, which in turn may be activated by different factors.

Our study establishes that high levels of p53 protein down-regulate tyrosinase mRNA, whereas transcriptionally active p53 upregulates tyrosinase mRNA. The lack of an identified p53 consensus sequence in the tyrosinase promoter (Bargonnetti *et al*, 1991) suggests that this regulation is indirect. For example, the initial downregulation of tyrosinase mRNA following p53 transfection of melanoma cells (Kichina *et al*, 1996) may result from p53 binding to the TATA-binding protein (Kern *et al*, 1991; Aoyama *et al*, 1992; Seto *et al*, 1992; Mack *et al*, 1993; Park *et al*, 1996), reducing the basal transcription rate; whereas the delayed tyrosinase upregulation may result from induction of one or more intermediary gene products by p53. Our data in a human melanoma line and in murine melanocytes *in vivo* validate the conclusions of Nylander *et al* (2000) in Saos-2 cells that p53 activation upregulates tyrosinase by demonstrating that this p53 effect is not an artifact of working with a nonmelanocyte cell line that might possibly express irrelevant p53-regulated gene products capable of modifying tyrosinase expression.

Regardless of the precise molecular mechanisms, our data demonstrate that p53 protein negatively regulates tyrosinase gene transcription, whereas activation of p53 increases tyrosinase mRNA level. Our results further demonstrate the essential role of p53 even in intact skin in the induction of pigmentation by pTpT, selected as the test agent because it appears to act by mimicking DNA damage specifically (Gilchrest and Eller, 1999), in contrast to UV irradiation that clearly activates cell membrane-associated signal transduction pathways (Rosette and Karin, 1996; Gilchrest *et al*, 1998) as well as nuclear pathways involving p53. In the context of previous studies (Eller *et al*, 1994; 1996; 1997; Goukassian *et al*, 1999), these data suggest that tanning should now be included in the broad array of DNA damage-induced p53-mediated adaptive differentiation responses that protect mammalian cells during subsequent exposure to DNA-damaging agents such as UV irradiation.

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