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Indispensable role of *Bcl2* in the development of the melanocyte stem cell

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Abstract

Bcl2 null mice display a characteristic loss of pigmentation demonstrating the importance of *Bcl2* in the melanocyte (Mc) lineage. It was recently reported that this abnormal phenotype is due to the failure of melanocyte stem cell (MSC) maintenance and that *Bcl2* is selectively important for the survival of MSCs. However, in our analysis of the same mouse, we observe a reduction in melanoblast (Mb) number in both epidermal and follicular populations. More importantly, there is a complete absence of MSCs. SCF downregulation in the epidermis is concomitant with the dramatic reduction in Mb numbers observed in the *Bcl2* null, suggesting that Bcl2 is indispensable for the survival of Mbs in the absence of c-Kit signaling. Consistently, abrogation of c-Kit signaling in *Bcl2* null mice depletes all Mbs and Mcs, whereas continuous expression of SCF in epidermal keratinocytes rescues the MSCs. Our results demonstrate that *Bcl2* has a general role in Mb and Mc survival and is essential for the emergence of MSCs. Moreover, the results indicate that the first wave of Mcs that provide hair pigmentation is derived directly from epidermal Mbs bypassing MSCs. Furthermore, a Bcl2-independent mechanism of action of SCF in the Mc lineage is revealed as SCF c-Kit signaling is functional in the absence of *Bcl2*.

Keywords: bcl2; bcl2 null; Melanoblast; c-Kit; Hair follicle development; Melanocyte stem cell; Melanocyte survival; Stem cell development; Stem cell factor

Introduction

In most parts of the adult mouse skin, melanocytes (Mcs) are present exclusively in hair follicles. In each follicle, the Mc lineage forms an independent stem cell system that generates differentiated Mcs in every cycle of hair regeneration, thereby supplying pigments to newly generating hair. This stem cell system consists of three compartments, the melanocyte stem cell (MSC), proliferating transit amplifying (TA) and differentiated melanocytes (DMc). MSCs have been identified as *Dct* expressing cells in the bulge/sub-bulge region (Nishimura et al., 2002), hereafter referred to as the lower permanent portion of hair follicle (abbreviated as LPP). Melanoblasts (Mbs) emerge from the neural tube at embryonic stage (E) 10.5 and migrate along the dorsolateral pathway in the dermis. At E12.5–E13.5, some of them enter the epidermis where they further proliferate. From E14.5, a proportion of the Mbs enter the developing hair follicles where they continue proliferation and differentiation (Schmidt-Ullrich and Paus, 2005). However, the development of MSCs and Mcs from Mbs is poorly understood.

Stem cell factor (SCF), also called steel factor (SLF) or mast cell growth factor (MGF), is the ligand of the receptor tyrosine kinase c-Kit. SCF/c-Kit signaling has been shown to be essential for the migration, proliferation and survival of both Mbs and Mcs (Murphy et al., 1992; Steel et al., 1992; Mackenzie et al., 1997). Null mutations of either the ligand (SCF) or the receptor (c-Kit) result in severe defects in hair pigmentation, indicating the importance of this signaling pathway during early embryonic stages (Cable et al., 1995; Wehrle-Haller and Weston, 1995). SCF mRNA has been shown to be expressed throughout E13.5 and E17.5 skin (Motro et al., 1991). However, the expression of an *SCF-β-galactosidase* transgene suggests that it is transiently expressed in the dermal area of skin at E12.5, and this expression is restricted by E15.5 to the dermal papillae (Yoshida et al., 1996). By P5.5, a high expression domain was found only in the matrix regions of the hair follicles, and no detectable expression was found after the

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first anagen. A humanized mouse model (hk14-SCF or Pao, Pigment all over) overexpressing membrane-bound SCF directed by a human K14 promoter maintains SCF expression in epidermal keratinocytes and results in the maintenance of Mbs and Mcs in the epidermis (Kunisada et al., 1998a,b). These results suggest that SCF is required for the maintenance of Mbs and Mcs in the epidermis. However, the details of the dynamic changes in the expression pattern of SCF within the postnatal skin have yet to be elucidated. While all Mbs and Mcs are dependent on SCF signaling through the c-Kit receptor, the MSCs in the LPP can survive in the absence of SCF/c-Kit signaling and have the ability to repopulate the melanocyte system in subsequent hair cycles (Nishimura et al., 2002).

Bcl2 is an anti-apoptotic molecule; its significance in the Mc lineage has been shown by the abrupt change in coat color of *Bcl2* homozygous null (*Bcl2* null) mice after entering the first hair cycle. The mice are born pigmented but become white from the onset of the first hair cycle (Veis et al., 1993; Kamada et al., 1995; Yamamura et al., 1996). It was recently reported that *Bcl2* is required for the survival of MSCs (Nishimura et al., 2005) based on the observation that MSCs are lost after colonization to the LPP. However, this conclusion appears to be inconsistent with a previous report from the same group (McGill et al., 2002) showing that *Bcl2* is activated by SCF/c-Kit signaling since c-Kit is not required for MSCs. This suggests that the mechanisms underlying Mc loss in the *Bcl2* null mice are more complicated than has been reported. Thus, we have performed a detailed analysis of phenotype of the Mc lineage in *Bcl2* null mice.

Our results demonstrate that melanoblasts in the LPP region of *Bcl2* null mice are lost before any cells fitting the criteria of the MSC (Dct^+, cKit $^{low/-}$ and SCF signaling independent) appear. This is not inconsistent with the observations of Nishimura et al. (2005) as they considered any Dct^+ cells in the LPP region as MSCs. It is, however, not consistent with their conclusion that Bcl2 is selectively required by MSCs. As overexpression of SCF in the epidermal keratinocytes of *Bcl2* null mice rescues the MSC, it is likely that Bcl2 plays a role in supporting the short-term survival of Mbs and Mcs during the stage when SCF is downregulated from the epidermis. Moreover, our result suggests that Bcl2 is not needed for the SCF/c-Kit-dependent survival pathway of Mcs. Furthermore, as the *Bcl2* null mice are black before the onset of the first hair cycle and there is no MSC in these mice, our results for the first time demonstrate that the neonatal matrix Mcs are not derived from MSCs.

Materials and methods

Experimental mice

Mouse husbandry and experimental procedures were conducted according to guidelines established by the animal facility in RIKEN, CDB (Japan). The morning when a vaginal plug was detected was counted as E0.5. ACK2 (anti-c-Kit antibody) treatment (Nishimura et al., 2002) was performed by subcutaneous injection of 0.2 mg per pup daily from P1.5 to P3.5.

Bcl2 null/*Dct*-LacZ mice were generated by mating *Bcl2* heterozygous knockout mice (C57BL/6J) (Yamamura et al., 1996) with *Dct*-LacZ transgenic mice (C57BL/6J) (Mackenzie et al., 1997). The resultant *Bcl2* heterozygous/*Dct*-LacZ (*Bcl2*-Z) mice were mated to produce *Bcl2* null, heterozygous and wild-type littermates. Genotyping was performed by PCR. *Bcl2* loci specific primers are GTCGCTACCGTCGTGACTTC (for wild-type loci), CAGGGGAGCAAAGCTACAAA (for neo inserted loci) and a common reverse primer AGGTCAGGGGAGCAAAGCTAC. Primers specific for the detection of LacZ are CGGCGGTGAAATTATCGATG and TCAACCACCGCACGATAGAGATTC. The PCR was run under the following conditions: pre-PCR incubation 95°C, 15 min followed by 30 cycles of 94°C, 30 s; 58°C, 30 s; 72°C, 1 min. All reactions used HotStarTaq (Qiagen) and were run in an ABI-PRISM 9700 PCR machine (Applied Biosystems, CA). The results were analyzed by electrophoresis in 2% agarose gel and observed under UV illumination.

To generate *Bcl2* homozygous *hk14*-SCF transgenic (*Bcl2*null-Pao) mice, *Bcl2*-Z heterozygous mice were bred with *hk14*-SCF (Pao) mice. Pao positive mice were identified by their extraordinary dark skin and ectopic pigmentation in limb pads and other non-hairy skin (Kunisada et al., 1998a,b). Genotyping of the *Bcl2* loci and LacZ were carried out as mentioned above. Mating between the *Bcl2*het-Pao littermates produced *Bcl2*null-Pao mice.

Histochemistry

The collected skin samples were washed in PBS (Ca^+ and Mg^+ free, autoclaved) and extra tissues removed. Then, the skin was put into a container containing freshly prepared 4% paraformaldehyde (PFA)/PBS and irradiated in a microwave oven at 600 W for 30 s followed by 30 min on ice. Excess fixative was removed by several washes with PBS. The samples were then incubated in a 10% sucrose solution overnight at 4°C. On the next day, the samples were mounted in plastic moulds with O.C.T. and snap-frozen in liquid nitrogen. Samples were stored at −80°C until use.

Whole-mount skin preparation

This method was adapted from the whole-mount preparation of mouse tail skin (Braun et al., 2003) and was modified for the dorsal skin. The dorsal skin was shaved (if necessary) with electric clippers and dissected from the mouse. The samples were cut into smaller pieces in PBS and floated on a 5 mM EDTA/PBS solution at 37°C for either 1 h (embryonic stages) or 2–4 h (postnatal stages). After incubation, the dermal layers of the skin pieces were carefully removed by a pair of fine forceps under a dissection microscope. The skin pieces consisting of only epidermis and hair follicles were fixed in 4% PFA/PBS on ice for 30 min. Excess fixative was removed by several PBS washes.

Immunostaining and confocal microscopy

Immunostaining of whole-mount and sectioned samples was the same, unless stated. The sample was blocked by 2% skimmed milk powder/PBST (1× PBS with 0.1% Triton X-100) for 1 h at room temperature (for sectioned samples) or overnight at 4°C (for whole-mount samples). Primary antibodies were applied, and the samples were incubated at 4°C overnight. The samples were incubated with the secondary antibodies for 1 h at room temperature (section) or overnight at 4°C (whole-mount). Non-bound antibodies were removed by three 15 min PBST washes at 4°C. Samples were mounted in Prolong antifade reagent (Molecular Probes) and cover-slipped.

Antigen retrieval was employed for SCF immunostaining. The sectioned samples were immersed in 1 mM EDTA (pH 9.0) and were heated to 121°C for 5 min in an autoclave machine. Slides were washed in PBS after cooling followed by standard staining procedures. To avoid mis-leading artifacts that might be produced after antigen retrieval, all the results were compared with negative controls, in which either the first or the secondary antibodies were not added. A sample of skin from Pao was used as a positive control.

The first antibodies used were: goat anti-LacZ (Biogenesis, 1:500) [3.3 mg/ml], rat anti-c-Kit (ACK4) (homemade 1:200) [19.4 mg/ml] (Kunisada et al., 1998a), goat anti-SCF (R&D Systems, 1:100) [100 μg/ml] and rabbit anti-K5 (Convance, 1:1000). The secondary antibodies used were donkey α-goat conjugated with Alexa-488 and 546 (Molecular Probes, 1:200) [2 mg/ml], donkey α-rat conjugated with Alexa-488 (Molecular Probes, 1:200) [2 mg/ml] and donkey α-rabbit conjugated with Alexa-546 (Molecular Probes, 1:200) [2 mg/ml]. All the samples were counter-stained with TO-PRO3 (Molecular

Probes, 1:1000) to visualize nuclei. Then, the slides were mounted with Prolong antifade reagents (Molecular Probes) and cover-slipped.

All the images were captured using a Carl Zeiss LSM510 system.

Results

Decrease in the LPP Mb population in the Bcl2 null mice from birth

As previously reported (Nishimura et al., 2005; Veis et al., 1993), we observed no *Dct*-LacZ$^+$ Mcs in the *Bcl2* null from the first hair cycle. In fact, there are no *Dct*-LacZ$^+$ cells in *Bcl2* null mice since the onset of the first anagen (P19, data not shown). In order to determine when the MSCs are lost from the hair follicles of the *Bcl2* null mice, we investigated the LPP Mb number from the day of birth (P0.5).

The analysis of postnatal hair development is complicated by the simultaneous appearance of several different types of hair whose development are not synchronized. In order to simplify our analysis, we focused our efforts primarily on the guard hair, and unless specifically indicated, all results refer to guard hairs exclusively.

We observed a reduction in the number of the LPP Mbs (which were identified as *Dct*-LacZ$^+$ cells in the lower part of the permanent portion of hair follicles) in the *Bcl2* null pups, starting from P0.5 (Fig. 1). At P0.5, wild-type littermates have on average 7 Mbs per LPP, whereas *Bcl2* null mice have around 1 Mb per LPP (Figs. 1A, C). The average number in the null mice further decreased by P4.5, and, by P18.5, we could not see any Mbs in the LPPs while there are on average 3 Mbs per LPP in the wild-type littermates (Figs. 1A, D). Only 80% of the hair follicles of the *Bcl2* null mice contain LPP Mbs at the time of birth, and this figure drops to 14% by P4.5 and to 0% by P18.5 (Fig. 1B). Moreover, the density of epidermal Mbs at P4.5 is lower in the *Bcl2* null than that in the wild-type mice (Fig. 1E).

Defects in the epidermal and follicular Mbs in Bcl2 null mice at embryonic stage (E) 18.5

As we observed a clear reduction in the LPP Mb population of the null mice, we extended our examination to E18.5. The density of the epidermal Mbs of the null mice is around half of that of the wild-type littermates (Figs. 2A and D). The Mb populations in two hair types: the non-bulb Mbs in the guard hair (which develop the earliest) and the total number of Mbs in the zigzag hair (the youngest hair type) were compared between the null and the wild-type littermates. While the numbers of Mbs in the zigzag hair follicles are not different between *Bcl2* null and wild-type fetuses (Fig. 2B), the Mb number is lower in the guard hair of *Bcl2* null fetuses (Fig. 2B) and a leftward shift in the distribution of the numbers of non-bulb Mbs in guard hairs of the *Bcl2* null fetus was observed. The peak of the distribution curve of the wild type is at 5–6 Mbs per follicle, whereas it is 3–4 in the null fetuses (Fig. 2C). We observed no obvious difference in the distributions of Mb numbers in zigzag hair follicles of null and wild-type fetuses (data not shown).

Complete absence of the MSC in the Bcl2 null mice

Although LPP localization is an essential characteristic of the MSC, it should be emphasized that not all LPP Mbs in the nascent hair follicle satisfy our functional criterion that only the MSC can survive in hair follicles after ACK2 injection (Nishimura et al., 2002). In order to measure the number of MSCs fulfilling this criterion, *Bcl2* null and wild-type neonates were subjected to ACK2 injection from P1.5–P3.5, and the number of Mbs in the LPP of hair follicles was counted. While some Mbs were detectable in the LPP of untreated *Bcl2* mice, we could not detect any Mbs in the LPP of *Bcl2* null mice (Figs. 3A and B). On the contrary, almost all of the hair follicles in the wild-type mice contain LPP Mbs. These results indicate that MSCs are not formed in *Bcl2* null mice. We also examined the hair follicles at P4.5 after whole-mount immunostaining of c-Kit and LacZ. We found that all the Mbs in the LPP of the *Bcl2* null mice are c-Kit$^+$ (Fig. 3C), while, in the LPP of wild-type mice, there is a mixture of the c-Kit$^+$ and c-Kit$^{low/-}$ Mbs (Figs. 3C and D).

Dynamic change in SCF expression in skin

The SCF/c-Kit signaling pathway has been implicated in Mb/Mc survival. While the expression of c-Kit in Mbs/Mcs in the skin has been studied extensively, the expression of its ligand during neonatal stages has not been reported in detail. We therefore investigated the expression pattern of SCF from E17.5 to P11.5 by immunohistochemistry.

At E17.5, SCF expression was found throughout the skin. It is expressed in the epidermis, developing hair follicles, dermal papilla and dermis (Fig. 4A). In the epidermis, its expression overlaps with K5 which marks epidermal keratinocytes. The expression in the epidermis and hair shaft (including the LPP) is reduced after birth. At P1.5, a reduction of SCF expression was observed in the skin, especially in the epidermis (Fig. 4B). The expression in the hair shaft further decreases by P3.5 (data not shown), though a high level of expression is maintained in the hair bulb. At P6.5, the expression of SCF was observed only in the bulb region (Figs. 4C and D). This expression pattern remains unchanged at least until P11.5, the latest stage of our analysis.

Rescue of MSCs in the Bcl2 null mice by overexpression of SCF

As SCF downregulation in the LPP is concomitant (P0.5–P4.5) with the dramatic reduction in Mb numbers observed in the *Bcl2* null, it seems possible that *Bcl2* is required for Mb survival under the situation in which SCF expression in the epidermis is downregulated. Provided that this is the case, it is expected that the Bcl2 dependency of Mbs will be overcome by sustained expression of SCF in the epidermis. In order to investigate this possibility, we generated *Bcl2* null mice expressing SCF in the epidermis and outer root sheath by mating mice bearing the *Bcl2* null allele with mice bearing a SCF transgene under the K14 promoter (Pao). In these mice, SCF expression in the epidermis and the LPP is maintained throughout their lifetime. We examined their coat color after the

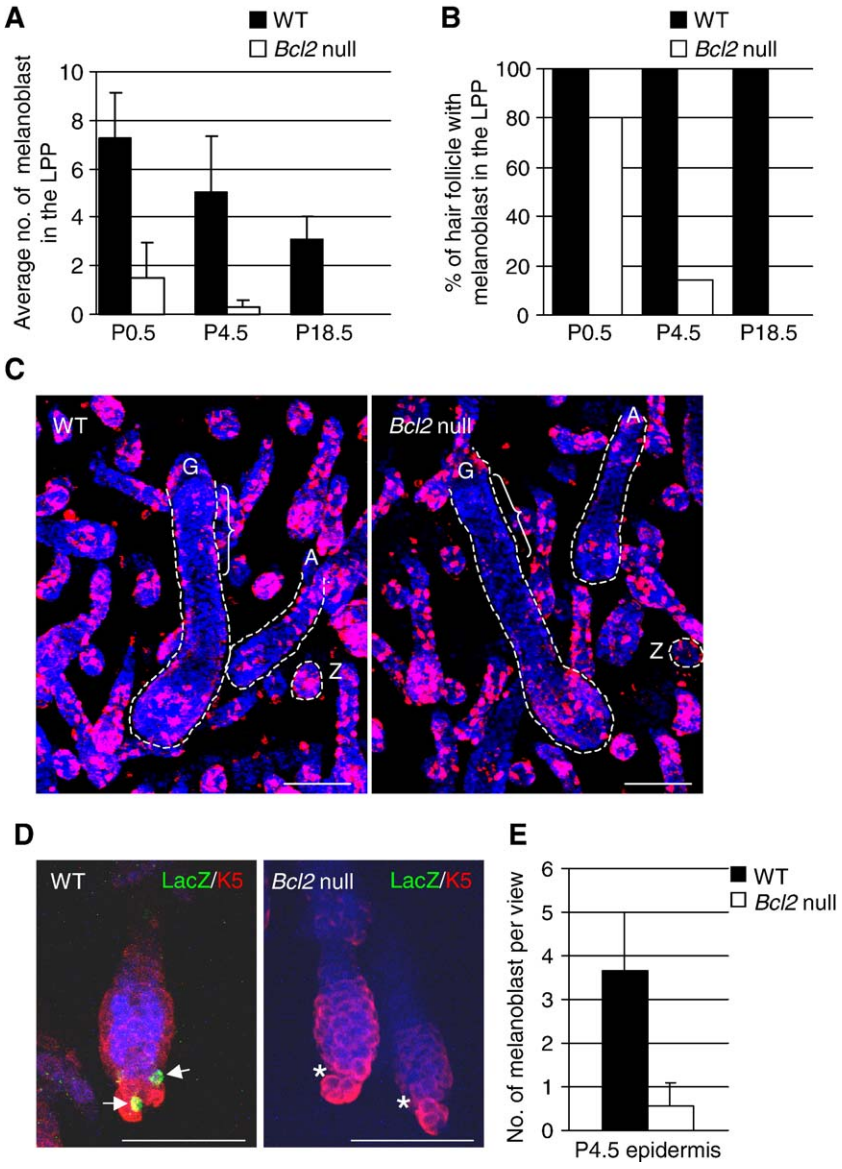

Fig. 1. Decrease of LPP Mb populations in *Bcl2* null mice from P0.5. (A) The average numbers of LPP Mbs in guard hair follicles at P0.5, P4.5 and P18.5. Mbs were counted after whole-mount immunostaining of LacZ (driven by *Dct* promoter, as the reporter of the Mc lineage). The average number of LPP Mbs at P0.5 is 3 times lower in the *Bcl2* null mice than in wild-type littermates, and, by P18.5, no LPP Mbs were observed. (B) The percentage of hair follicles that contain LPP Mbs at P0.5, P4.5 and P18.5. In the null mice, the percentage decreases dramatically from P0.5 to P4.5 and eventually to 0% by P18.5. The total number of hair follicles counted at P0.5 is 20 (wild type) and 60 (null); at P4.5, 64 (wild type) and 42 (null); and at P18.5, 36 (wild type) and 40 (null). Error bars show the standard deviation within each group. (C) Whole-mount immunostaining of skin at P0.5 with LacZ (red). A reduction in the number of LPP Mbs in the null hair follicle is observed (right) when compared to the wild-type littermate (left). White brackets indicate the approximate LPP in the guard hairs. Guard hair, awl hair and zigzag hair are marked as G, A and Z respectively. Scale bars represent 50 μm. (D) 3D reconstructed images of whole hair follicles from P18.5 wild-type (left) and null (right) mice. The empty LPPs in the null mice (*) show that there is a complete loss of MSCs in the mice. On the contrary, LPP Mbs are present in wild-type hair follicles and are indicated by white arrows. The hair follicles were stained with LacZ (green) and K5 (red) to show the Mbs and the outer root sheath of the hair follicle. Scale bars represent 50 μm. (E) Plot of the density of epidermal Mbs in P4.5 skin of wild-type and the null mice. A dramatic reduction of epidermal Mbs in the null skin is observed.

induction of the hair cycle by shaving at P18.5. At 15 days after shaving, most of the hairs in the *Bcl2*null-Pao mice are black (Fig. 5A), indicating a rescue of the hair pigmentation defect in the *Bcl2* null mice. Immunostaining of skin sections reveals the presence of numerous LPP Mbs in the *Bcl2*null-Pao mice (Fig. 5B). We also analyzed the skin at P18.5 by whole-mount immunostaining of the hair follicles, and the result shows that there are c-Kit^{+} as well as c-Kit$^{low/-}$ Mbs in the LPP (Fig. 5C). Hence, this rescue of melanocyte survival in *Bcl2*null-Pao mice is contributed by the rescue of MSCs rather than just a continuous proliferation of non-stem Mbs, indicating that Bcl2 is essential for the survival of Mbs at the neonatal stage when SCF expression is downregulated.

Discussion

A recent study (Nishimura et al., 2005) suggested that *Bcl2* is important for the survival of MSCs in the niche. This conclusion

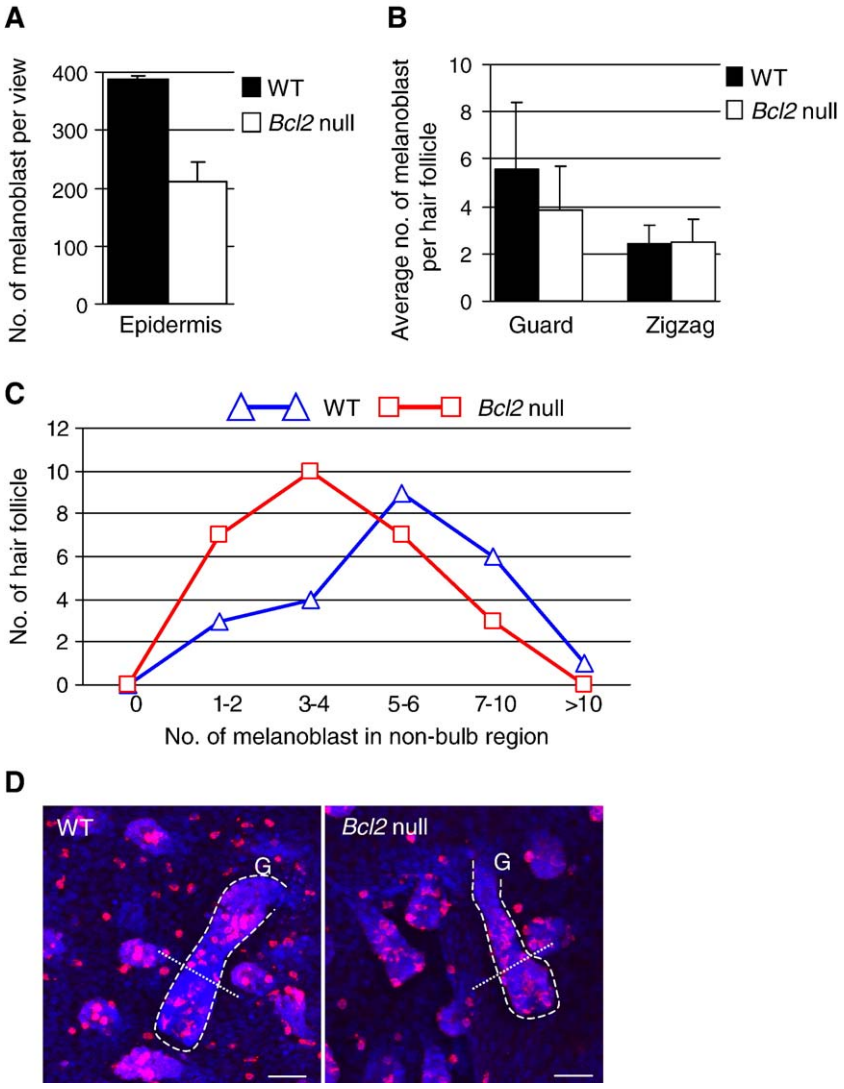

Fig. 2. Defects in the epidermal and follicular Mbs in E18.5 *Bcl2* null mice. (A) Plot of the density of epidermal Mbs in wild-type and null skin at E18.5. Skins of the null fetuses have only half the density of wild-type littermates. (B) Average number of non-bulb Mbs in guard hairs and total follicular Mbs in zigzag hairs at E18.5. A slightly reduced number of the non-bulb Mbs were observed in the guard hairs of the null fetus, but no difference in the total Mb number in the zigzag hairs was observed. (C) Distribution plot of the number of non-bulb Mb in guard hair follicles at E18.5. A leftward shift in the peak of the distribution curve of the null mice is observed. The peaks of the distribution curves are at 5–6 Mbs per hair follicle in wild-type but only 3–4 Mbs per hair follicle in the null mice. The numbers of whole hair follicle counted are 24 (wild-type) and 28 (null). (D) 3D reconstructed images of E18.5 skin from wild type (left) and the null (right). A reduced epidermal Mb density can be observed in the null mice skin. Mbs were stained with LacZ (red), and guard hairs are indicated as (G). Dotted lines indicate the estimated boundary of the bulb region. Error bars represent the standard deviation within each group. Scale bars represent 50 μm.

was based on the observation that, while Mbs colonize the LPP at P6.5, they are rapidly lost before P8.5 in *Bcl2* null mice. Since *Bcl2* has been implicated in the survival of many different cell types, it is conceivable that *Bcl2* may play a role in the survival of the MSC. However, a previous report from the same group (McGill et al., 2002) proposed that *Bcl2* expression is activated by c-Kit signaling which is likely to be inactive in MSCs since these cells express low or undetectable levels of c-Kit and are not dependant on SCF for their survival (Nishimura et al., 2002). These previous findings suggest that *Bcl2* is expressed and required by Mbs/Mcs during the c-Kit-dependent stage, but not by the c-Kit-independent MSC.

As shown by the previous report (Nishimura et al., 2005) and in this paper, Mbs in the LPP of *Bcl2* null mice are completely lost from the first anagen; therefore, *Bcl2* must function during neonatal stages. In this study, our results demonstrate that (1) the reduction in Mb numbers in *Bcl2* null mice can be observed from E18.5 in the epidermis and developing hair follicles and that the decrease becomes progressively more obvious after birth (P0.5), (2) we cannot detect any Mbs/Mcs that fit our definition of the MSC (Dct^{+}, c-$Kit^{low/-}$ and SCF/c-Kit signaling independent) in *Bcl2* null mice, suggesting that there is a complete absence of MSCs in these mice and (3) the hair pigmentation, together with MSCs in the LPP, can be rescued by SCF overexpression throughout the skin.

Nishimura et al. (2005) reported that Mbs of *Bcl2* null mice first colonize the LPP normally but then disappear after P6.5 and proposed that *Bcl2* is selectively required for MSC

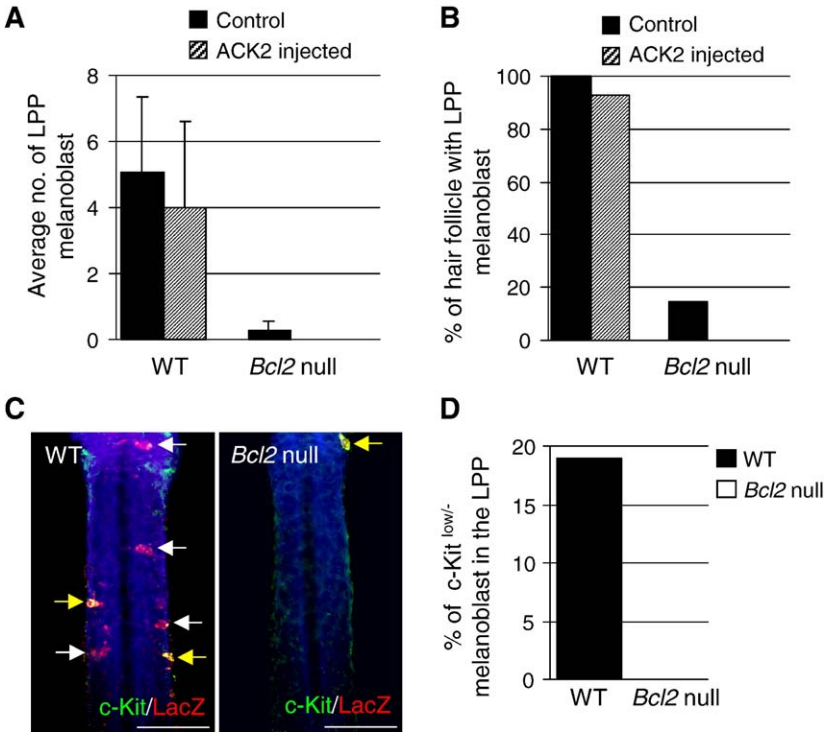

Fig. 3. Complete absence of the MSC in the *Bcl2* null mice. Wild-type and *Bcl2* null littermates were subjected to neonatal ACK2 treatment. The skins were harvested at P4.5, and the number of LPP Mbs was counted. (A) Average number of LPP Mbs with or without ACK2 treatment. The average number of LPP Mbs in the wild-type is slightly decreased after the treatment, but no LPP Mbs remained in the *Bcl2* null hair follicles. Error bars represent the standard deviation. (B) The percentage of hair follicles containing LPP Mbs with or without ACK2 treatment is shown. After treatment, more than 95% of hair follicles contain LPP Mbs in the wild type. However, no hair follicles contain LPP Mbs in the null mice. The total numbers of hair follicles counted in the no treatment groups are: 61 (wild type) and 40 (null); and that in the ACK2 treated groups are 37 (wild type) and 50 (null). (C) 3D reconstructed images of the LPP of a wild-type (left) and the null (right) hair follicles at P4.5. The samples were analyzed by whole-mount immunostaining of c-Kit (green) and LacZ (red). White arrows indicate the c-Kit$^{low/-}$ Mbs, and yellow arrows indicate the c-Kit^{+} Mbs. Scale bars represent 50 μm. (D) Percentages of c-Kit$^{low/-}$ LPP Mbs in P4.5 hair follicles. There is c-Kit$^{low/-}$ Mbs in the LPP of wild-type hair follicles, though we were unable to detect any c-Kit$^{low/-}$ Mbs in *Bcl2* null mice. The numbers of whole hair follicle counted are 28 (wild type) and 20 (null).

maintenance in the LPP. In contrast, we have found a reduction in the LPP Mb number in the epidermis and non-bulb region of hair shafts already at E18.5. More importantly, we could not detect MSCs in the LPP of *Bcl2* null mice. The discrepancies between our results and the previous report (Nishimura et al., 2005) can be explained partly by the means by which the defect was quantified and the difference in the definition of the MSC used. Nishimura et al. (2005) considered any melanoblasts present in the LPP region as MSCs, whereas we also considered their requirement for SCF signaling and their expression of c-Kit. Unlike in adult mice, the development of the different types of hair follicles is not synchronized in embryonic and neonatal skin (Schmidt-Ullrich and Paus, 2005). In order to avoid unnecessary confusion that may be caused by the complicated situation of the neonatal skin, we focused mainly on guard hairs so as to compare hair follicles at similar developmental stage at a given time point. Additionally, we exploited whole-mount preparations of skin samples to attain a precise measurement of the number of Mbs/MSCs in the LPP.

However, probably the most important reason for this discrepancy is the difference in the definition of the MSC. While Nishimura et al. (2005) define MSCs solely as *Dct*-LacZ^{+} cells localized in the LPP, we exploited additional criteria. It has been shown that the LPP of newborn hair follicles contain both MSCs and non-stem Mbs and that non-stem Mbs can be eliminated by ACK2 injection from P0.5 to P4.5 (Nishimura et al., 2002). In order to verify the stem identity of Mbs present in the LPP of P4.5 hair follicles of *Bcl2* null mice, we performed ACK2 treatment during this period. No LPP Mbs remained after ACK2 treatment of *Bcl2* null mice, while almost all hair follicles in wild-type littermates possessed Mbs in the LPP. Moreover, immunohistochemistry of P4.5 skin revealed that most of the Mbs in the LPP of *Bcl2* null mutants were c-Kit^{+}, while both c-Kit^{+} and c-Kit$^{low/-}$ cells were detected in the LPP of the control mice at the same age. Based on these observations, we conclude that there are no MSCs in *Bcl2* null mice and hence that Bcl2 must be required prior to or during the development of the MSC. Given this, it is not possible to determine the role of *Bcl2* in the maintenance of MSCs from the phenotype of these null mice.

Despite an absence of MSCs, *Bcl2* null mice have black hair until the first hair cycle. This observation indicates that the Mcs that function in the first hair follicles arise directly from embryonic precursors bypassing the MSC stage. Moreover, as we have shown that the hair bulb is the only region where SCF expression is maintained in postnatal life, it is plausible that Bcl2 is dispensable in the region where SCF is available. This is consistent with the fact that the development of Mcs in the bulb

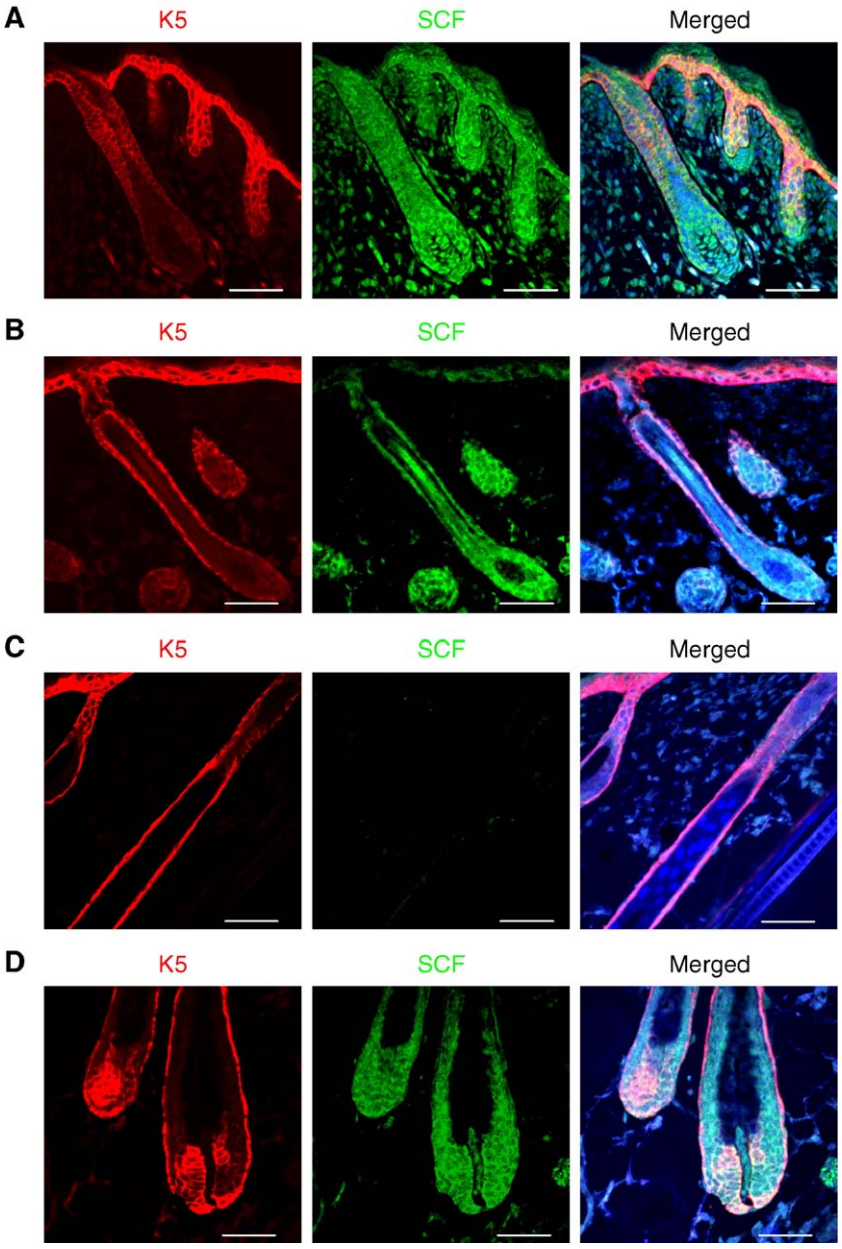

Fig. 4. SCF expression in the neonatal epidermis and hair follicles. (A) Sectioned immunostaining of E17.5 skin with K5 (red) and SCF (green). High expression of SCF was detected in the epidermis, hair shaft and bulb region of developing hair follicles and the dermis at E17.5. (B) SCF and K5 expression at P1.5. The SCF expression is reduced in the epidermis and the hair shaft compared to earlier stages but is maintained in the bulb region. (C and D) SCF expression at P6.5 skin is shown. SCF expression in the epidermis and hair shaft was not detectable (C), while the bulb expression remained high (D). Images of P6.5 follicles were captured with the same settings of the confocal microscope to allow direct comparisons. Scale bars represent 50 μm.

region is complete, and these Mcs are functionally normal in *Bcl2* null mice.

However, we also noted that the number of epidermal and non-bulb Mbs in *Bcl2* null mice is already lower than that of the wild-type littermates at E18.5. This suggests a general role for Bcl2 in the suppression of cell death throughout the development of the Mc lineage, though its role is more overt in the microenvironment where SCF is absent. Indeed, SCF expression in the epidermis and the LPP is gradually down-regulated after birth, and this coincides with the stage when the difference between the *Bcl2* null mutant and wild-type mice becomes overt. As SCF is essential for the survival of all Mbs and Mcs except MSCs in the LPP, this downregulation results in depletion of Mbs from the non-bulb area and is responsible for the development of the Mc distribution pattern in adult mice in which Mbs and MSCs are restricted to the LPP and hair bulb. Moreover, this restriction of MSCs to the LPP suggests that other factors produced in this region provide signals that are necessary for the survival in the absence of SCF. In wild type, the downregulation of SCF results in the death of Mbs, but a small number of Mbs are able to survive for a sufficient period of time to colonize the developing niche and become MSCs. In contrast, in the absence of Bcl2, Mbs die before MSCs are able to develop. Taken together, we hypothesize that Bcl2 is a cell

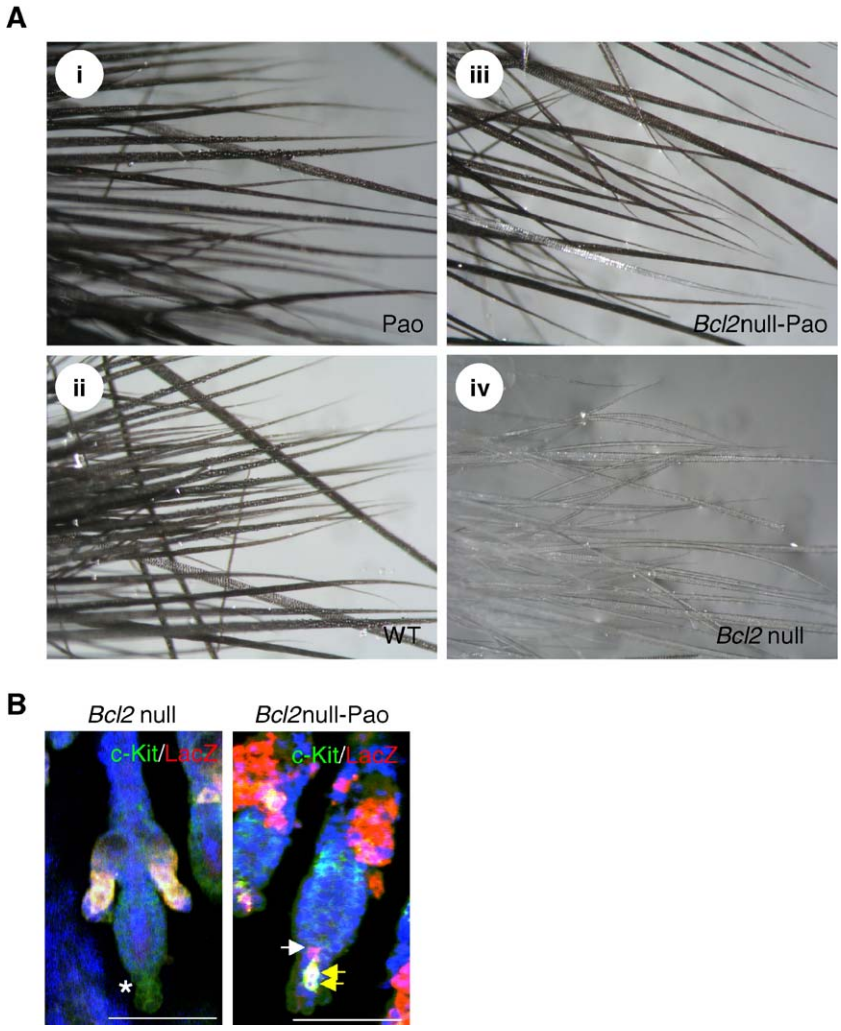

Fig. 5. Rescue of hair pigmentation and MSCs in *Bcl2* null mice by overexpression of SCF. (A) Hair color of *Bcl2*-Pao littermates at 15 days after induction of the first hair cycle. Wild-type littermates have black hairs regardless of the presence (i) or the absence (ii) of SCF overexpression. In *Bcl2*null-Pao, almost all the hairs are black (iii) in contrast to the *Bcl2* null littermates (iv). Magnifications are 100×. (B) Whole-mount immunostaining of hair follicles at P18.5. The presence of c-Kit$^{low/-}$ LPP Mbs in the *Bcl2*null-Pao mice (right) also indicates that there is a rescue of MSCs in these mice. White arrow indicates c-Kit$^{low/-}$ Mbs, and yellow arrows indicate c-Kit^{+} Mbs in the LPP. On the other hand, no Mb can be found in the *Bcl2* null littermates (left). The empty LPP in the *Bcl2* null littermates are indicated by asterisk (*). Scale bars represent 50 μm.

survival factor that is complementary to SCF that supports Mb survival in a Bcl2-independent manner. Thus, its effect becomes overt only when SCF becomes unavailable.

In Fig. 6, we illustrate our hypothesis of the development of the MSC. After birth, the expression of SCF is downregulated in the epidermis and the upper part of hair follicles, resulting in the cell death of Mbs in these areas. Under normal circumstances, the life span of Mbs is sufficiently long to allow the formation of MSCs in all hair follicles through interaction with the newly formed niche. In the *Bcl2* null mutant, the rate of Mb death exceeds the speed of their maturation to MSCs. Consequently, MSCs do not develop in any hair follicles of *Bcl2* null mice. However, as the Bcl2 role can be superseded by SCF, Mbs are expected to differentiate to MSCs in the *Bcl2* null mutant if SCF expression is maintained in the epidermis.

To examine this hypothesis, we generated *Bcl2*null-Pao mice, in which SCF is continuously expressed in the epidermis and the outer root sheath (ORS) of the hair follicle (including the LPP). The hair after the induction of the first hair cycle in these mice is mostly black. Although it is possible that the rescue of the pigmentation defect in these mice results from a continuous survival and proliferation of differentiated Mcs, the presence of unpigmented and c-Kit$^{low/-}$ Mbs in the LPP suggests strongly that some Mbs mature to MSCs even in the continuous presence of SCF. In *Bcl2*null-Pao mice, epidermal Mbs are also maintained after birth. This indicates that the loss of Mbs from the neonatal epidermis is solely due to the programmed downregulation of SCF in the neonatal epidermis. Moreover, this observation shows clearly that *Bcl2* is not necessary for SCF-dependent Mb survival in the epidermis. It has been suggested that there are Dct^{-} multipotent stem cells that reside in the LPP region that can give rise to Mcs (Sieber-Blum et al., 2004). While we think it is unlikely that MSCs are derived from this or other Dct^{-} population, we cannot exclude this possibility and it should be addressed in future studies. Rescue of hair pigmentation in the *Bcl2* null mice was also

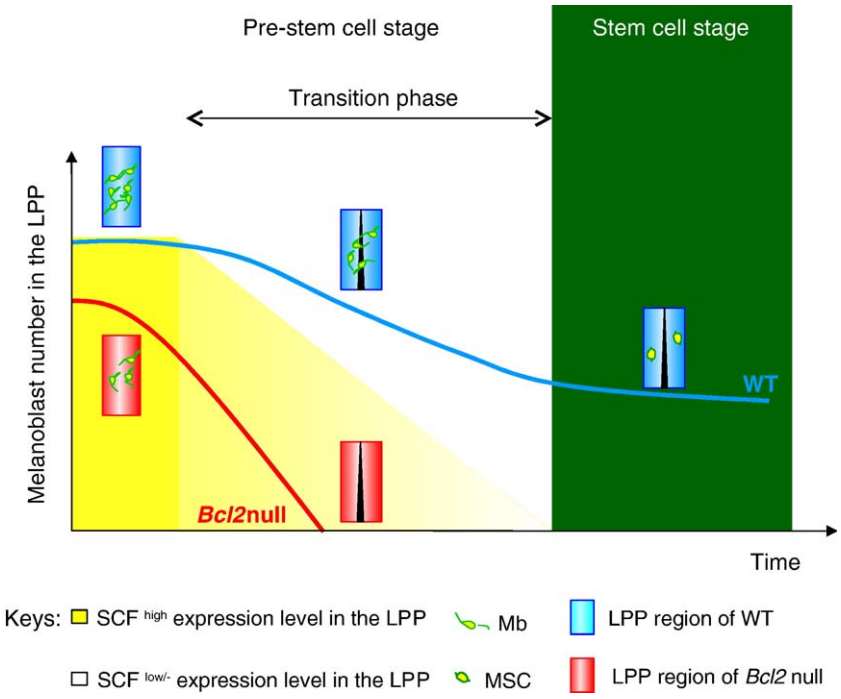

Fig. 6. Proposed model of *Bcl2* function during the development of the MSC. The MSC system can be divided into the pre-stem cell stage, before the MSC has appeared, followed by the stem cell stage (green area). During the pre-stem cell stage, SCF is expressed (indicated by yellow) at a high level throughout the skin. During this time, active Mb migration and proliferation occur along the epidermis and inside the developing hair follicles. We refer to the time of the transition from this stage to the stem cell dominant stage as the transition phase. The transition phase is characterized by the downregulation of SCF expression in both the epidermis and the hair shaft (including the LPP). In the absence of SCF, the LPP Mbs are completely dependent on *Bcl2* for their survival. In the wild type (blue line), *Bcl2* expression in the LPP Mbs allows the cells to survive until they can be recruited into the niche and become MSCs. In the absence of *Bcl2* (red line), the LPP Mbs cannot survive through this transition phase, and they die before they can differentiate to mature stem cells.

observed when *Bim* was simultaneously knocked out (Bouillet et al., 2001). It seems possible that, in the complete absence of *Bim*, the pro-apoptotic counterpart of *Bcl2*, pre-stem Mbs can survive through the transition phase and allow the formation of MSCs to occur in these double null mice.

In summary, our data demonstrate that *Bcl2* is required for extending the life span of Mbs at early neonatal stages when SCF is deprived. Thus, *Bcl2* expression is up-regulated by SCF/c-Kit signaling during the c-Kit-dependent phase but exerts its primary effect only after SCF is deprived. Thus, SCF signaling has a two-pronged effect in Mbs; it provides an immediate survival signal as well as promotes the accumulation of the *Bcl2* protein, which allows Mbs to survive for a limited period after the withdrawal of SCF. Indeed, the extended life span supported by *Bcl2* is essential for the development of MSCs in all hair follicles. While this result is consistent with the first conclusion of Fisher and his colleagues that *Bcl2* is activated downstream of c-Kit and Mitf, it is not consistent with their second conclusion that *Bcl2* is a requisite for the maintenance of MSCs in the LPP.

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