Manipulation of outer root sheath cell survival perturbs the hair-growth cycle

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Transgenic mice that overexpress the anti-apoptotic gene *bcl-x_L* under the control of the keratin 14 promoter have significantly shorter hair than non-transgenic littermates. The deficit in hair length correlated with a decrease in the duration of anagen, the growth phase of the hair cycle. A prolongation in telogen, the resting phase of the hair cycle, was also observed in adult animals. In the developing hair bulb, *bcl-x_L* transgene expression was observed exclusively in the outer root sheath (ORS) cells. *Bcl-x_L* expression enhanced the survival of ORS cells treated with apoptotic stimuli. The results suggest that preventing the apoptotic death of ORS cells during anagen leads to a more rapid termination of progenitor cell commitment/proliferation, while the increased survival of ORS cells during telogen delays the initiation of a new hair cycle. ORS cells produce fibroblast growth factor-5 (FGF-5), which acts in a paracrine fashion to terminate precursor cell division during anagen. The short hair phenotype of *bcl-x_L* transgenic mice was substantially reversed in FGF-5-deficient mice. Thus, the production of growth inhibitory factors by ORS cells may provide a mechanism through which the hair-growth cycle is regulated by cell survival.

Keywords: apoptosis/Bcl-x_L/growth inhibitory factors/hair

Introduction

Hair is the cumulative, physical result of a coordinated process of cellular proliferation and differentiation within a hair follicle (for review see Hardy, 1992). Hair follicles are epidermally derived appendages which arise as a result of inductive events between specialized dermal fibroblasts acting on bipotential epithelial stem cells. The stem cells which commit to a hair follicle fate enter a period of massive proliferation that culminates in the formation of a mature hair follicle. The hair cycle is comprised of three sequential stages: anagen, during which the hair bulb is actively producing precursor cells that differentiate into the individual types of hair cells; catagen, during which production of precursor cells ceases and the hair bulb rapidly involutes; and telogen, the resting phase of the hair cycle (Dry, 1926).

The anagen hair follicle consists of six distinct cellular layers (Sperling, 1991). The five internal follicle layers are composed of cells derived from precursor cells in the matrix region which, under the inductive influence of the dermal papilla, undergo a highly ordered process of cell death that results in the production of a hair. The cells of the outer layer in the growing hair follicle, termed the outer root sheath (ORS) cells, survive the differentiation process and line the hair shaft of the mature follicle. The ORS cells are morphologically similar to the basal keratinocytes of the interfollicular epidermis with which they form a continuous cell layer that lines the epidermal basement membrane and hair follicle dermal sheath throughout the hair cycle. Like basal keratinocytes, ORS cells express keratins 5 and 14 (Coulombe *et al.*, 1989; Byrne *et al.*, 1994).

Recently, insight into the control of the growth phase of the hair cycle has been provided by studies of fibroblast growth factors (Hebert *et al.*, 1994). ORS cells have been shown to generate a signal for the termination of anagen, thus initiating catagen. Mice bearing naturally occurring (Angora, go/go) or site-directed inactivating mutations of fibroblast growth factor-5 (FGF-5) display an abnormal long hair phenotype. Histological analysis of the FGF-5 knockout and Angora mouse hair follicles demonstrated that the duration of anagen had been lengthened, resulting in the increased hair length. *In situ* experiments have localized the production of FGF-5 expression to the ORS. These data suggest that FGF-5 produced by ORS cells plays a role in regulating the length of anagen.

Despite the prolonged anagen phase in FGF-5-deficient mice, the hair follicles enter catagen in both wild-type and Angora mice. During catagen the lower portion of the follicle undergoes involution. The mechanisms by which the proliferative and maturational zones of the follicle are eliminated have not been fully characterized (Straile *et al.*, 1961; Strutton, 1980). However, histological and radiolabeling studies reveal that a significant population of keratinocytes contiguous with the ORS survive catagen and become incorporated into a structure known as the epithelial bulge (Cotsarelis *et al.*, 1990). The bulge of the ORS remains as the hair follicle enters telogen and is thought to contain cells that are required to re-initiate subsequent hair-growth cycles (Wilson *et al.*, 1994b). During telogen, the hair follicle is quiescent and is comprised of a layer of ORS cells surrounding a completely formed hair. In adult hair follicles, telogen can persist for 30–40 days terminating with the initiation of a new anagen phase (Wilson *et al.*, 1994a). The factors which induce the reinitiation of anagen are unknown. Hair stalk removal can cause reinitiation of hair growth (Ebling, 1976). In a significant percentage of hair follicles, new hair production begins before the old hair is lost (Chase, 1954).

The factors that regulate cell survival during hair

production are not known. There are reasons to suspect that anti-apoptotic genes may play a regulatory role during this process (Cotsarelis, 1997). Among the best-characterized genes which regulate apoptosis are the members of the Bcl-2 family (Chao and Korsmeyer, 1998). Bcl-2 family proteins such as Bcl-2 and Bcl-x_L have been shown to be important regulators of cell survival (Yang and Korsmeyer, 1996). Overexpression of these genes increases the resistance of cells to a variety of apoptotic stimuli. In contrast, other family members appear to enhance a cell's sensitivity to apoptosis. Bcl-2 has been shown to be induced in cells within the hair matrix during anagen with expression declining when catagen is initiated (Stenn *et al.*, 1994). *In situ* analyses have demonstrated the upregulation of pro-apoptotic Bax mRNA transcripts during the early stages of catagen (Lindner *et al.*, 1997).

To investigate how Bcl-2-related proteins might regulate epithelial cell differentiation, transgenic mice that overexpress the anti-apoptotic gene *bcl-x_L* under the control of the keratin 14 (K14) promoter were produced (Pena *et al.*, 1997). The terminal differentiation program of the skin was not disrupted in the *bcl-x_L* transgenic mice. However, Bcl-x_L conferred a distinct survival advantage to basal keratinocytes in response to stress-inducing events. We now report that these animals also exhibit a striking alteration in hair growth. Transgenic animals had a significant shortening of the anagen phase of the hair cycle with a reciprocal prolongation of telogen. These effects appear to correlate with the ability of the transgene to increase the survival of ORS cells, the one hair cell type that survives the differentiative process of hair formation. The ability of Bcl-x_L to cause a shortening of anagen was partially dependent on the ability of ORS cells to produce FGF-5, suggesting that one mechanism by which ORS cells may terminate anagen is through the ability of these cells to produce factors that suppress further progenitor cell proliferation and/or differentiation. These data suggest that differentiated cell survival may be an important parameter that contributes to the feedback suppression of progenitors during normal tissue homeostasis.

Results

Bcl-x_L is overexpressed and functions in the ORS

CD1 transgenic mice that express Bcl-x_L in the epidermis under the control of the K14 promoter were generated to examine the effect of apoptotic inhibitors on the epidermal maturation process (Pena *et al.*, 1997). While the differentiation program of the skin was not disrupted, the K14-*bcl-x_L* transgenic mice contained a noticeable alteration in the length of hair relative to littermate controls. Previous studies have shown that the K14 promoter is active within the hair follicle (Vassar *et al.*, 1989; Byrne *et al.*, 1994). Immunohistochemical detection of Bcl-x_L confirmed that the K14 promoter was active within the ORS cells of the hair follicle throughout the hair cycle (Figure 1; Pena *et al.*, 1997) whereas Bcl-x_L was not detectable in wild-type follicles during the hair cycle. Bcl-x_L was functional within the ORS cells as these cells displayed increased resistance to programmed cell death (PCD) in response to UV light. Wild-type and *bcl-x_L* transgenic skin were exposed briefly to UV-B irradiation and the number of observed apoptotic cells within hair

Fig. 1. The *bcl-x_L* transgenic mice express Bcl-x_L in the ORS cells of the hair follicle. Dorsal skin from a post-partum day 12 *bcl-x_L* transgenic mouse contained hair follicles that were in the anagen phase of the primary hair cycle. (A) Immunohistochemical localization (visualized in orange) of Bcl-x_L within the hair follicles demonstrated that Bcl-x_L expression is confined to the outer root sheath of the hair follicle (100×). Bcl-x_L expression is contiguous with the basal keratinocytes that comprise the interfollicular epidermis. (B) The ORS cells, but not the hair matrix precursor population, which surround the hair bulb express Bcl-x_L during anagen (200×).

follicles in telogen was determined 24 h later using established morphological criteria (Danno and Horio, 1987). The hair follicles from *bcl-x_L* transgenic animals displayed ~5-fold reduction in the number of apoptotic cells when compared with follicles of wild-type mice (2.8 ± 0.9 versus 14.9 ± 2.9 apoptotic cells per cm of cross-sectioned tissue, transgenic versus wild-type, respectively).

Anagen terminates prematurely in Bcl-x_L transgenic hair follicles

The murine hair coat comprises four different hair types: the guard, auchene, awl and zig-zag (Gibbs, 1941; Fraser, 1951). The hair of wild-type and *bcl-x_L* transgenic mice from four separate litters was removed from an equivalent region of the mid-dorsal area, measured and analyzed according to specific type. The measurement data revealed that all four hair types from the *bcl-x_L* transgenic mice were 20–30% shorter than those of wild-type CD1 mice

Fig. 2. The hair from 7-week-old *bcl-x_L* transgenic mice is shorter in length than wild-type littermate controls. Hair was taken from both male and female wild-type and transgenic mice and measured. The hair was removed from equivalent regions of the dorsal skin in all the mice. All four hair types from the *bcl-x_L* transgenic mice are shorter in length than the corresponding littermate controls.

(Figure 2). The hair did not appear to be brittle as macroscopic analysis did not reveal the breakage of hair tips nor were there any gross abnormalities in the hair structure or pigmentation. The short hair phenotype was present in the transgenic mice starting with the primary hair cycle and persisted throughout the life of the animal. Quantitative measurements of hair taken from the progeny of an independent transgenic line confirmed the observations determined in the offspring from the original transgenic animals and demonstrated the heritable nature of this change in hair coat.

Histological analysis was performed on hematoxylin and eosin (H&E)-stained posterior dorsal skin sections from wild-type and *bcl-x_L* transgenic mice at 3 day intervals starting at embryonic day 10 and continuing at 24 h intervals beginning at day 10 post-natally through the primary and secondary hair cycles. Since hair coat growth varies regionally, care was taken to examine sections from the same skin location for each mouse. The histological analysis of the early inductive events revealed that hair follicle initiation during the first hair cycle occurred at equivalent timepoints in both the wild-type and *bcl-x_L* transgenic embryonic mice (data not shown). Furthermore, the hair generated from these follicles erupted through the epidermal surface at approximately the same time post-natally. These data suggest that the deficit in length is not attributable to a delay in early hair morphogenesis.

Sections of day 12 post-partum skin from both the wild-type and *bcl-x_L* transgenic mice showed that the hair follicles were in the anagen phase of the hair cycle (Figure 3, top). At day 16 post-partum during the primary hair cycle, the wild-type hair follicles remained in anagen with hair formation continuing within the hair shaft (Figure 3, middle, left). In contrast, the *bcl-x_L* transgenic hair follicles had ceased generating hair and entered catagen as demonstrated by the involution of the lower portion of the hair follicle (Figure 3, middle, right). Wild-type hair follicles were not observed to enter catagen until day 19 while *bcl-x_L* transgenic animals had already completed catagen and were in telogen (Figure 3, bottom). Sequential analysis of the first hair cycle was completed on at least three groups of wild-type and transgenic mouse pairs, each exhibiting the premature termination of anagen.

Fig. 3. The *bcl-x_L* transgenic mouse hair length is shorter due to a premature termination of the anagen phase. Skin sections were made from wild-type and *bcl-x_L* transgenic mice at 12, 16 or 19 days post-partum. The follicles pictured are from similar mid-dorsal regions on both groups of mice and are in the first hair cycle. At day 12 (d12) post-partum both the wild-type (top left panel) and transgenic (top right panel) hair follicles are in anagen as the hair follicles are deeply invaginated into the subcutaneous region and contain all six cellular layers of a mature follicle. At day 16 (d16) the *bcl-x_L* transgenic hair follicles (middle right panel) have almost completed catagen and have regressed into the upper dermis. In contrast, the wild-type follicles remain in anagen (middle left panel). At day 19 (d19) post-partum the wild-type follicles (lower left panel) have almost completed catagen. The hair follicles from the *bcl-x_L* transgenic mouse (lower right panel) are in telogen.

Measurements of the time period that the hair follicles of each animal spent in catagen and telogen suggest that the duration of these stages in the first hair cycle were comparable between transgenics and littermate controls. Taken together, these studies show that the *bcl-x_L* transgenic animals enter the anagen phase of the second synchronous hair cycle 2–3 days earlier than littermate controls.

Analysis of sequential histological sections from the second hair cycle demonstrated that the anagen phase was again 2–3 days shorter in the transgenic animals. The net result was that the hair follicles in the *bcl-x_L* transgenic mice appeared to enter telogen at 37–38 days post-partum, on average 4 days before the follicles in littermate controls. Telogen in the wild-type hair follicles was achieved at 42 days post-partum. The premature termination of anagen and entry into catagen was observed when the *bcl-x_L* transgene was bred onto two genotypic backgrounds and in both *bcl-x_L* transgenic founder lines. These data demonstrate that the anagen phase in both the primary and secondary hair cycles is shortened by ~2 days each, suggesting that this may account for the presence of shorter hair on the *bcl-x_L* transgenic mice. Shortened anagen phase comparable with that observed in the second

hair cycle occurred again in the third hair cycle. Hair length in telogen was observed to be consistently shorter in *bcl-x_L* transgenics than in littermate controls throughout the life of the animals.

The hair-cycle interval is prolonged in adult *Bcl-x_L* transgenic mice

The first two hair cycles of *bcl-x_L* transgenic mice were significantly shorter than those of littermate control mice. This shortening of the hair cycle could be almost entirely accounted for by a decrease in the anagen growth phase. In contrast, the duration of catagen and telogen appeared grossly normal. Depilation experiments in adult mice confirmed that reinduction of hair follicle growth was still accompanied by a significantly shortened anagen phase and grossly normal catagen. Adult transgenic animals exhibited a shortened hair phenotype in all four major hair types. However, depilation did not allow the examination of telogen duration in adult animals.

Previous work has demonstrated that after the first hair cycle, telogen is either skipped or confined to a brief 1 to 2 day interval with the secondary hair cycle initiating synchronously in all hair follicles (Dry, 1926). However, a major change occurs in the control of telogen after the first hair cycle. In contrast to the first telogen, subsequent hair cycles are marked by a prolonged telogen phase. In addition, re-initiation of the hair cycle, even between neighboring follicles, is asynchronous. This suggests that the hair cycles in adult mice are regulated by mechanisms that differ from those that control the initial hair cycles of neonatal mice.

To address whether the length of telogen in hairs from adult mice was altered by the expression of the *Bcl-x_L* transgene, a hair coloring experiment was performed utilizing the *bcl-x_L* transgenic CD1 mice. This experiment capitalized on the fact that the hair on CD1 mice is white and that a significant percentage of hair from the previous hair cycle is lost as a new hair is generated during the subsequent hair cycle. Eight wild-type and eight *bcl-x_L* transgenic mice were colored with a permanent brown hair dye and the percentage of brown hair within an equivalent region of dorsal skin was serially examined in all mice over time. After the mice were colored, hair was plucked at 4 and 8 weeks from both wild-type and *bcl-x_L* transgenic mice and the relative proportion of brown and white hair was determined (Figure 4A). At both timepoints, the *bcl-x_L* transgenic mice retained a significantly higher proportion of colored hair compared with wild-type mice ($P < 0.001$). This difference did not appear to be attributable to an increased retention of dyed hair following a subsequent hair cycle, as neither the number of hair follicles with more than one hair club during telogen nor the number of hair clubs per follicle during telogen were significantly different between *bcl-x_L* transgenics and littermate controls (Figure 4B).

Precursor cell proliferation rate does not account for hair length deficit

Although histological examination of the hair cycle demonstrated the premature termination of anagen, it was possible that the *bcl-x_L* transgene could affect hair length through additional mechanisms. Recent reports have indicated that the Bcl-2 protein family members can

Fig. 4. *Bcl-x_L* overexpression influences the length of telogen.

(A) Wild-type and *bcl-x_L* transgenic mice that were 12–14 weeks of age were dyed with a coloring agent (see Materials and methods). At the timepoints indicated after dye treatment, hair was removed from the dorsal region of wild-type and *bcl-x_L* transgenic mice. The number of brown hairs as a percentage of total hairs counted was tabulated (mean $\pm$ SD). Eight mice per group consisting of equally males and females were evaluated in this data set. The experiment was repeated twice with equivalent results. Differences in the means of the two groups were statistically significant ($P < 0.001$ at 4 weeks; $P < 0.001$ at 8 weeks). (B) Representative cross-sections of *bcl-x_L* transgenic mice and control littermates were evaluated for the percentage of follicles containing one, two, three and more than three hair clubs. In neither group were hair follicles containing more than three hairs observed. No statistically significant differences were observed between the two groups.

influence the cellular proliferation rate (O'Reilly *et al.*, 1996). To determine whether *Bcl-x_L* overexpression affected the proliferation rate within the matrix precursor cells, we performed segment analysis of the zig-zag hair of the mouse. The zig-zag hair contains three defined constriction points, as a result of the dermal papilla shifting at specified time intervals, dividing the hair into three segments and a tail portion (Straile, 1965). As hair comprises vertically aligned cells generated from the matrix region, the length is directly dependent on the total number of cells and proportional to the cellular proliferation rate. Therefore, the length of each segment in the zig-zag hair represents the proliferation rate over a defined period of time, specifically, the time interval between each shift of the dermal papilla.

A comparative analysis of segment lengths from zig-zag hair from 7-week-old *bcl-x_L* transgenic and wild-type mice

Fig. 5. The hair from the *bcl-x_L* transgenic mice is shorter due to a decrease in the length that is confined to the tail portion of the hair. (A) Schematic drawing of the zig-zag hair with the three zig segments and tail labeled in the diagram. (B) The first two segments (left) and the last segment and tail (right) were measured and the lengths indicated were combined. Only the terminal portion of the zig-zag hair is shorter in the *bcl-x_L* transgenic versus the wild-type mice. These data are representative of both male and female mice from at least three different litters comprised of >10 mice per group.

was performed. Hair was removed from the mid-dorsal region of age- and sex-matched mice and each segment was measured. The length of the first two segments and the last segment and tail were combined to simplify the analysis. The last segment and tail portion of the hair from transgenic animals was significantly shorter than the hair segments from control mice. In contrast, the lengths of the first two hair segments were not statistically different (Figure 5). The shortening of the terminal segments appears to account for the observed shortening in overall hair length. These quantitative results are consistent with the histological observations of the hair cycle: a shortened anagen phase of the hair cycle accounts for the deficit in hair length.

FGF-5 deficiency can reverse the *Bcl-x_L*-induced phenotype

The above data suggested that *Bcl-x_L* overexpression in the ORS of the hair follicle promotes premature termination of anagen. The duration of anagen has been demonstrated to be partially regulated by the presence of a secreted factor, FGF-5. In naturally occurring (Angora) or site-directed FGF-5 knockout mice, the hair is dramatically longer than wild-type mice due to an increased length of the anagen phase (Pennycuik and Raphael, 1984). As FGF-5 is produced by the ORS cells it is possible that the *bcl-x_L* transgene enhances the accumulation and/or survival of the ORS cells that produce FGF-5. Increased survival of ORS cells during the early stages of anagen might result in growing follicle reaching anagen VI, the point where ORS production equals loss, earlier than normal. If the subsequent production of FGF-5 by ORS cells is timed relative to entry into anagen VI, then FGF-5 production would occur earlier than normal, resulting in the premature termination of anagen. To determine whether

Fig. 6. FGF-5 deficiency reverses the premature termination of anagen. Skin sections were taken from day 16 post-partum mice from the genetic backgrounds indicated. The wild-type hair follicles (upper left panel) remain in anagen as indicated by the presence of mature follicles that extend into the subcutaneous region. In contrast, the *bcl-x_L* transgenic mice with a functional FGF-5 allele have terminated catagen and entered telogen, consistent with previous data. The Angora (FGF-5^{-/-}) mice in the absence of the *bcl-x_L* transgene (lower left panel) demonstrate anagen hair follicles at day 16 post-partum. Finally, Angora mice with the *bcl-x_L* transgene bred onto the Angora background (lower right panel) display hair follicles that remain in anagen.

the effects of the *bcl-x_L* transgene on anagen are dependent on FGF-5, the K14-*bcl-x_L* transgenic mice were bred onto the Angora (FGF-5^{-/-}) mouse strain that carry a deletion in the FGF-5 gene.

Sixteen days post-partum, FGF-5^{+/-} (wild-type) and FGF-5^{-/-} mice with or without the K14-*bcl-x_L* transgene were sacrificed and H&E stained skin sections of the dorsal region were examined (Figure 6). The hair follicles from FGF-5^{-/-}/*bcl-x_L* transgenic mice were still in anagen (Figure 6, lower right). In contrast, the *bcl-x_L* transgenic mice hair follicles were at the end stages of catagen at this time point, similar to the finding in CD1 *bcl-x_L* transgenic mouse hair follicles (Figure 6, upper right). These observations demonstrate that the absence of FGF-5 prolongs the length of anagen in *bcl-x_L* transgenic animals.

To assess whether the *Bcl-x_L*-induced short hair phenotype is affected by breeding to the FGF-5^{-/-} animals, the hair length in adult FGF-5^{-/-}/*bcl-x_L* transgenic, FGF-5^{-/-} and wild-type mice was compared. The guard hairs from the FGF-5^{-/-} and FGF-5^{-/-}/*bcl-x_L* transgenic mice were found to be of equivalent length (Figure 7), both significantly longer than the wild-type guard hairs. This finding suggests that the follicles producing the guard hair on FGF-5^{-/-} mice with or without the *bcl-x_L* transgene remain in anagen for an equivalent period of time. In contrast to the guard hair, the remaining three hair types from FGF-5^{-/-}/*bcl-x_L* transgenic mice were shorter compared with those of the FGF-5^{-/-} mice (Figure 8A). However, despite being shorter than the equivalent hair type from FGF-5^{-/-} mice, the hairs from the FGF-5^{-/-}/*bcl-x_L* transgenic mice were still longer than the wild-type hair showing at least a partial reversal of the *Bcl-x_L* hair-growth phenotype.

Segment length analysis comparing the zig-zag hair from the FGF-5^{-/-} and FGF-5^{-/-}/*bcl-x_L* transgenic mice was performed to determine if the deficit in overall hair length could be attributed to a shortened anagen phase.

Fig. 7. The guard hair from Angora ($FGF-5^{-/-}$) mice with or without the $bcl-x_L$ transgene have equivalent lengths. The guard (overcoat) hair from Angora or Angora/ $bcl-x_L$ transgene-positive mice were plucked from equivalent dorsal regions of the skin and measured. No statistical difference in length was observed between the two groups. The measurements are representative of three different litters from female and male mice. The wild-type guard hair length is shown for comparison.

Fig. 8. The three undercoat hairs from Angora ($FGF-5^{-/-}$) mice with the $bcl-x_L$ transgene demonstrate partial reversal of the short hair phenotype and is confined to the tail portion. Hair from Angora mice with or without the $bcl-x_L$ transgene was removed from the dorsal region and measured. (A) All three undercoat hairs from the Angora/ $bcl-x_L$ transgenic mice were shorter than the Angora mice without the transgene. The length of each hair type from wild-type mice with a similar genetic background is represented by the dashed line in the figure and provided for comparison. (B) Segment length analysis of zig-zag hair from Angora mice with or without the $bcl-x_L$ transgene was completed. The lengths of the first and the second segmental pairs were combined and the tail portion was kept separate to simplify analysis. Similarly to the previous zig-zag hair analysis, the deficit in length from the Angora/ $bcl-x_L$ transgenic mice was confined to the tail portion of the hair.

Zig-zag hair from $FGF-5^{-/-}$ mice contains an additional segment due to the increase in anagen phase and the $FGF-5^{-/-}/bcl-x_L$ transgenic mice were found to have the additional segment as well. For this analysis, the lengths of the first and second segments and of the third and

fourth segments were combined while the tail measurement was kept separate (Figure 8B). As in the previous analysis of zig-zag segment length, the shortened hair length was attributable entirely to the tail portion of the hair. This suggests that the absence of FGF-5 does not completely reverse the premature termination of anagen in all hair types of the $bcl-x_L$ transgenic mouse. Histological analysis of sequential H&E stained skin sections from the first hair cycle revealed that the $FGF-5^{-/-}/bcl-x_L$ transgenic mouse follicles of zig-zag hairs entered catagen slightly earlier than hair follicles from the $FGF-5^{-/-}$ mice (data not shown).

Discussion

Animals bearing a K14- $bcl-x_L$ transgene have an altered hair-growth cycle which includes a shortened growth phase (anagen) and in later hair cycles, prolonged hair retention. These alterations are heritable and appear to be due to functional Bcl- x_L overexpressed in the ORS of the hair follicle, where the promoter is active. Unlike the hair cell types that contribute to the structural formation of the hair, a portion of the ORS cells remains viable throughout all phases of the hair-growth cycle (Straile, 1964). However, a significant number of ORS cells have been reported to die by apoptosis within the neck of the developing hair bulb during the anagen phase of the hair cycle (Lindner *et al.*, 1997). In addition, during telogen ORS cells are likely to be exposed to a variety of apoptotic stimuli including thermal stress and UV irradiation. Expression of the $bcl-x_L$ transgene increased the apoptotic threshold of ORS cells resulting in enhanced resistance to PCD. Increasing the apoptotic threshold of ORS cells is likely to enhance the survival of the ORS cells produced during the growth phase and increase their persistence during telogen. One possible explanation for the observed effects of Bcl- x_L on the hair-growth cycle is that the absolute number of ORS cells (the number produced minus the number which die in an interval) directly modulates the initiation and duration of the growth phase of the hair cycle. Alternatively, the overexpression of the pro-survival protein Bcl- x_L may directly promote premature entry into catagen, resulting in a shorter hair phenotype. In this case, the apparent lengthening of telogen could be the result of a decreased propensity of shorter hair to be removed during grooming.

These studies provide insight into how the size and integrity of a biological structure such as a hair is regulated. Hair plays important roles in both barrier protection and thermal homeostasis (Bressler and Bressler, 1989). Therefore, the control of hair length and the integrity of the hair follicle are important biological issues. Since hair is expressed on the epidermal surface, hairs are susceptible to damage and loss. Therefore, hair must be maintained by regeneration in adult animals. Most evidence suggests that the adult hair follicle initiates a regenerative cycle autonomously. However, the factors that lead to the reinitiation of anagen have been poorly understood (Ogawa and Hattori, 1982). Traumatic loss of hair leads to the reinitiation of hair growth. However, in most instances, hair regeneration often begins prior to the loss of the previous hair. Thus, hair loss is not a common mechanism by which hair growth is reinitiated.

The hair follicle is lined by the ORS cell layer which, along with the basal layer of the epidermis, provide a continuous layer of viable cells at the base of the epidermis. A decrease in the number of ORS cells, or the integrity of the epidermal layer to which ORS cells contribute, could serve as a signal to initiate a regenerative hair cycle. Consistent with this, we have found that increasing the apoptotic threshold of the ORS cells between transgenics and littermates could prolong the length of time a hair is retained. Differences in hair retention result from susceptibility to loss during grooming. However, the fact that there is no difference in the percentage of hair follicles with more than one club hair nor in the absolute number of club hairs per follicle suggests the differences observed in retention are not entirely accounted for by differences in traumatic hair removal. Many instances in which hair growth is reinitiated, including irradiation and thermal injury hair removal, could potentially be explained if loss of ORS cells served as a trigger for the reinitiation of anagen (Collins, 1918; Malkinson and Keane, 1981; Ley and Applegate, 1987). All of these are conditions which would be expected to induce loss or damage of ORS cells. The death of ORS cells might then lead to reinitiation of hair cell growth.

Current evidence suggests that the length of hair is controlled by the period of time the hair spends in anagen. Consistent with this, the shortened hair phenotype of Bcl- x_L animals directly correlates with a shortening of the anagen phase of the hair cycle. In as much as it is difficult to imagine how the size of a non-viable structure such as a hair can be monitored, it is interesting that hair growth is affected by a transgene that functions to promote the resistance of ORS cells to apoptosis. One mechanism by which hair-forming progenitor cells, the matrix cells at the base of the anagen hair follicle, could monitor the status of hair growth is through the function of ORS cells. This hypothesis is supported by experiments investigating the role of FGF-5 in hair length transgenic mice. FGF-5 has previously been shown to be produced in ORS cells and serves to signal the termination of anagen by inhibiting further stem cell proliferation. Bcl- x_L function in promoting cell survival may directly or indirectly contribute to the ability of ORS cells to function in the feedback control of hair growth. For example, by inhibiting cell death, anagen VI may be reached earlier resulting subsequently in a premature entry into catagen. Alternatively, the anti-apoptotic protein Bcl- x_L may promote an enhanced or accelerated ability of ORS cells to produce factors such as FGF-5. Consistent with this possibility, the shortened hair phenotype associated with the bcl- x_L transgene is significantly reduced in the absence of FGF-5. However, additional factors beyond FGF-5 are likely to play a role in the ability of ORS cells to regulate the hair-growth cycle. FGF-5 is not absolutely required for the termination of anagen as the anagen/catagen transition still occurs in go/go (FGF-5^{-/-}) mice. The fact that awl, auchene and zig-zag hairs continue to show partially shortened stalk segments in the Angora/bcl- x_L transgenic mice suggests that other factors, in addition to FGF-5, may play a role in the early termination of anagen in K14-bcl- x_L animals. Previous studies have demonstrated the isolation of additional factors in crude protein extracts isolated from cells in telogen that can negatively regulate

hair growth (Paus *et al.*, 1990). Thus, the control of hair growth and retention is a complex process regulated at a number of distinct levels. Our data add to a growing list of genes that can affect hair growth by demonstrating that apoptotic regulation genes can have important effects on hair-growth control.

Materials and methods

Generation of transgenic mice and mouse related work

The generation of bcl- x_L transgenic mice was as described previously (Pena *et al.*, 1997). Briefly, the bcl- x_L cDNA was placed downstream of a 1.7 kb DNA fragment that contained the K14 promoter and enhancer which provided epidermis-specific expression (Boise *et al.*, 1993). An intronic sequence followed by a polyadenylation signal was cloned directly downstream of the bcl- x_L cDNA. The construct was excised from the pBluescript vector, isolated by gel extraction, the DNA extracted, and injected into CD1-fertilized ovum. Two founders which expressed significant levels of Bcl- x_L in the skin were identified and utilized for these experiments. Once founder lines were established and confirmed by Southern blot analysis, PCR was used to identify bcl- x_L transgenic progeny. To examine embryonic hair-follicle development, matings were set with the plug date considered as day 0.5, with the pregnant mice being sacrificed at the appropriate time after identification of the plug. For analysis of the hair cycle post-partum, the ensuing day after birth was considered day 0.5 and mice were sacrificed at the appropriate timepoints after birth. Angora mice were obtained from Jackson laboratories (Bar Harbor, ME) and genotyped by PCR using FGF-5 primers 5'-CCGAATTCGAGACCATGTCCACCCTG and 3'-CCGAATTCGACGATGAGCATCATCC (Operon, Alameda, CA) to the first exon which is deleted in Angora mice. The bcl- x_L transgene from the CD1 background was bred onto the Angora mouse strain by mating bcl- x_L transgenic CD1 mice to Angora (FGF-5 homozygote-deficient) mice. The resultant Angora heterozygous bcl- x_L transgenic mice were then bred back to Angora. The F₂ generation from these crosses was examined for the hair experiments detailed below.

Histology and immunohistochemistry

For the histology performed to examine the hair cycle, animals were sacrificed at the timepoints indicated after the birth date. Briefly, the dorsal skin was excised and fixed in 10% formalin (Fisher Scientific, Pittsburgh, PA) for a 24 h period, paraffin-embedded and H&E stained. The hair-cycle status was determined by morphological examination. Confirmation of the shortened hair cycle was completed by observing the hair cycle at 15–21 days post-partum in both bcl- x_L CD1 transgenic founder lines. For immunohistochemistry, skin sections were fixed in 10% formalin or 4% paraformaldehyde (Sigma, St Louis, MO, pH 7.2) and paraffin-embedded. Sections (5 μ m thick) were deparaffinized with xylenes and the citrate antigen retrieval method was used for Bcl-x detection (Pena *et al.*, 1997). After washing briefly in phosphate-buffered saline, slides were stained with an anti-Bcl- x_L polyclonal antibody at a 1:500 dilution as a primary dilution. A biotin-avidin peroxidase staining method (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) was used to detect primary antibodies using 3-amino-9-ethyl-carbazol (Sigma, St Louis, MO) as the substrate; counterstaining was performed with Gill III formula hematoxylin (Surgipath Medical Industries, Grayslake, IL).

UV irradiation

Bcl- x_L transgenic and littermate control CD1 mice 6–8 weeks of age were used for the irradiation experiments. Mice were irradiated directly beneath a hand-held UV-B light emitting 302 nm wavelength (UVP, Upland, CA) at a dose rate of 16.7 J/m²/s. Vertical and horizontal movement of the mice was restrained by placing the animal in a confining unit with a wire mesh over the animal. The mice were sacrificed at 24 h after irradiation and the dorsal skin was excised and fixed in 10% formalin, paraffin-embedded and H&E stained. Sunburn cell determination was made by morphological examination of the skin sections under light microscopy. Three sections per irradiated mouse were counted to determine the average sunburn cell formation per mouse in the telogen hair follicles.

Hair measurements

In order to determine hair length, hair was removed at the indicated time periods in each experiment. In general, hair from wild-type or

bcl-x_L transgenic mice was removed at 7 weeks of age from the mid-dorsal region of each animal to reduce the potential of regional variation, although this is not known to occur in the dorsal region of the mouse. Hair from the Angora or the Angora with the *bcl-x_L* transgene was removed from the mid-dorsal region at 8 weeks of age. This represents an age when the majority of hair follicles are in telogen and this fact was confirmed by histological examination. The hair was placed on a glass slide and measured utilizing a light reflecting dissecting microscope (Nikon) at a 100× magnification. Hair was measured from at least 15 mice per group from both the wild-type and *bcl-x_L* transgenic mice and from three different litters that included males and females. Hair from both founder lines was measured. However, the data presented is representative of one founder line. The data from the Angora mice is representative of the same founder line bred back to the Angora genotype and from at least 10 mice per group.

Telogen experiments

To determine if the length of time between hair cycles was affected in the *Bcl-x_L* transgenic compared with wild-type mice, hair dyeing experiments were performed (L'Oreal, New York, NY). The CD1 mice are white and accordingly were dyed with glossy black hair coloring dye and monitored for the loss of color, representing the time of new hair growth. Prior to dye application, wild-type and *bcl-x_L* transgenic male and female mice were washed with water to ensure that impurities were not present in the dorsal hair. Coloring application was performed as described. Basically, dye was mixed, applied to mouse hair and allowed to set for 20 min. Mice were again washed to remove excess dye and hair was allowed to dry. Hair was removed from one region of the dorsal skin to determine the percent hair that was colored or white, indicating the replacement of the colored hair. Animals were also photographed to track the overall progression of new hair growth over the entire mouse. This experiment was repeated twice with at least four males and four females in both the wild-type and *bcl-x_L* transgenic groups.

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