**Short Communication**

p53 Involvement in the Control of Murine Hair Follicle Regression

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p53 is a transcription factor mediating a variety of biological responses including apoptotic cell death. p53 was recently shown to control apoptosis in the hair follicle induced by ionizing radiation and chemotherapy, but its role in the apoptosis-driven physiological hair follicle regression (catagen) remains to be elucidated. Here, we show that p53 protein is strongly expressed and co-localized with apoptotic markers in the regressing hair follicle compartments during catagen. In contrast to wild-type mice, p53 knockout mice show significant retardation of catagen accompanied by significant decrease in the number of apoptotic cells in the hair matrix. Furthermore, p53 null hair follicles are characterized by alterations in the expression of markers that are encoded by p53 target genes and are implicated in the control of catagen (Bax, Bcl-2, insulin-like growth factor binding protein-3). These data suggest that p53 is involved in the control of apoptosis in the hair follicle during physiological regression and imply that p53 antagonists may be useful for the management of hair growth disorders characterized by premature entry into catagen, such as androgenetic alopecia, alopecia areata, and telogen effluvium. (Am J Pathol 2001, 158:1913–1919)

During postnatal development, the hair follicle (HF) shows cyclic activity with periods of active growth (anagen), involution (catagen), and resting (telogen).1–6 Every stage of the hair cycle is characterized by different intensity of the signaling exchange between HF keratinocytes and dermal papilla fibroblasts, which is maximal in anagen HFs and minimal during telogen.5,6

In anagen HFs, dermal papilla fibroblasts secrete numerous growth factors that maintain active proliferation and differentiation of keratinocytes of the proximal hair bulb leading to the hair fiber formation.4–6 HF transition from anagen to catagen is associated with a sudden decline in secretion of growth factors by the dermal papilla resulting in the dramatic reduction of proliferation and differentiation of hair matrix keratinocytes with cessation of hair shaft production. This is accompanied by massive apoptosis in the proximal HF epithelium and shortening of HF length by up to 70% during its transition from anagen to telogen.7–10

Increasing evidence suggests that apoptosis during HF involution is well coordinated and regulated differentially in each distinct HF compartment. It was shown that during catagen, the HF outer root sheath, inner root sheath, and hair matrix show distinct expression patterns for growth factor receptors [p55-kd tumor necrosis factor receptor (p55TNFR), p75-kd neurotrophin receptor (p75NTR), Fas/Apo-1, transforming growth factor-β receptor type II] that mediate apoptosis.10–12 In particular, neurotrophins and transforming growth factor-β1 were implicated in apoptosis control and shortening of the outer root sheath during catagen.11,12

p53 is a transcription factor that mediates apoptosis after a variety of stresses including growth factor withdrawal and DNA damage.13–15 During the cellular reaction to stress, p53 alters expression of multiple p53-responsive genes [Fas, Fas-ligand, Bax, Bcl-2, insulin-like growth factor binding protein-3 (IGF-BP3), insulin-like growth factor I receptor (IGF-IR)], the activity of which is responsible for cell survival or apoptotic death.16–21 Interestingly, proteins whose transcription is encoded by

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the genes listed above are all expressed in the HF during anagen-catagen transition.\textsuperscript{10,11,22}

Recently we showed that p53 is essential for the HF response to DNA damage induced by chemotherapy. Specifically, we reported that HFs in p53 knockout mice show neither apoptosis nor hair loss and instead continue active hair production after administration of cyclophosphamide.\textsuperscript{23} However, a possible role for p53 in mediating apoptosis in the HF during spontaneous HF anagen-catagen-telogen transition remains to be elucidated. In this article, we analyze distribution of p53 and its co-localization with apoptotic markers during catagen in C57BL/6 mice and compare the dynamics of apoptosis-driven HF regression between wild-type and p53 knockout mice. Furthermore, we define putative mechanisms of p53 involvement in catagen control by comparing the expression of p53 targets (Bax, Bcl-2, Fas, IGF-BP3) between wild-type and p53 null HFs. Taken together, our data suggest involvement of p53 in the control of apoptosis during catagen in the normal hair cycle.

Materials and Methods

Animal Models and Tissue Collection

Eight-week-old C57BL/6 female mice ($n = 10$), 8- to 10-week-old p53 knockout mice ($n = 6$), and wild-type mice ($n = 6$) were purchased from Charles River (Boston, MA) and Jackson Laboratory (Bar Harbor, ME). p53 knockout mice generated on C57BL/6 background were viable, showed apparently normal fur, and developed spontaneous tumors 12 to 18 weeks after birth.\textsuperscript{24} We, after the end of the experiment. Mice were housed in community cages at the animal facilities of the Boston University School of Medicine and University of Illinois at Chicago. All mice were fed water and murine chow \textit{ad libitum}, and were kept under 12-hour light/dark cycles. Active hair growth (anagen) was induced in the back skin by application of the wax-resin mixture with subsequent depilation, as described before.\textsuperscript{25,26} Skin samples were harvested at days 17 to 20 after depilation, ie, during catagen. Harvesting of skin and cryosectioning were performed using a special technique to obtain longitudinal sections of the HFs, as described previously.\textsuperscript{27}

Immunohistochemistry and Terminal dUTP Nick-End Labeling (TUNEL) Technique

Expression of p53 protein, p55TNFR, IGF-R1, and IGF-BP3 were assessed using rabbit polyclonal antiserum against murine p53 (Novocastra, Newcastle-upon-Tyne, UK), rat monoclonal antibody against murine p55TNFR (Pharmingen, San Diego, CA), and rabbit polyclonal antisera against IGF-R1 and IGF-BP3 (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. The tyramide amplification method was used for the immunovisualization of these antigens, as described previously.\textsuperscript{28} Briefly, after blocking of endogenous peroxidase and nonspecific avidin/biotin binding, sections were incubated in TNB buffer (Dupont-NEN, Boston, MA) followed by application of one of the corresponding primary antisera listed above (1:1000) overnight. Then the corresponding biotinylated goat anti-rabbit or goat anti-rat antisera (1:200, 30 minutes), diluted in TNB blocking buffer (Dupont-NEN), were applied. The reaction product was developed using a commercial tyramide amplification kit (Dupont-NEN). Sections were incubated in streptavidin-horseradish peroxidase (1:100 in TNB, 30 minutes), washed with TTN buffer (Dupont-NEN), followed by a 10-minute application of TRITC-tetramethyl-rodamine-isothio-cyanate-tyramide (1:50 in Amplification Diluent, Dupont-NEN). Sections were washed three times with Tris buffer, and then counterstained by TO-PRO3-iodide for visualization of cell nuclei. Cyclophosphamide-treated murine skin samples with high p53 levels\textsuperscript{23} were used as a positive control for p53 immunostaining, and skin samples of p53 knockout mice were used as a negative control. Double immunovisualization of p53 and TUNEL was performed, as described previously.\textsuperscript{29,30} Immunodetection of Fas, p75NTR, Bax, and Bcl-2 was performed using corresponding monoclonal antibodies and the avidin-biotin-alkaline phosphatase method, as described previously.\textsuperscript{10,28}

Histomorphometry

Immunoreactivity patterns were scrutinized by studying at least 50 different HFs per mouse, and five mice were assessed per hair-cycle stage. For the precise identification of the defined stages of HF cycling, histochemical detection of endogenous alkaline phosphatase activity was used as described, because this allows visualization of the morphology of dermal papilla as a useful morphological marker for hair-cycle staging.\textsuperscript{31} The percentage of HFs in different catagen stages was assessed and calculated in p53 knockout ($/-/-$) and their corresponding age-matched wild-type mice. All evaluations were performed on the basis of accepted morphological criteria for HF classification.\textsuperscript{2,5,7} Only every tenth cryosection was used for analysis to exclude the repetitive evaluation of the same HF, and 2 to 3 cryosections were assessed from each animal. All together, 250 to 300 HFs in 50 to 60 microscopic fields derived from six animals (40 to 50 follicles per animal) of distinct age were analyzed and compared to that of a corresponding number of HFs from the appropriate, age-matched wild-type mice. The distance between the stratum corneum and the subcutis/panniculus carnosus border was measured for assessing the skin thickness in p53 knockout and wild-type animals 18 and 20 days after depilation. In total, 40 to 50 such measurements were performed in 50 to 60 microscopic fields derived from three animals per mutant and wild-type group. Number of TUNEL-positive cells was assessed in the hair matrix of catagen I-IIH HFs in p53 knockout and wild-type animals. In total, 40 to 50 such measurements were performed in 50 to 60 microscopic fields derived from three animals per mutant and wild-type group. All sections were analyzed at $\times 200$ to $\times 400$ magnification, and means and SEM were calculated from pooled data. Differences were judged as significant if the
Results p53 Protein is Up-Regulated and Co-Localized with Apoptotic Cells in the HF during Catagen

To analyze p53 protein localization and co-localization with apoptotic cells in the regressing HF compartments during anagen-catagen-telogen transition, double immunovisualization of p53 and TUNEL was performed on skin cryosections (8-µm thickness) of 8-week-old C57BL/6 mice 15 to 19 days after hair cycle induction by depilation. Cyclophosphamide-treated C57BL/6 mouse skin and skin samples of p53 null mice were used as positive and negative controls for p53 immunostaining, respectively. Immunovisualization of p53 and TUNEL was performed on skin cryosections (8-µm thickness) of 8-week-old C57BL/6 mice 15 to 19 days after hair cycle induction by depilation. The observed patterns of p53 immunoreactivity and TUNEL are documented by the representative examples in Figure 1, A to F, and are schematically summarized in Figure 1G. Positive and negative controls for p53 immunostaining are shown in Figure 1, H and I.

In anagen VI HFs, p53 immunoreactivity was seen in the proximal outer and inner root sheaths and dermal papilla (Figure 1, A and G). Relatively weak p53 immunostaining was also found in the hair matrix of anagen VI.
HFs (Figure 1A). During HF transition from anagen VI to catagen II stage, p53 protein was increased in the proximal outer and inner root sheaths and in the hair matrix keratinocytes closely adjacent to the dermal papilla (Figure 1, B and G). In catagen III HFs, a further increase of p53 immunoreactivity was found in the proximal outer and inner root sheaths, perifollicular connective tissue sheath, and in the hair matrix (Figure 1, C and G). Co-localization of p53 protein and TUNEL was also observed in the hair matrix of catagen III-IV HFs (Figure 1, C and D). In catagen IV-VI HFs, p53 immunoreactivity was detected in the regressing outer and inner root sheaths perifollicular connective tissue sheath, whereas dermal papilla became p53-negative (Figure 1; D, E, and G). During catagen VII-VIII, prominent p53 immunostaining and p53 co-localization with TUNEL was seen in the outer root sheath and bulge (Figure 1F). In addition, p53 protein was found in the arrector pili muscle, dermal cells, and in the epidermis (Figure 1F).

Taken together, these phenomenological data suggest that p53 protein plays a role in the control of apoptosis-driven HF regression. This would be consistent with recent reports that p53 mediates apoptosis induced by growth factor withdrawal in different model systems.33–35 We therefore further explored the role of p53 in catagen control in functional assays.

Deletion of p53 Leads to the Retardation of HF Regression

To define whether constitutive p53 deletion alters spontaneous catagen development, postnatal p53 knockout (−/−) mice24 were compared for the speed of spontaneous HF regression after hair-cycle induction by depilation, using age-matched wild-type mice as a control. p53 knockout mice showed a significant retardation of catagen development (P < 0.05), compared to wild-type controls. At day 18 after depilation, ~80% of the HFs in p53 null skin were still at catagen I-II, while more than half of the HFs in wild-type skin were already at catagen III-V (Figure 2, A–C). In addition, as an important indicator of advanced catagen development,36,37 skin thickness was significantly reduced (P < 0.05) in wild-type mice (438.1 ± 54.5 μm), compared to p53 mutants (594.7 ± 43.1 μm; Figure 2, B and C). Furthermore, at day 20 after depilation, all HFs in wild-type skin were already at late catagen stages (catagen VII-VIII), while ~50% of the HFs in p53 null skin were still at catagen IV-VI (Figure 2, D and E).

Importantly, catagen II HFs in p53 knockouts, compared to wild-type mice, were characterized by the significantly reduced (P < 0.05) number of TUNEL-positive cells in the hair matrix (Figure 2, F–H), whereas number of TUNEL-positive cells in the outer and inner root sheaths appeared to be unaltered (data not shown). This suggests that deletion of p53 is associated with a significant retardation of catagen development, and with specific reduction of apoptosis in the hair matrix, where p53 was found to be co-localized with TUNEL (Figure 1C).

Catagen HFs in p53-Deficient Mice Are Characterized by Down-Regulation of Bax and Insulin-Like Growth Factor Binding Protein-3 and by Increase of Bcl-2

To identify factors that might contribute to the catagen retardation observed in p53 knockout mice, we compared the expression of number of molecules (Bcl-2, Bax, Fas, IGF-BP3, IGF-R1, p55-kd TNFR, p75-kd neurotro-
and in hair matrix, and was relatively unchanged in p53 null HFs, compared to that of wild-type mice (data not shown). The immunoreactivity patterns of p75NTR and p55TNFR also showed no differences between wild-type and p53 null HFs (not shown).

**Discussion**

HF transition from anagen to telogen is a physiological process of programmed organ involution, which is characterized by tightly coordinated apoptosis in the cyclic portion of the HF epithelium.\(^9\)\(^{-}\)\(^6\) During spontaneous HF regression, apoptosis first occurs in the HF matrix keratinocytes closely adjacent to the dermal papilla, and then, in more advanced catagen stages, is seen in the regressing outer and inner root sheaths and in the epithelial strand.\(^10\) This leads to the rapid shortening of cyclic portion of the HF associated with dermal papilla relocation from the subcutis to the dermis/subcutis border, where it contacts secondary hair germ and bulge.

In this article, we show that p53 plays a role in apoptosis during HF regression: p53 protein is increased in the regressing HF compartments during catagen (Figure 1), and p53 knockout mice are characterized by significant catagen retardation, compared to wild-type mice (Figure 2). Furthermore, we show that compared to the wild-type controls, p53-deficient HFs display a decrease in Bax and IGF-BP3, and an increase of Bcl-2 proteins (Figure 3, A–D).

Numerous indications suggest that p53 mediates apoptosis followed by growth factor withdrawal without DNA damage in hemopoietic cells and neurons.\(^33\)\(^{-}\)\(^35\),\(^40\) During anagen, dermal papilla fibroblasts secrete numerous growth factors (IGF-I, keratinocyte growth factor, stem cell factor, hepatocyte growth factor/scatter factor) maintaining active proliferation and differentiation in hair matrix keratinocytes.\(^39\),\(^41\)\(^{-}\)\(^45\) Because HF transition from anagen to catagen is characterized by the decline in secretion of growth factors by dermal papilla for hair matrix keratinocytes,\(^3\)\(^{-}\)\(^6\) we speculate that p53 may serve as a candidate molecule mediating apoptosis in hair matrix keratinocytes after growth factor withdrawal.

It was reported previously that steady-state levels of the p53 transcripts are found in murine skin during HF anagen-catagen-telogen transition.\(^9\) In other models, it was demonstrated that during apoptotic cell death, p53 protein is dramatically up-regulated mostly because of the posttranslational regulation.\(^46\) We show here that p53 protein is increased in hair matrix keratinocytes closely adjacent to the dermal papilla in catagen II HFs (Figure 1B) and that p53 is co-localized with TUNEL in the hair matrix during catagen III (Figure 1C). We also show that, in contrast to wild-type HFs, hair matrix of p53 null catagen II-III HFs is characterized by a significant decline of TUNEL-positive cells (Figure 2, F–H), as well as by down-regulation of Bax and up-regulation of Bcl-2 (Figure 3, A–D). Both Bax and Bcl-2 proteins are encoded by genes, where expression is regulated by p53.\(^38\),\(^39\) Other
data indicate that during catagen hair matrix keratinocytes do not show pro-apoptotic Fas or p75NTR and are characterized by relatively weak immunoreactivity for p53. Together these observations suggest that p53 may indeed mediate early steps of growth factor withdrawal-induced apoptosis in hair matrix keratinocytes during catagen, most likely via an increasing Bax/Bcl-2 ratio. However, p53 protein is also increased in the proximal outer and inner root sheaths of catagen HF s and we cannot exclude p53 involvement in mediating apoptosis in those HF compartments as well. In the outer root sheath keratinocytes, p53 may mediate apoptosis induced by neurotrophins interacting with p75NTR, in the same way as in neurons. In addition, in the proximal outer and inner root sheaths, p53 may regulate expression of IGF-BP3, which prevents interaction of dermal papilla-derived IGF-I, known by its anagen-promoting activity, with IGF-R1 expressed on hair matrix keratinocytes, thus accelerating HF anagen transition. Indeed, we show strong down-regulation of IGF-BP3 protein in catagen HF s of p53 knockout mice, compared to wild-type HFs (Figure 3, E and F).

Taken together, our data suggest that p53 is required for mediating apoptotic cell death in the HF during physiological HF regression in mice. This suggests p53 as a universal modulator of apoptosis in the HF cells induced either by growth factor withdrawal (in the absence of DNA damage), or after DNA damage induced by ionizing radiation or chemotherapy. It was recently shown that a small molecule acting as a p53 inhibitor efficiently reduces the side effects of ionizing radiation in mice, presumably by suppressing p53-mediated apoptosis. We thus presume that a local pharmacological blockade of p53 by synthetic antagonists may provide a new therapeutic strategy for the management of hair growth disorders with premature entry into catagen, such as androgenetic alopecia, alopecia areata, and telogen effluvium.

References

2. Paus R: Control of the hair cycle and hair diseases as cycling disorders. Curr Opin Dermatol 1996, 3:248–258
dependent hematopoetic cells following IL-3 withdrawal. EMBO J 1994, 13:1368–1374
43. Hibberts NA, Messenger AG, Randall VA: Dermal papilla cells derived from beard hair follicles secrete more stem cell factor (SCF) in culture than scalp cells or dermal fibroblasts. Biochem Biophys Res Commun 1996, 222:401–405