

Hair Follicle Apoptosis and Bcl-2

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Hair follicle (HF) morphogenesis and cycling are characterized by a tightly controlled balance of proliferation, differentiation and apoptosis. The members of the bcl-2 family of proto-oncogenes are important key players in the apoptosis control machinery of most cell types. Bcl-2, an apoptosis inhibitor, and Bax, an apoptosis promoter, show tightly regulated, hair cycle-dependent expression patterns: during catagen, the distal ORS of the HF remains strongly positive for Bcl-2 and Bax; in contrast, the proximal epithelial part of the HF loses most Bcl-2 expression while it remains strongly positive for Bax. In Bcl-2 null mice, skin becomes markedly hypopigmented during the first postnatal anagen probably due to increased melanocyte apoptosis. Reportedly, these mice also show a retardation of the first anagen development after birth. Transgenic mice overexpressing Bcl-2 under the

control of the keratin-1 promoter display multifocal epidermal hyperplasia and aberrant expression of keratin-6, while alterations of HF cycling have not been investigated. Surprisingly, Bcl-2 overexpression under the control of the keratin-14 promoter leads to accelerated catagen progression and increased chemotherapy-induced apoptosis, HF dystrophy and alopecia. Transgenic mice overexpressing Bcl-X(L), another anti-apoptotic bcl-2 family member, under the control of the K14 promoter, reportedly also display accelerated catagen development. These and other Bcl-2 transgenic and null mice are now available to further dissect the as yet unclear, and likely complex, role of Bcl-2 in HF growth and pigmentation. Key words: pigmentation/morphogenesis/transgenic mice/TUNEL/anagen/catagen/Bax/keratin. *Journal of Investigative Dermatology Symposium Proceedings* 4:272-277, 1999

One of the most intriguing features of hair follicle (HF) biology is that this miniorgan undergoes three different processes of pattern formation. First, during HF morphogenesis, two clusters of epithelial and mesenchymal cells develop into a complex fiber production machinery. Second, during HF regression (catagen) this complex miniorgan undergoes a tightly controlled process of organ involution that leads to a dramatic reduction of the follicle length and a reconstruction of a very small HF, which enters into the so-called resting phase (telogen) at the end of this regressive or inverse pattern formation. Third, the HF enters into anagen and starts again to develop into a fiber production machinery (Hardy, 1992; Paus and Cotsarelis, 1999; Stenn *et al*, 1996, 1998; Philpott and Paus, 1998; Paus *et al*, 1999). During these three distinct processes of HF pattern formation, one major principle at work is the balance of epithelial cell proliferation and apoptosis.

Apoptosis or programmed cell death, in which cells commit suicide by fragmenting themselves into membrane-packed bits, is

one of the hottest topics in current biology (Locksley *et al*, 1998; Watters and Lavin, 1999; Evan and Littlewood, 1996). Because human and murine HF morphogenesis (Polakowska *et al*, 1994; Magerl *et al*, 1998) and murine HF catagen (Lindner *et al*, 1997) are characterized by substantial apoptosis, this special form of cutaneous growth control is of particular interest in hair research (Paus *et al*, 1993; Paus, 1996; Stenn *et al*, 1996, 1998; Philpott and Paus, 1998). Modulating the balance of follicular proliferation and apoptosis may be a key strategy for the control for hair growth and regression and may lead to more effective therapies of many hair diseases, which can be viewed as cycling disorders characterized by an abnormal control of catagen (Paus, 1996).

PATTERNS OF PROLIFERATION AND APOPTOSIS DURING HAIR FOLLICLE GROWTH AND REGRESSION

Hair follicle morphogenesis in humans (Polakowska *et al*, 1994) and in mice (Magerl *et al*, 1998) is characterized by a tightly controlled balance of proliferation, differentiation and apoptosis. Interestingly, even throughout the earliest stages of murine HF development substantial apoptosis can be detected by transmission electron microscopy (TEM) and high-resolution light microscopy (HRLM), which escape recognition by the TUNEL technique (Magerl *et al*, in press).

Catagen-associated regression of the HF is a unique process of regressive or inverse pattern formation, which is driven by massive

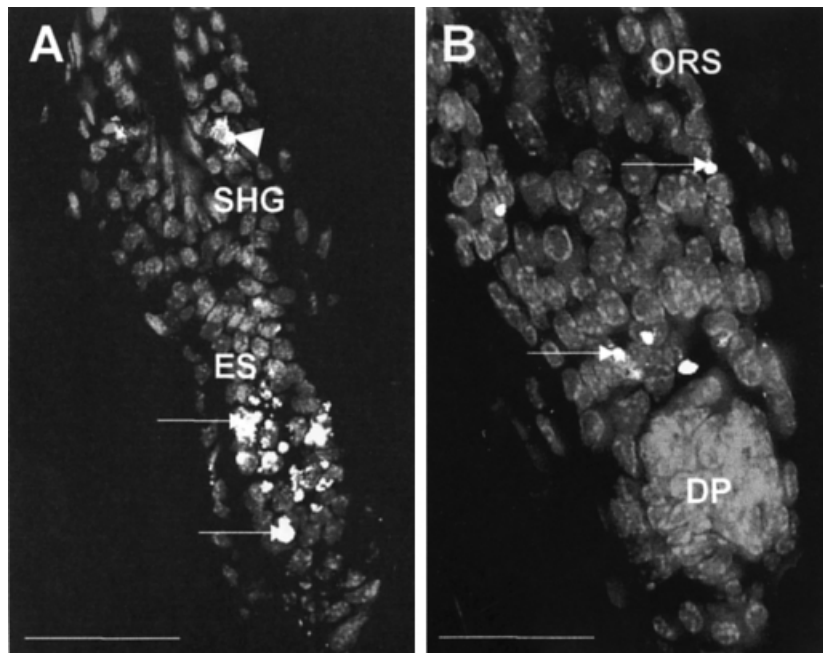
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Abbreviations: DP, dermal papilla; HF, hair follicles; HK1.Bcl-2, mice human keratin-1-driven Bcl-2 overexpressing mice; IR, immunoreactivity; K14-Bcl-2 mice, human keratin-14-driven Bcl-2 overexpressing mice.

Figure 1. Patterns of DNA-fragmentation in keratinocytes assessed by TUNEL method revealed accelerated catagen development in Bcl-2 overexpressing mice.

Ten micrometer cryostat sections of mouse back skin were stained by the TUNEL method to determine DNA-fragmentation during apoptotic cell death in different stages of the hair cycle using FITC-TUNEL kit (Oncor). (A) Numerous TUNEL-positive keratinocytes were located in the trailing epithelial strand (arrowhead) and in the secondary hair germ in catagen VII hair follicles (arrow). (B) Catagen V HF displayed single TUNEL-positive keratinocytes in the regressing hair matrix around the dermal papilla (bright dots, arrows). ORS, outer root sheath; DP, dermal papilla; ES, epithelial strand; SHG, secondary hair germ. Scale bars: (A) 100 μ m, (B) 50 μ m.



apoptosis of hair bulb keratinocytes (Lindner *et al*, 1997) (Fig 1). Multiple factors have been implicated in the control of catagen and catagen-associated HF apoptosis such as Fas, TGF β , TNF α , FGF-5, IGF-1, PTHrp, neurotrophins, and many others (for reviews see Paus, 1996; Stenn *et al*, 1996, 1998; Paus *et al*, 1999). In order to clarify which role any of these factors play in the control of HF apoptosis, we have recently characterized the location of apoptotic hot spots during HF regression (Fig 2A) and have correlated this with the expression of key parameters likely to be relevant in the control of keratinocyte apoptosis, such as TUNEL-staining, Bcl-2, Bax, interleukin-1 β converting enzyme (ICE, caspase 1), and the apoptosis receptors Fas/Apo-1, p55^{TNFR}, and p75^{NTR} (Lindner *et al*, 1997).

TUNEL/HOECHST 33342 double labeling revealed two important hot spots of apoptosis in fully developed HF during late stages of neonatal HF morphogenesis (Magerl *et al*, in press) and mid-anagen VI (Lindner *et al*, 1997). The central part of the inner root sheath (IRS) and the distal outer root sheath (ORS), including the bulge and isthmus region, displayed clusters of TUNEL-positive cells (Lindner *et al*, 1997; Magerl *et al*, in press). Most interestingly, the isthmus/bulge region displayed large numbers of TUNEL positive cells with epithelial phenotype throughout catagen (Lindner *et al*, 1997). During catagen development, another two apoptosis hot spots with large clusters of TUNEL-positive cells were found in the regressing proximal hair matrix, namely the epithelial strand of the regressing HF and the secondary hair germ (Lindner *et al*, 1997) (see Fig 1A,B).

These findings contradict conventional hair research concepts in several respects. Most notably, it reveals that HF apoptosis in normal murine skin commences much earlier than previously appreciated (mid-anagen VI, i.e., long before the regression of anagen HF becomes morphologically visible) and occurs not only in the regressing proximal hair bulb but also in three additional apoptosis hot spots (central IRS, distal ORS, secondary hair germ) including the epithelial stem cell region of the HF (Fig 2A). Thus, even the so-called "permanent" distal portion of the follicle that contains these stem cells (Cotsarelis *et al*, 1990) undergoes extensive remodeling by the induction of controlled apoptosis (Lindner *et al*, 1997).

In this context, Bcl-2 and related proteins are of special interest, since they operate as key regulators of apoptosis (Chinnaiyan *et al*, 1996; White, 1996; Adams and Cory, 1998). The spatiotemporal

distribution of the antiapoptotic protein Bcl-2 is strictly hair cycle-dependent (Stenn *et al*, 1994; Lindner *et al*, 1997): throughout the hair cycle, the dermal papilla (DP) is the only region that is consistently Bcl-2 positive in all phases of the hair cycle. Whereas the HF epithelium displays no Bcl-2 immunoreactivity (IR) during telogen, it becomes Bcl-2 positive during early anagen. During anagen VI, the bulb, the basal cells of the ORS, and the bulge/isthmus region express Bcl-2. Interestingly, during catagen, the staining intensity decreases along an apparent gradient from the bulb towards the distal HF epithelium; in telogen all epithelial Bcl-2 expression ceases. A detailed analysis of the Bcl-2/Bax ratio during the anagen-catagen transformation (Lindner *et al*, 1997) (Fig 2B) revealed that Bcl-2 is expressed by the DP and the distal ORS during all examined cycle stages; keratinocytes of the bulb and the isthmus region are Bcl-2 positive and Bax-negative, whereas cells in the proximal IRS, ORS, and hair matrix in late anagen VI display both Bcl-2 and Bax IR. Only in the epidermis, DP, and distal ORS (including bulge and isthmus region) does Bcl-2 remain strongly expressed throughout catagen development, whereas no Bax IR was seen in these regions. Double labeling of Bcl-2/Bax expression shows a sudden, progressive decline of Bcl-2 expression, associated with a simultaneous increase of Bax IR in the proximal hair matrix during catagen II to VIII, compared with that in anagen VI. During catagen VI, virtually all keratinocytes of the epithelial strand displayed a strong predominance of Bax expression, and only a few isolated epithelial cells in the secondary hair germ were Bcl-2/Bax double positive. In DP fibroblasts, no Bax expression was found during all examined hair cycle stages (Lindner *et al*, 1997) (Fig 2B).

These phenomenologic studies summarized above raised more questions than expected: The expression of Bcl-2 in the basal epidermal layer suggested that Bcl-2 may serve to maintain the stem cell pool by protecting the stem cells from postmitotic differentiation, senescence, and death (Hockenberry *et al*, 1991; LeBrun *et al*, 1993; Stenn *et al*, 1994). It was a confusing finding that Bcl-2 expression in the follicular bulge region, which harbors a major epithelial stem cell population (Cotsarelis *et al*, 1990), was absent during the resting phase of the hair cycle (telogen) (Stenn *et al*, 1994). It was even more surprising that this region should be a hot spot of apoptosis during catagen (Lindner *et al*, 1997), because the distal ORS prominently expresses Bcl-2 and hardly any bax (Lindner *et al*, 1997). This pattern would be expected to suppress

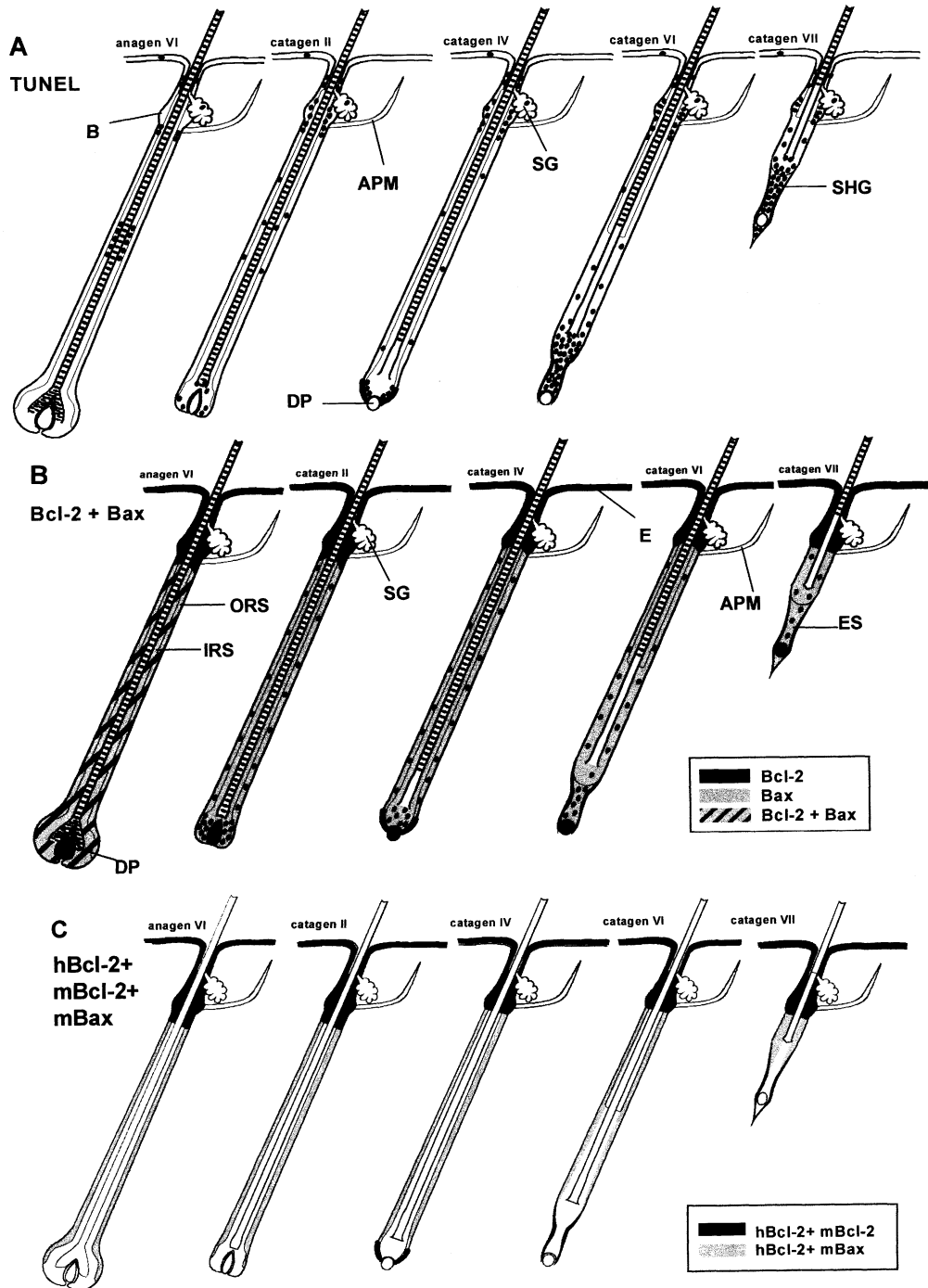


Figure 2. Schematic representation of the topographic distribution of TUNEL-positive cells, Bcl-2/Bax-IR, and K14-Bcl-2 expression during different stages of murine hair follicle regression (catagen). Those keratinocytes or hair follicle compartments with TUNEL-positive staining (A), Bcl-2-IR (B), or constitutive expression of murine Bcl-2 and K14-driven over-expression of human Bcl-2 (mBcl-2 + hBcl-2) (C) are in black. Those hair follicle compartments or individual cells with Bax-IR (B) or hBcl-2 + Bax (C) are in gray, whereas Bcl-2/Bax-double labeled areas or keratinocytes are hatched (B). The different stages of hair cycle are indicated according to Straile *et al* (1961). Modified after Lindner *et al*, 1997. APM, muscle arrector pili; B, bulge/isthmus; E, epidermis; ES, epithelial strand; SHG, secondary hair germ; SG, sebaceous gland; ORS, outer root sheath; IRS, inner root sheath; DP, dermal papilla; ES, epithelial strand.

apoptosis (Chinnaiyan *et al*, 1996; White, 1996; Adams and Cory, 1998) in this region. In addition, this follicle region expresses strongly and homogeneously the pro-apoptotic interleukin-1 β converting enzyme (ICE) and the apoptosis receptors Fas/Apo-1 and p75^{NTR} throughout the entire anagen-catagen transformation (Lindner *et al*, 1997).

Thus, the epithelial stem cells in the bulge region have to escape cell death in the midst of a tissue environment characterized by intense apoptotic activity. Furthermore, the prominence of

keratinocyte apoptosis in the ORS distal to the insertion of the arrector pili muscle reveals that this so-called noncycling segment of the HF is not as permanent as traditionally assumed (cf. Chase, 1954; Cotsarelis *et al*, 1990; Stenn *et al*, 1996). Due to the velocity of programmed cell death, the visualization of a few apoptotic cells within a tissue compartment indicates that actually more apoptosis occurs here than the mere number of TUNEL-positive cells suggests (cf. Cotter and Martin, 1996) – in particular because the TUNEL method is less sensitive in the detection of follicular

apoptosis than TM or HRLM (Magerl *et al*, in press). Therefore, our data imply that a substantial portion of the distal HF epithelium is deleted during catagen, including significant tissue remodeling. This implies that catagen development affects the entire murine HF, and not only its proximal part, and questions the concept of a noncycling portion of the HF (Linder *et al*, 1997; Paus and Cotsarelis, 1999).

If the balance of follicular proliferation, differentiation and apoptosis is that important for HF morphogenesis and regression, in principal the molecular targets for the development of novel hair drugs may include topically administered agents that selectively modulate the ratio of pro-apoptotic IIB (e.g., Bax, Bcl-x_s) and antiapoptotic factors (e.g., Bcl-2, Bcl-x_l) (Paus *et al*, 1993). However, recent findings in mouse mutants suggest more complex functions of Bcl-2 than previously appreciated.

BCL-2-DEFICIENT MICE

Bcl-2 null mice have been created by three different laboratories. One group created Bcl-2 α -deficient mice (Veis *et al*, 1993; Sorenson *et al*, 1995) whereas two other groups generated Bcl-2 $\alpha\beta$ -deficient mice (Nakayama *et al*, 1994; Kamada *et al*, 1995; Yamamura *et al*, 1996) displaying a similar phenotype. Briefly, Bcl-2 deficient mice show a multitude of abnormalities including fulminant lymphoid apoptosis, polycystic kidneys, distorted small intestine, retarded first anagen development, and hypopigmented hair (Veis *et al*, 1993; Nakayama *et al*, 1994; Kamada *et al*, 1995; Sorenson *et al*, 1995; Yamamura *et al*, 1996).

Bcl-2-deficient mice appeared normal at birth and were initially indistinguishable from their littermates (Veis *et al*, 1993). At approximately 1 wk of age, Bcl-2 α and Bcl-2 $\alpha\beta$ null mice displayed a decreased size, small external ears, and immature facial features (Veis *et al*, 1993; Nakayama *et al*, 1994). The initial skin phenotype of Bcl-2 α null mutants on both black and agouti coat color backgrounds was indistinguishable from their normal littermates during neonatal HF morphogenesis (falsely termed "the first hair cycle", c.f., Paus *et al*, 1999) (Veis *et al*, 1993; Nakayama *et al*, 1994). At approximately 4 wk post partum the wild-type (WT) mice reportedly initiate the first anagen (falsely termed "the second hair cycle"). Instead Bcl-2 α mutant mice were small and developmentally delayed and the first anagen occurred later than normal, at 5–6 wk (these data were not assessed by quantitative histomorphometry, c.f., Botchkarev *et al*, 1999) (Veis *et al*, 1993). During the first anagen, the coat of the Bcl-2 α and Bcl-2 $\alpha\beta$ mutant mice became markedly hypopigmented (Veis *et al*, 1993; Kamada *et al*, 1995; Yamamura *et al*, 1996). Graying occurred on both the black and the agouti background (Veis *et al*, 1993; Nakayama *et al*, 1994; Kamada *et al*, 1995; Yamamura *et al*, 1996). The hypopigmentation in all mutants developed in a wave-like manner (Veis *et al*, 1993; Nakayama *et al*, 1994; Kamada *et al*, 1995; Yamamura *et al*, 1996), e.g., in Bcl-2 α null mice the hypopigmentation began at the nose and proceeded caudally over 3–4 d leading to gray hair all over the body at approximately 6 wk post partum (Veis *et al*, 1993). Because anagen development in C57BL/6 mice starts at the flanks and proceeds from tail to neck while catagen development proceeds from neck to tail (Paus *et al*, 1994c), the observed pattern reflects a catagen wave rather than an anagen wave. The hair shafts of Bcl-2 α mutant mice still displayed differentially pigmented regions (white at the base, black in the middle, brown at the tip) but were markedly lightened throughout (Veis *et al*, 1993). All hairs still displayed some melanin granules and there was no mixture of light and dark hairs (Veis *et al*, 1993). Unfortunately the authors did not analyze the ratio of melanocytes/hair follicle and the morphology of the remaining melanocytes in order to test a potential increase of the physiologic rate of melanocyte apoptosis in mice (Tobin *et al*, 1998; Tobin *et al*, 1999).

Kamada *et al* (1995) and Yamamura *et al* (1996) demonstrated that Bcl-2 $\alpha\beta$ -deficient mice show a substantial retardation of HF cycling compared with WT mice: whereas the first anagen in WT after the completion of HF morphogenesis (falsely termed "the

second hair cycle") started reportedly at approximately 4 wk in WT mice, Bcl-2 $\alpha\beta$ null mice showed a retarded anagen development (1–2 wk later) (Kamada *et al*, 1995; Yamamura *et al*, 1996). Furthermore, Bcl-2 $\alpha\beta$ null mutants reportedly displayed retarded anagen development after depilation: in WT mice early anagen HF were found at day 1 after depilation (a.d.); at day 4 a.d., dopa-positive melanocytes appeared in the HF; at day 7 a.d., fully developed anagen VI HF with thick pigmented hair shafts were found (Yamamura *et al*, 1996). In contrast, null mutants displayed early anagen HF seen at day 2 a.d. and fully developed anagen VI HF only at day 10 a.d. (Yamamura *et al*, 1996). Reportedly, hair shafts in mutant mice were produced 1.2–1.4 times more slowly compared with WT mice.

Additionally, even at day 14 a.d. nearly all HF in Bcl-2 $\alpha\beta$ -deficient mice displayed neither dopa-positive melanocytes nor melanin granules (Yamamura *et al*, 1996). In contrast to the report by Veis *et al* (1993) on Bcl-2 α null mice with a homogeneous hypopigmentation of all hair shafts, the skin color in Bcl-2 $\alpha\beta$ null mice was less dark after the first catagen due to a lower percentage of pigmented hair shafts. Furthermore, depilation-induced hair shafts were white, and melanocytes were almost absent in depilation-induced anagen HF (Yamamura *et al*, 1996). Because in murine skin many club hairs are not shed and remain in the hair canal during telogen and the consecutive anagen, the spontaneously developing anagen skin is composed of old hair shafts that have been generated during neonatal HF morphogenesis and that are regularly pigmented. Thus, by depilation all hair shafts generated during neonatal HF morphogenesis are removed, and all newly generated hair shafts are white (i.e., the entire skin is white in the depilated region). This suggests that Bcl-2 deficiency of melanocytes leads to massive but not total melanocyte apoptosis during the first catagen (c.f., Tobin *et al*, 1999).

Massive graying in a short time is reminiscent of so-called "overnight graying" during diffuse alopecia areata (AA). Overnight graying is interpreted as losing the majority of all pigmented hair shafts while pigment-free (i.e., white) hair follicles remain unaffected, possibly due to a disturbed MHC class I-driven presentation of melanogenesis-associated antigens (Paus *et al*, 1994b). In contrast to this model of AA, Bcl-2-deficient mice reportedly do not show signs of follicular dystrophy and the graying seems to be caused by a reduced number of melanocytes in the HF. In both systems graying appears when the pigmented hair shafts are removed either by follicular degeneration during AA or by physiologic shedding/depilation in Bcl-2 mutants. During AA, existing white hair shafts remain in the skin whereas black hair shafts are lost. In Bcl-2 mutants, white hair shafts develop for the first time and pigmented hair shafts are shed.

In summary, several important questions have been raised by the studies on Bcl-2-deficient mice:

- 1 Is the first catagen after completion of neonatal HF morphogenesis (falsely termed "the first hair cycle" later initiated or is its progression retarded in Bcl-2 mutant mice – leading to retarded anagen development (falsely termed "the second hair cycle")?
- 2 Does Bcl-2 deficiency retard spontaneous and induced anagen by increased apoptosis?
- 3 Do melanocytes in Bcl-2-deficient mice undergo massive apoptosis during catagen? If yes, what is the trigger?

BCL-2-OVEREXPRESSING MICE

Several lines of Bcl-2 overexpressing mice have been generated (Martinou *et al*, 1994; Hsu *et al*, 1996; Rodriguez *et al*, 1996; Jäger *et al*, 1997; Rodriguez-Villanueva *et al*, 1998; Müller-Röver *et al*, in press). Here, we will focus on mutant mice overexpressing Bcl-2 in skin (Rodriguez-Villanueva *et al*, 1998; Müller-Röver *et al*, in press) because the other lines are not reported to display a skin phenotype.

Rodriguez-Villanueva *et al* (1998) generated mouse mutants that overexpress human Bcl-2 driven by the human keratin-1 promoter (HK1.Bcl-2 mice). The transgene was expressed at high levels

specifically in the epidermis extending from the stratum basale through the stratum granulosum. The epidermis of the mutant mice exhibited multifocal hyperplasia without hyperkeratosis and aberrant expression of keratin-6. No increase in proliferation was found in the mutant mice compared with WT controls, although suprabasal BrdU incorporating cells were only present in mutant epidermis. No substantial differences in TUNEL positivity in normal have been found. Bcl-2 overexpression reduced UVB-induced apoptosis in these mutant mice (Rodriguez-Villanueva *et al*, 1998). HF morphogenesis and cycling have not been analyzed by quantitative histomorphometry (Botchkarev, 1999), thus, the influence of suprabasal Bcl-2 expression in the epidermis on HF development, regression, and anagen development is still not clear.

Recently, we have generated mutant mice that express human Bcl-2 driven by the human keratin-14 promoter in order to analyze the influence of Bcl-2 overexpression on HF morphogenesis and the first catagen/telogen/anagen transformation. Furthermore, we analyzed the influence of the transgene on the development of chemotherapy-induced HF apoptosis, dystrophic catagen, and the resulting alopecia (Paus *et al*, 1994a).

The full-length cDNA of human Bcl-2 was ligated into the keratin 14/hGH expression vector, and transgenic mice were generated by microinjection of the construct into the pro-nuclei of fertilized ova of white FVB/N mice. The presence of the transgene was detected by Southern blotting and polymerase chain reaction. Protein expression was confirmed by immunohistochemistry. Western blotting under reducing conditions revealed a transcript of about 27 kDa. In order to test the *in vivo* function of the transgene, 8–9-wk-old mice were irradiated with UVB at a wavelength of 312 nm and apoptotic cells (“sunburn cells”) were counted in both the basal and the suprabasal layers of interfollicular epidermis. For *in vitro* induction of apoptosis with UVB, epidermal cell cultures were irradiated with UVB light (290–330 nm). HF morphogenesis was compared between transgenic (TG) and WT littermates during neonatal HF morphogenesis, during late postnatal HF development, during the first catagen, and during the first anagen. In order to study the effects of Bcl-2 overexpression on cyclophosphamide (CYP)-induced alopecia and follicle dystrophy (Paus *et al*, 1994a), adolescent WT and TG mice were depilated to induce a new anagen wave. Nine days after depilation 120 mg per kg CYP were injected intraperitoneally. TG and WT mice were sacrificed 36 h and 6 d after CYP treatment. Cryostat sections of standardized back skin regions were analyzed by quantitative histomorphometry to determine differences in the velocity of HF morphogenesis, spontaneous catagen development, and CYP-induced dystrophic catagen (Maurer *et al*, 1997). Intrafollicular apoptosis was determined by TUNEL/Hoechst 33342-double staining (Lindner *et al*, 1997).

Keratin 14-driven expression of human Bcl-2 in murine skin was found in the basal epidermal layer, the ORS, and within the regressing epithelial strand of HF (Fig 1C). Thus, the transgene was also expressed in those regions that have been found to display hot spots of apoptosis (Fig 1A). In contrast to HK1.Bcl-2 mice (Rodriguez-Villanueva *et al*, 1998), K14-Bcl-2 overexpressing mice did not show any overt abnormalities of the skin phenotype – neither by macroscopic nor by histomorphologic criteria. No significant differences in the velocity and quality of HF development until day 8 *post partum* was found by histomorphometric analysis of WT and TG mice.

In order to test if the transgene is functional we tested the influence of Bcl-2 overexpression on UVB-induced apoptosis in skin. Transgenic mice (8–9 wk old) irradiated with UVB showed about a 5–10-fold reduced number of sunburn cells in the basal layer of the epidermis compared with age-matched WT mice. Cultures of primary keratinocytes from transgenic mice were completely resistant to UVB-induced histone formation, at doses that readily induced histone release from WT keratinocytes.

Contrary to our expectations, the first catagen development in TG mice was significantly ($p < 0.01$) accelerated (Fig 2B) com-

pared with WT littermates (Fig 2A) and the consecutive anagen was also significantly ($p < 0.01$) accelerated. Furthermore, CYP-treated TG mice displayed massive, macroscopically visible alopecia 2 d earlier than WT mice and showed increased follicular dystrophy compared with WT control mice. TUNEL staining revealed a significantly higher rate of apoptotic cells in the HF of TG mice than in the controls ($p < 0.01$). Because the Bcl-2 overexpressing mice had a very marked reduction in UVB-induced sunburn cells compared with WT mice, specifically in those keratinocyte subpopulations in which the transgene was expressed, the transgene was active as expected, despite the overexpression of a human protein in mouse tissue. Unexpectedly, however, the overexpression of Bcl-2 does not protect HF keratinocytes from spontaneous (catagen) or experimentally induced apoptosis (CYP-induced alopecia).

Thus, strikingly, this mutant mouse strain overexpressing an apoptosis inhibitor shows accelerated apoptosis-driven catagen induction and/or progression. Our data complement the findings in Bcl-2 null mutants (Veis *et al*, 1993; Nakayama *et al*, 1994; Kamada *et al*, 1995; Yamamura *et al*, 1996): Bcl-2 deficiency reportedly leads to a retardation of the first anagen development, whereas K14-Bcl-2 overexpression accelerates the first anagen development.

Recently, Pena *et al* (1999) have published a transgenic mouse line overexpressing Bcl-X(L), another anti-apoptotic bcl-2 family member, under the control of the K14 promoter. Similar to our K14/Bcl-2 mice, an acceleration of catagen development was found. Crossbreeding of these transgenic mice with FGF-5-deficient mice reversed most of the HF phenotype, suggesting that overexpression of Bcl-X(L) in the ORS of these transgenic mice leads to prolonged survival of FGF-5 producing ORS keratinocytes. Since FGF-5 promotes catagen development (Hebert *et al*, 1994), a similar principle might underlie the catagen acceleration in our K14/Bcl-2 mice. Furthermore, it has previously been suggested that Bcl-2 may not be limited to the regulation of apoptosis but may also, directly or indirectly, act as a key factor during differentiation and may have a cell cycle-inhibitory function distinct from its apoptosis-inhibitory function (Marthinuss *et al*, 1995; Vairo *et al*, 1996).

Several questions remain to be dissected:

Does Bcl-2 overexpression in the basal layer or in suprabasal layers (Rodriguez-Villanueva *et al*, 1998) of the epidermis and/or the outer root sheath lead to premature anagen termination/catagen induction at the end of the first genuine anagen period?

By which mechanism are catagen progression yet also the subsequent anagen development, accelerated in Bcl-2 transgenic mice?

Is the retardation of the first catagen/telogen/anagen transformation in Bcl-2-deficient mice correlated with the acceleration in Bcl-2 overexpressing mice?

Does Bcl-2 upregulation lead to a counterregulatory upregulation of pro-apoptotic molecules such as bax or Bcl-x_s?

Rather than to affect primarily keratinocyte apoptosis, does Bcl-2 overexpression or deficiency in keratinocytes alter the control of keratinocyte differentiation (in contrast to apoptosis-inhibition) during anagen? And how important exactly is Bcl-2 for melanocyte survival in the cycling HF?

In summary, the phenomenologic studies and the studies on Bcl-2 mutant mice reviewed here raise more questions than they provide answers, and suggest that we have as yet barely scratched the surface of the true role of Bcl-2 in the control of hair follicle apoptosis.

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