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Research Article

Melanocyte fate in neural crest is triggered by Myb proteins through activation of c-kit

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Abstract. The c-*myb* proto-oncogene and its oncogenic derivative v-*myb*^{AMV} encode transcriptional regulators engaged in the commitment of hematopoietic cells. While the c-Myb protein is important for the formation and differentiation of various progenitors, the v-Myb^{AMV} oncoprotein induces in chicks a progression and transformation of the single (monoblastic) cell lineage. Here we present the first evidence of cell fate-directing abilities of c-Myb and v-Myb^{AMV} proteins in avian neural crest (NC), where both proteins determine melanocytogenesis. The increased concentration of c-Myb induces progression into dendritic melanocytes and differentiation. The v-*myb* oncogene converts essentially all NC cells into melanocytes and causes their transformation. Both Myb proteins activate in NC cells expression of the c-*kit* gene and stem cell factor c-Kit signaling – one of the essential pathways in melanocyte development. These observations suggest that the c-myb-c-kit pathway represents a common regulatory scheme for both hematopoietic and neural progenitors and establishes a novel experimental model for studies of melanocytogenesis and melanocyte transformation.

Keywords. c-*myb* proto-oncogene, v-*myb*^{AMV} oncogene, neural crest, cell fate determination, melanocytes, c-kit signal.

Introduction

Transcription factors c-Myb and v-Myb were originally recognized as key regulators in hematopoietic cells. c-Myb sustains proliferation of all types of immature hematopoietic cells (reviewed in [1]). It is required for the development of definitive erythroid and myeloid lineages [2] as well as for the survival and differentiation of specific stages of T- and B-cells [3, 4]. In addition, c-Myb regulates stem/progenitor cells in colonic crypt and adult neurogenic zones (reviewed in [5]) and in the neuroectoderm of the early chick embryo [6]. The gene is also expected to function in vascular smooth muscle cells [7, 8]. Graded intracellular concentrations of c-Myb significantly influence its biological activity, including the lineage choice process. Higher c-Myb concentrations in definitive murine hematopoietic progenitors accent the development of erythroid cells, while low concentrations result in the preferential development of megakaryocytes and macrophages [9]. Graded levels of c-Myb also participate in the formation, survival and motility of avian neural crest (NC), with higher concentrations activating the epithelio-mesenchymal transition in the neuroectoderm and the formation of migratory NC cells [6].

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The oncogene of avian myeloblastosis virus, *v-myb*^{AMV}, is the truncated version of the c-*myb* gene. It contains only the central part of c-*myb* coding sequences with several point mutations, and its constitutive expression is driven by the retroviral promoter. Similarly to c-Myb, the v-Myb oncoprotein binds specifically to the Myb recognition DNA element (MRE) and regulates transcription of adjacent genes (reviewed in [10]). In contrast to c-Myb, however, it distorts the development of common myeloid progenitors by favoring the monocyte/macrophage lineage at the expense of other lineages [11], blocks terminal differentiation of immature macrophage precursors – monoblasts – and induces their malignant transformation (reviewed in [10]). In cooperation with bFGF, v-Myb can also promote exclusive erythroid development in primitive progenitors [12].

The second *myb* allele, v-*myb*^{E26}, composed of fused gag-myb-ets sequences, transforms myeloid and erythroid cells as well as their multipotent progenitors. The E26 oncogene also has the potential to affect the developmental fate of the progeny of multipotent hematopoietic progenitors [13]. In addition, it has been shown to transform melanocytes in chick embryo cultures in cooperation with the exogenous EGF (epidermal growth factor) receptor [14].

The NC cells develop in early vertebrate embryos at the border between the neural plate and the non-neural ectoderm. These multipotential cells delaminate from the dorsal neural tube in a temporary rostrocaudal wave and migrate throughout the embryo. NC cells derived from the trunk region of the neural tube differentiate into neurons and glia of the sensory and sympathetic ganglia, neuroendocrine cells of the adrenal medulla and melanocytes [15–17]. A fraction of pluripotent NC cells persists in adult tissues [18, 19]. Formation of NC cells and specification of their developmental fates are controlled by complex signalling events that are not yet completely known (reviewed in [20–25]).

NC-derived melanocyte precursors in chicken embryos emerge relatively late and enter the dorsal path of migration [26–28]. In addition to Wnt signals [29], their formation also requires signals from stem cell factor (SCF) and endothelin 3, which activate their cognate receptors c-Kit and endothelin B – ET (B) – respectively [30–32]. These signals activate intrinsic determinants of melanocytic differentiation, including Pax3 and several members of the Sox gene family, and activate synthesis and function of the microphthalmia-associated transcription factor (Mitf), which is a central factor of the melanogenic cascade that is essential for the maturation of pigment cells [33, 34].

We found that both Myb proteins support the development of melanocyte lineage in chick embryo neural tube explants. In contrast to c-Myb, the v-Myb oncoprotein strongly interferes with the differentiation of NC cells, inducing formation of transformed pigment cells at the expense of the neuroglial progeny. We show that a significant part of the molecular mechanism that Myb proteins trigger to determine melanocyte cell fate is activation of the SCF/c-Kit signal.

Materials and methods

Embryos, cell cultures and viruses. Brown Leghorn chick blastoderms and neural tube explants were prepared, infected with c-myb and v-myb retroviruses, and cultivated in liquid or semisolid media as described elsewhere [6, 11]. The v-myb mutations N118D [35], L237A [36] and L3,4A [11] were prepared by a mutated primer/PCR approach. v-mybER and N118D v-mybER were constructed from v-*myb* and N118D v-*myb* genes by replacing the TAG stop codon with the fusion sequence encoding two hemagglutinin epitopes and the mouse estrogen receptor containing the G525R mutation [37]. 200 nM 4-hydroxytamoxifen (Sigma) was used for induction of v-MybER proteins. In the control Δmyb virus genome the *myb* gene was deleted. Proportions of melanocytes in the cultures were obtained by counting pigmented and total cells in the cultures. Images of 10 randomly selected fields of each culture were captured for cell counting using a reticle. Data are expressed as a sample mean. Error bars represent standard deviations.

Antibodies and chemicals. The rabbit polyclonal anti-Myb and neutralizing anti-SCF antibodies were described [6, 38]. Monoclonals 1E8, MelEM and kit2c75 were from DSHB (Iowa City, Iowa, USA) and SouthernBiotech (Birmingham, AL, USA), respectively. Affinity-purified $F(ab')_2$ fragments of goat anti-mouse FITC (fluorescein isothiocyanate)-conjugated or anti-rabbit Cy3-conjugated IgGs (immunoglobulin) were from Jackson Immunoresearch Laboratories (West Grove, PA, USA). The AG1296 inhibitor was purchased from Calbiochem (Darmstadt, Germany).

Immunofluorescence and flow cytometry analyses. Cells for immunofluorescence analysis or Giemsa staining were processed as described [11]. Fluorescence images were captured using a DMIRB microscope, DFC480 camera and IM500 software (Leica Microsystems, Wetzlar, Germany) and processed using Adobe software (Adobe Systems, San Jose, CA, USA). Growing NC cells and CEFs for flow

cytometry were washed 3 times with culture media+1% BSA and exposed sequentially to kit2c75 and the secondary FITC-conjugated antibodies (1 h each) in the media+0.5% BSA at 37 °C. Unreacted antibodies were removed by 3 washes with the media+0.5% BSA. Cells were detached by 1% collagenase (PAA Laboratories, Linz, Austria) for 30–45 min at 37 °C, until approximately 90% of cells were released, collected by low-speed centrifugation suspended in buffered saline and analyzed in FACSort (Becton Dickinson, San Jose, CA, USA). Analysis of data and histogram overlays were performed with WinMDI software. All histograms were gated on propidium iodide-negative cells.

Western blot analysis. Western blot analyses were performed as described previously [11]. Total proteins were compared on Coomassie-stained gels. Equivalent protein samples were loaded. PageRuler prestained protein ladder (Fermentas) was used as marker.

RT-PCR. Total RNA was isolated from cell cultures using RNAwiz (Ambion). Carefully quantitated RNAs (for semiquantitative RT PCR) were reverse-transcribed to cDNA (complementary DNA) using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Semiquantitative PCR reactions contained 200 μM dNTPs, 200 nM primers, 0.7 μl of cDNA and 0.02 U/μl of Taq polymerase in the magnesium-containing buffer (Promega) in 20 μl. Samples of all PCR reactions were checked electrophoretically at various numbers of PCR cycles to ensure that all the reactions are observed in the exponential phase. Cycling parameters for electrophoretograms shown in figures were as follows: 95 °C 20 s, annealing temperature (2 °C below the calculated T_m of a given pair of primers) 20 s, 72 °C 45 s: 28 cycles with MITF-F (5′-CGGCTCTGAATACCCACTCACG) and MITF-R (5′-CGAGCTTGCATCTCAAGTTCC TG), resulting product 580 bp; 27 cycles with CKIT-F (5′-GCAAAGGGCATGAGCTTCC TGG) and CKIT-R (5′-GGCTCCTCTTCTTCCAGATGCCAC), resulting product 624 bp; 30 cycles with SCF-F (5′-ACCAGAGAATGATTCCAGAGTCGCTG) and SCF-R (5′-GCAAACATGGAACTGTTACCTGCCAG), resulting product 406 bp (these reactions also contained, in addition to standard components, 1 M betain); and 22 cycles with GAPDH-F (5′-CCATGACAACTTTGGCATTG) and GAPDH-R (5′-TCCCCACAGCCTTAGCAG), resulting product 164 bp. The primer pairs were derived from different exons.

Real-time PCR. For quantitation of c-kit cDNA sequences, primers 5KITAE (5′-GTCGAGAGCACTGTGAACGCCAGC) and 3KITAE (5′-CTATGATGCACATCAGTCCAGCGGC) yielding the 187-bp product were used in addition to the above GAPDH-F and GAPDH-R. Reactions were performed in triplicate using DyNAmo SYBR Green qPCR kit (Finnzymes) on the DNA Engine Opticon2 (MJ Research) according to the manufacturer's protocol.

Results

c-Myb and v-Myb proteins induce accumulation of pigment cells in chicken blastoderm cultures; v-Myb causes their transformation. We previously demonstrated that c-Myb is synthesized in the majority of cells of the early chicken embryo and is significantly involved in the formation of the trunk NC [6]. In the present work we analyzed the influence of c-Myb and v-Myb proteins on early chick embryo cells, including NC.

First, c-*myb* or v-*myb* genes were expressed in explanted blastoderms of the developmental stage HH10 [39] using MAV-1-based retroviruses, and the synthesis of Myb proteins was determined (Fig. 1c). Infected explants were either grown in liquid cultures or dispersed and seeded into semisolid media. The liquid c-myb and v-myb cultures differed after 7 days of cultivation from cultures infected with the control (Δmyb) virus by high counts of hematopoietic blasts and by the morphology of cells attached to dishes (Fig. 1a). In c-myb cultures, hematopoietic cells were represented mainly by myeloblasts, which frequently differentiated into macrophages. Up to 5% of attached cells were pigmented melanocytes characterized by a rather high content of pigment granules and differentiated morphology (Fig. 1b, c-myb panels). v-myb cultures contained mainly typical v-*myb*-transformed monoblasts and usually over 5% of attached cells were melanocytes characterized by rather immature appearance, with a lower content of pigment granula (Fig. 1b, v-myb panels). In uninfected or control retrovirus-infected cultures virtually no pigmented cells were found (Fig. 1a, Δmyb panel).

Only v-myb monoblasts and melanocytes formed colonies in semisolid media (Fig. 1d-i). Some colonies of pigmented cells reached a 1-mm diameter (Fig. 1d-ii) and contained over 10^5 cells (16 generations). Cells isolated from such colonies continued proliferating in liquid cultures for an additional 20–30 generations with a doubling time of 32–36 h. In dense cultures, pigmented cells detached from the dish surface, adopted a rounded shape and adhered to one another,

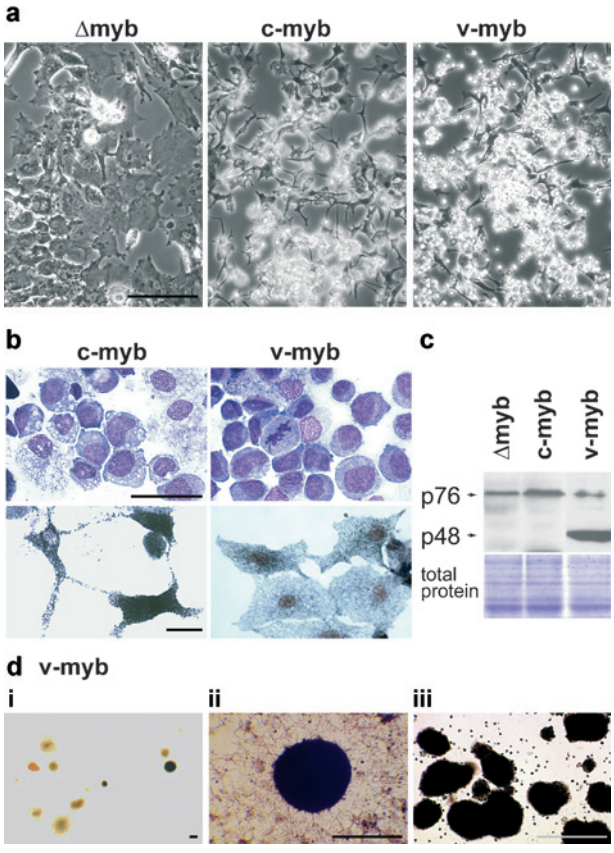

Figure 1. c-*myb* and v-*myb* genes promote accumulation of immature myeloid cells and melanocytes in blastoderm cultures. (*a*) Phase contrast images of cultures infected with the control Δmyb, c-myb and v-myb retroviruses. Bar, 50 μm. (*b*) Giemsa-stained hematopoietic cells (upper panels) and melanocytes (lower panels) in cultures infected with c-myb or v-myb retroviruses. Bar, 10 μm. (*c*) Western blot detection of c-Myb (76 kD) and v-Myb (48 kD) proteins in the lysates of cultured cells shown in (*a*) selected by G418. (*d*) Transformation of hematopoietic cells and melanocytes by v-*myb*; (i) light colonies of hematopoietic cells and dark colonies of melanocytes in semisolid media. (ii) The large melanocyte colony with outgrowing unpigmented melanoblasts. (iii) Floating clusters of v-*myb*-transformed melanoblasts in a liquid culture. Bar, 0.5 mm.

forming large floating clusters (Fig. 1d-iii). After some time, these clusters readhered, and pigmented cells grew out from them and multiplied until they reached a density activating their detachment and formation of new floating clusters. After a total of 40–50 generations cells stopped proliferating, turned into adherent dendritic melanocytes and finally decomposed. Repeated experiments clearly showed that both c-*myb* and v-*myb* genes activate the occurrence of pigment cells in whole-embryo cultures, and that the v-*myb* oncogene transforms these cells and induces their anchorage-independent growth and long-term proliferation.

Myb proteins activate formation of NC-derived melanocytes. Two types of pigment cells are easily detectable in developing embryos – retinal pigment epithelium and NC-derived skin melanocytes. In order to confirm that analyzed pigment cells are NC derivatives, trunk neural tube fragments containing unfused neural folds were isolated, infected with concentrated retroviral stocks (c-myb, v-myb or Δmyb) and placed onto collagen-coated plates. Following 12–16 h pioneer cells started to emigrate from successfully adhered explants. The emigrated cell populations were analyzed 7–12 days later. The results from six independent sets of cultures showed that while Δmyb virus-infected and non-infected populations contained at most 20% melanocytes, c-myb and v-myb cultures invariantly contained about 40 and 95% of pigment cells, respectively, documenting the capability of Myb proteins either to activate melanocyte cell fate in NC cells or to support the proliferation of committed melanocytes.

Myb proteins commit NC cells to the melanocyte cell lineage. To find out whether Myb proteins can determine melanocyte lineage choice, c-*myb* and v-*myb* genes were introduced into naive neural plates which contain only prospective NC cells and no committed melanocyte progenitors. Retroviruses used in this work efficiently transduce *myb* genes into undifferentiated neural tissues, including NC cells [6]. In neural plate explants both *myb* genes caused cell delamination and formation of pigmented melanocytes (Fig. 2). Thus, the v-*myb* oncogene, like c-*myb* [6], can activate very early steps in the formation of trunk NC, and both Myb proteins appear to sustain differentiation of early NC cells to the melanocyte cell lineage.

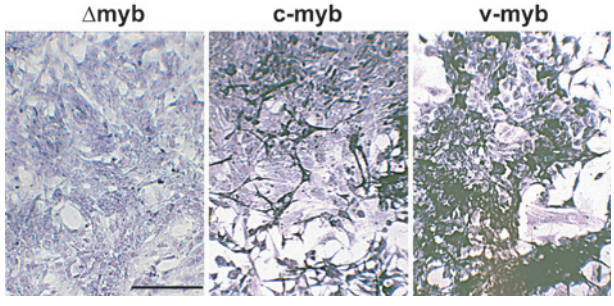

Figure 2. *myb* genes induce formation of melanocytes (dark pigmented cells in c-myb and v-myb panels) in naive neural plate explants. Phase contrast images of representative fields of cultures infected with the control Δmyb, c-myb and v-myb retroviruses.

The early NC cells emigrating from trunk neural tube fragments containing unfused neural folds were used for further experiments. In the embryo, trunk NC

cells delaminate from neural folds and invade surrounding tissues in waves. Cells of the early wave differentiate mainly into neurons and glial cells of the peripheral nervous system. Cells of the second delayed wave differentiate into melanocytes [27, 40]. This mode of NC emigration/differentiation is to some extent also recapitulated in tissue culture. Cells of the early wave spontaneously differentiate *in vitro* mainly into glial cells. However, as they contain oligopotent precursors, including bipotential glial-melanocytic progenitors [40, 41], they have the potential to develop along the melanocyte cell lineage when induced, for example, by bFGF or bFGF+SCF, which both participate in the induction of melanogenesis [30, 42]. We isolated early wave NC cells and used them to study the effects of Myb proteins on cell lineage determination. As expected, the vast majority of these cells contained the HNK-1 epitope (Fig. 3a), which is typical for undifferentiated migrating NC cells [43]. As shown in Figure 3b, which summarizes results of four independent experiments, Δmyb virus-infected cells differentiated into non-pigmented cells (mainly glial), while after 10 days such cultures treated with bFGF or bFGF+SCF contained 25 or 80% of pigment cells, respectively. The early wave cells were then infected with c-myb and v-myb retroviruses and analyzed 7–12 days later. Both c-myb and v-myb directed early wave cells to the melanocyte cell lineage. In c-myb cultures pigment cells represented about 50% of cells, while in v-myb cultures typically 90% of the cells were melanocytes.

The strong melanocyte-promoting activity of v-Myb was strictly dependent on the integrity of DNA binding, transactivation and leucine zipper domains. As shown in Figure 3b, the N118D mutation, which completely eliminates the specific DNA binding of Myb proteins [35], and the L237A mutation, which inactivates Myb transactivation domain [36], eliminated melanocyte formation. Similarly, replacement of crucial leucine residues L325 and L332 within the zipper domain by alanines, referred to as the L3,4A mutation, significantly reduced v-Myb ability to redirect glial development to melanocyte cell lineage. The L3,4A mutation was previously shown to strongly reduce the ability of v-Myb to direct the commitment of common myeloid progenitors to monocyte/macrophage lineage and to transform monoblasts [11]. Since none of the mutations significantly affected the steady-state concentration of v-Myb proteins (Fig. 3b), it is evident that the same v-Myb domains (functions) necessary for the commitment of hematopoietic progenitors into the monocyte/macrophage lineage and transformation of monoblasts are also required for the direction of NC progenitors to melanocyte cell lineage and transformation of melanocytes.

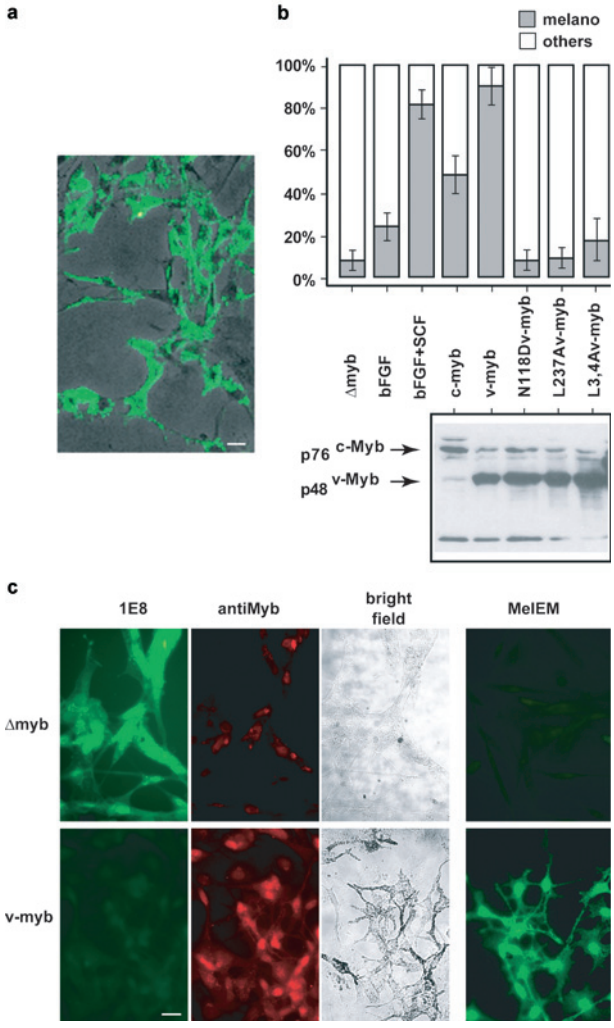

Figure 3. *myb* genes direct the development of early neural crest (NC) cells into melanocytes. (*a*) Adobe Photoshop overlay of the phase contrast and HNK-1 fluorescence (green) images (the same field) of cells emigrating in an early wave from neural fold explants. (*b*) The ratios of non-melanocytes (others) and pigmented melanocytes (melano) in cultures of early wave NC cells infected with Δmyb and treated with bFGF or bFGF+SCF, or infected with retroviruses carrying c-*myb*, wt v-*myb*, DNA-binding domain mutant of v-*myb* (N118Dv-myb), transactivation domain mutant of v-*myb* (L237Av-myb) and the leucine zipper mutant of v-*myb* (L3,4Av-myb). The data were obtained in four independent experiments. The abundance of Myb proteins in selected cultures was determined by Western blot using antiMyb antibody. (*c*) The presence of lineage-specific antigens in differentiating early wave NC cells infected with Δmyb and v-myb viruses. 1E8 and MelEM antibodies reveal glial cell- and melanocyte-specific antigens, respectively. The 1E8, antiMyb and bright field images are taken from the same field. Bars, 10 μm.

Antibodies against specific antigens were used to prove the identity of the cells studied. Early-wave progenitors infected with the Δmyb retrovirus differ-

entiated into 1E8-positive glial cells (Fig. 3c), just like non-infected progenitors (not shown). On the other hand, cells in myb-infected cultures (exemplified by the v-myb cells in Fig. 3c) did not synthesize the 1E8 antigen in accordance with their melanocyte phenotype and strongly stained with the melanocyte-specific MelEM antibody, recognizing the glutathione S-transferase subunit synthesized specifically in NC melanoblasts committed to produce the pigment [44, 45]. The control glial cells were negative. In v-myb melanoblasts the MelEM antigen was distributed essentially as described [46]. In mid-early cells (7 days after emigration) the antigen was mostly scattered in the cytoplasm; later (12 days after emigration), it was found concentrated around the nuclei (Fig. 3c). In highly pigmented differentiated melanocytes no immunoreactivity with the MelEM was observed (not shown). It is worth noticing that the melanocytogenic activity of *myb* genes did not require additional exogenous inductors (bFGF, SCF).

The induction of melanocyte formation by c-Myb, v-Myb or by bFGF+SCF was possible only when early wave cells were treated by these factors soon after emigration while they were 1E8-negative. Later infection or addition of factors resulted in progressively lower amounts of melanocytes in cultures; almost no melanocytes were obtained when *myb* genes or cytokines were introduced to 1-week-old cultures in which the majority of cells were 1E8-positive (not shown). This supports the notion that the early wave cell population we used is composed mainly of uncommitted progenitors, which gradually lose their ability to differentiate into melanocytes along with their progression into glial cells. Importantly, total cell counts in cultures infected with viruses (c-myb, v-myb or Δmyb) or treated with cytokines steadily increased, while the massive changes of the ratio of melanocytes/other cells took place. No signs of cell death were observed. This suggests that pigment cells were generated by the conversion of progenitors and not by the outgrowth of a few pigment cells that have spontaneously arisen nor by the death of non-pigmented cells.

Myb proteins activate expression of key regulators of the melanogenic cascade. We analyzed expression of selected crucial factors engaged in melanocytogenesis.

The introduction of c-*myb* and v-*myb* genes into early wave cells correlated with the accumulation of transcripts of the *mitf* gene (Fig. 4a) – the pivotal factor for the progression of melanocyte development [47]. mRNA (messenger RNA) of the tyrosinase gene – the target gene of the Mitf factor – was also strongly upregulated in c-myb and v-myb cells (not shown). The activity of the Mitf protein is controlled in several ways. Among them, Mitf modifications induced by SCF/c-Kit signaling represent an activating cue important for the development of melanocyte cell lineage [48, 49]. Since c-*kit* expression depends on c-Myb in immature erythroid cells [50], we asked whether there is a link between c-Myb and c-*kit* expression in NC cells as well. As documented in Figure 4a, infection of early wave cells with c-myb and v-myb resulted in the accumulation of c-kit mRNA, while no striking changes were observed in the levels of SCF mRNA. To find out whether *myb* genes also promote accumulation of the c-Kit protein at the cell surface, NC cells emigrating in early and late waves from the HH10 neural tube explants were pooled and infected with Δmyb, c-myb and v-myb retroviruses and analyzed by flow cytometry with the kit2c75 anti-chicken c-Kit antibody. The overlays in Figure 4b document a detectable level of c-Kit in control NC cells (present on late wave cells, which contain committed melanocytes) and its significant increase following introduction of c-*myb* and v-*myb* genes. The overlays also reveal that while there are still some c-Kit-negative cells in the c-myb-infected population, almost no such cells can be found in v-myb cultures. This corresponds well to the proportion of melanocytes in c-myb and v-myb cultures.

While the importance of c-*myb* for c-*kit* expression has been already documented [50], no such data are available for v-*myb*. To find out whether the increase of c-kit mRNA depends on v-Myb and is not solely caused by altered composition of cells in a differentiating cell culture, first wave NC cells were exposed for 32 h to retroviruses transducing the 4-hydroxytamoxifen (4-OHT)-inducible v-mybER and N118Dv-mybER (unable to bind the Myb recognition sequence) variants and then treated with 4-OHT for 7 or 24 h. cDNAs were assayed for the relative concentrations of c-kit sequences by semiquantitative and quantitative PCR (Fig. 5a) and protein lysates (prepared from cells immediately prior to 4-OHT treatment) for the presence of Myb fusion proteins (Fig. 5b). At the time of analysis not all the cells were productively infected, and the amounts of synthesized fusion proteins were only similar to that of the endogenous c-Myb (Fig. 5b). Nevertheless, it was critical to analyze cells well before any signs of differentiation became apparent and any significant changes in culture composition could take place. The results of the real-time PCR (columns) as well as the semiquantitative PCR (electrophoretograms) shown in Figure 5a document the increase in c-kit mRNA concentration already 7 h after 4-OHT induction, which became more pronounced after 24 h of 4-OHT treatment. It is apparent that v-MybER displays some

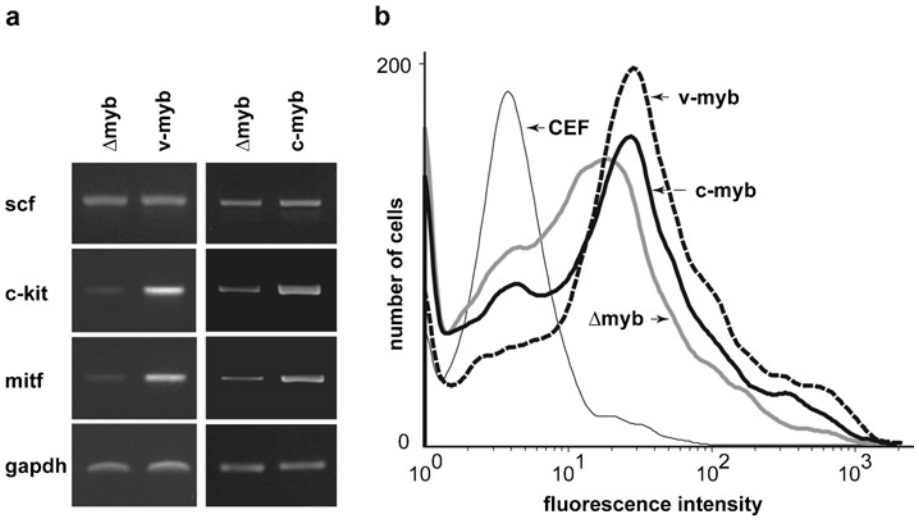

Figure 4. Upregulation of melanocyte-specific factors in pooled early and late wave NC cells. (*a*) Semiquantitative RT-PCR determination of relative concentrations of scf, c-kit, mitf and gapdh transcripts in cells infected with Δmyb, v-myb or c-myb retroviruses. (*b*) Flow cytometry quantification of the cell surface c-Kit protein. Chick embryo fibroblasts (CEF) were used as the negative control.

basal activity and increases c-kit mRNA even in the absence of 4-OHT, as indicated by comparison of v-mybER unstimulated cells with control cells (infected with Δmyb virus). Importantly, both the basal and 4-OHT-inducible effects of v-MybER were completely eliminated by the N118D mutation in the v-Myb DNA binding domain. This supports the concept of direct involvement of v-Myb in the activation of c-*kit* expression.

As early NC cells produce SCF [51], the increased levels of the c-Kit receptor caused by Myb proteins are likely to result in enhanced c-Kit signaling. This might represent at least some of the mechanism through which Myb proteins activate melanocytogenesis in NC cells.

Myb proteins activate SCF/c-Kit signaling in NC cells. In order to learn whether the melanocyte-promoting activity of Myb proteins is mediated by the SCF/c-Kit signal, early wave cells were infected with c-myb, v-myb and Δmyb retroviruses and treated with inhibitors of SCF/c-Kit signaling, namely the neutralizing anti-SCF antibody [38] and the AG1296 inhibitor of c-Kit tyrosine kinase activity [52], which were applied to cultures 12 h after retroviral infections. The average cell counts obtained 10 days after infection in two independent experiments are plotted in Fig. 6. Both the antibody and the AG1296 tyrphostine significantly reduced the number of melanocytes in c-myb and v-myb cultures to approximately 50–30 % of values obtained in the absence of these compounds. The melanocytogenic activity of c-Myb was slightly more sensitive to c-Kit signal inhibitors than the activity of v-Myb. Concomitant with the decrease in melanocytes, the number of other, mainly glial cells in these cultures increased. We conclude that at least some of the melanocytogenic activities of c-Myb and v-Myb proteins are dependent on the SCF/c-Kit signal. This signal is likely activated by the Myb-dependent increase in the cell surface concentration of the c-Kit receptor. Thus, the c-myb–c-kit pathway is involved in cell fate determination not only in hematopoietic progenitors [50] but also in NC cells.

Discussion

We identified c-Myb and v-Myb^{AMV} transcription factors as powerful inducers of the melanocytogenesis in avian neural tube explants. Both factors efficiently promoted melanocyte development in explanted neural folds, where the formation of NC cells has already been activated and where the early stages of lineage specification programs have been launched. Furthermore, Myb proteins induced melanocytogenesis in neural plate epithelium, where these programs have not been initiated. These results confirm our recent observations that c-Myb is involved in early inductive events in the neural tube leading to NC formation [6] and reveal similar activity of the v-Myb oncoprotein. Moreover, they document the capability of both Myb proteins to specify development of melanocyte cell lineage. At least a part of the molecular mechanism(s) underlying these Myb activities could be activation of c-*kit* transcription or c-kit mRNA stability, leading to accumulation of c-kit mRNA and to the rise in c-Kit receptor concentration. It can be expected that higher c-Kit levels in NC cells result in activation of the SCF-c-Kit melanocytogenic signal, since NC cells produce SCF [51]. The promoter of the c-*kit* gene has been long considered a candidate Myb target promoter [53, 54]. However, there is no evidence that Myb proteins directly regulate c-*kit* gene promoter in NC cells, and further work is needed

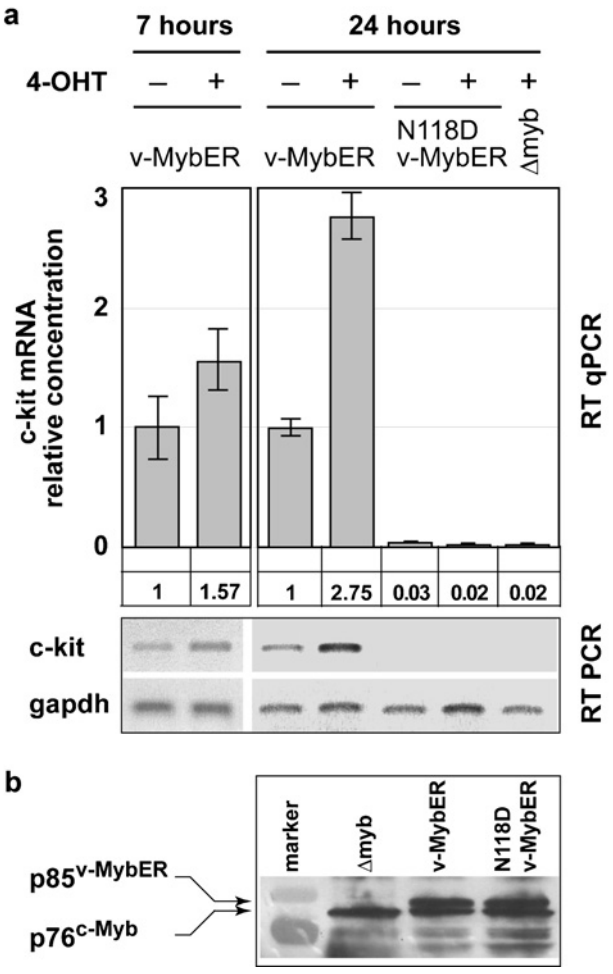

Figure 5. Increase in c-kit mRNA depends on v-Myb. (*a*) Early wave NC cells were infected with v-mybER-, N118Dv-mybER- or Δmyb retroviruses; cultures were split 32 h post-infection and half of them treated with 200 nM 4-OHT. In two independent experiments RNAs were isolated either 7 or 24 h later, and cDNAs were subjected to PCR with c-kit or gapdh primers. The column diagram summarizes results of real-time PCR (RT qPCR). The electrophoretograms show results of semiquantitative PCR (RT PCR) with CKIT-F and CKIT-R and GAPDH-F and GAPDH-R primers. (*b*) Western blot analysis of first wave cells infected for 32 h with Δmyb, v-mybER and N118D v-mybER retroviruses using antiMyb antibody.

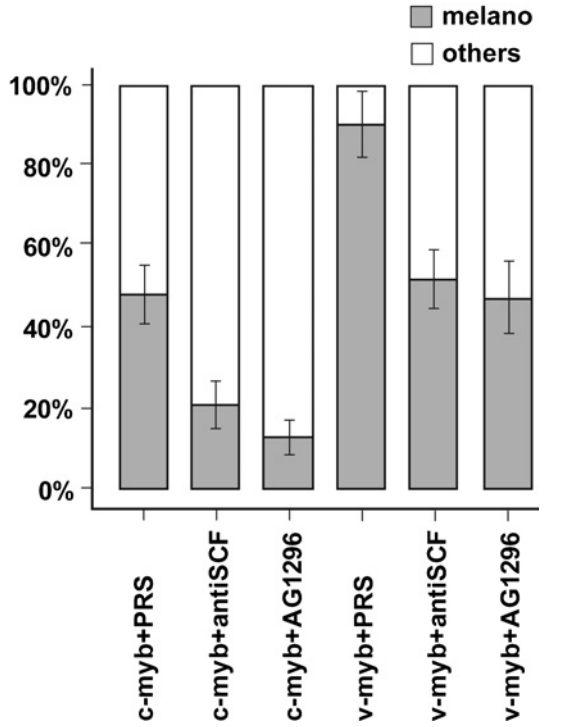

Figure 6. Inhibition of c-myb and v-myb melanocytogenesis by compounds interfering with SCF/c-Kit signaling. Early wave NC cells infected with c-myb or v-myb retroviruses were treated with preimmune rabbit serum (PRS), neutralizing anti-SCF rabbit antibody or with AG1296 inhibitor of c-Kit phosphorylation. The amounts of pigmented melanocytes and other cells in individual cultures were determined as in Figure 3. The data were obtained in two independent experiments.

to prove their interaction both in hematopoietic and NC cells. The promoter of the SCF gene could also be directly regulated by c-Myb, as suggested by analyses of SCF production in fetal liver stroma in c-*myb*$^{-/-}$ mice [55]. However, in our experiments with NC cells no changes in SCF mRNA were observed in response to elevated c-Myb or v-Myb proteins.

Activation of the c-Kit signal *per se* is probably not sufficient to induce *mitf* transcription. However, Myb proteins display a rather complex integration with the signaling network of progenitors. We observed that introduction of c-Myb into neural tube epithelium treated with BMP4, SCF or bFGF leads to differing results. For example, elevated concentrations of c-Myb combined with bFGF result in massive delamination and outgrowth of melanocyte precursors and pigmented melanocytes, while in cooperation with BMP4, rather undifferentiated progenitors accumulate in culture [V. Karafiat, unpublished observation]. Thus, other pathways, in addition to the c-Kit signal, are likely modulated by Myb proteins. One of them might be the EGF receptor signal [14].

In neural tube epithelium the v-Myb oncoprotein induces development of cells of the melanocytic lineage at the expense of other lineages. In melanocyte lineage-restricted precursors it causes a partial block of their terminal differentiation, activation of their proliferation and induction of transformation. Thus, c-Myb and v-Myb activate similar events – commitment of progenitors to the same cell lineage and activation of a set of genes responsible for melanogenesis. This is in contrast to conclusions based on expression profiles [56]. The discrepancy is likely due to the cell type used for the experiments and to the inferential spectrum of surrounding regulatory factors. We suggest that in cells that naturally use c-Myb for regulation, v-Myb can operate in the same pathways, although with

eventually different phenotypical outcome caused by the deregulated nature of the oncoprotein.

The complex effect of both *myb* genes on NC formation and melanocyte development recalls their effects on hematopoietic cells. The results of this work support the idea of overlapping genetic programs and similar cell fate specification regulatory pathways in both cell compartments [57, 58]. We hypothesize that at least in birds, c-Myb is involved in molecular mechanisms controlling proliferation, survival and fate determination programs related in both hematopoietic and NC progenitors. One of these mechanisms might be activation of c-Kit signaling, as suggested by recent experiments in mice [50] and data in the work described here. If activation of c-Kit signaling is a common c-Myb property, then other cell types dependent on the SCF/c-Kit signal may also require c-Myb as a part of this pathway.

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