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This article has been published in *Developmental Biology, 2006*
doi : 10.1016/j.ydbio.2006.09.032 ; Elsevier
The publication is available online at
http://www.sciencedirect.com/

Research Article; Submission to ***Developmental Biology***

Clonally cultured differentiated pigment cells can dedifferentiate and generate multipotent progenitors with self-renewing potential

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Running title: Melanocyte reversion to neural crest-like stem cells

Key words: quail embryo, in vitro culture, self-renewal, melanocyte, glia, myofibroblast, stem cell, differentiation, neural crest

Abbreviations: CNS, central nervous system; d, culture day; E, embryonic day; ET3, endothelin 3; ETRB, endothelin receptor B; MelEM, melanoblast/melanocyte early marker; Mab, monoclonal antibody; NC, neural crest; NCCs, neural crest cells; PNS, peripheral nervous system; □SMA, alpha smooth muscle actin; SMP, Schwann cell myelin protein.

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Abstract

The differentiation of a given cell should be irreversible in order to ensure cell-type specific function and stability of resident tissue. However, under stimulation in vitro or during regeneration, differentiated cells may recover properties of immature cells. Yet the mechanisms whereby differentiated cells can change fate or reverse to precursor cells are poorly understood. We show here that neural crest (NC)-derived pigment cells that have differentiated in quail embryo, when isolated from the skin and clonally cultured in vitro, are able to generate glial and myofibroblastic cells. The phenotypic reprogramming involves dedifferentiation of dividing pigment cells into cells that reexpress NC early marker genes *Sox10*, *FoxD3*, *Pax3* and *Slug*. Single melanocytes generate multipotent progenitors able to self-renew along serial subcloning, thus exhibiting stem cell properties. The presence of endothelin 3 promotes the emergence and maintenance of multipotent progenitors in melanocyte progeny. These multipotent cells are heterogeneous with respect to marker identity, including pigmented cells and dedifferentiated cells that have reacquired expression of the early NC marker HNK1. These data provide evidence that, when removed from their niche and subjected to appropriate culture conditions, pigment cells are phenotypically unstable and can reverse to their NC-like ancestors endowed with self-renewal capacity.

Introduction

The notion that cell differentiation is unidirectional and irreversible has been challenged in several biological systems. This is the case of the vertebrate nervous system, where precursors and stem cells exhibit cell-fate plasticity under the influence of environmental factors (Bjornson et al., 1999; Clarke et al., 2000; Doetsch et al., 2002; Kondo and Raff, 2000, 2004). These findings, together with classical examples of the recruitment of already differentiated cells to produce new cell types in regenerating amphibian eye and limb, have shed a new light on the plasticity of the differentiated state (Okada, 1991; Brockes and Kumar, 2002; Gardiner et al., 2002; Henry, 2003). It has even been proposed that stem cell properties might be retained (or acquired) by virtually any cell including those considered as highly differentiated ones (reviewed in Blau et al., 2001; Zipori, 2004). The view that stem cells can cross lineage boundaries is presently the subject of controversies, essentially due to non-reproducibility of certain published data (for references, see Anderson et al., 2001; Weissman et al., 2001; Wagers et al., 2002). Moreover, in normal physiological conditions, change in cellular phenotypes seems to be extremely rare if it exists at all.

In the work presented here, we have addressed the problem of the stability of the differentiated state by using a well-defined model, the derivatives of the neural crest (NC). This system allows manipulating individual cells and identifying with specific molecular markers their various differentiation states and developmental options. Diversification of the multiple phenotypes arising from the NC in the vertebrate embryo is spatially and temporally controlled by the interplay of cell intrinsic changes and local environmental cues that NC cells (NCCs) encounter during migration and proliferation (reviewed in Le Douarin and Kalcheim, 1999; Le Douarin et al., 2004). As attested by accumulating evidence from clonal cell cultures devised in birds (Baroffio et al., 1988; Baroffio et al., 1991; Sieber-Blum and Cohen, 1980; Sieber-Blum, 1989) and mammals (Stemple and Anderson, 1992; Ito et al., 1993; Hagedorn et al., 1999; Morrison et al., 1999), a subset of early migratory NCCs is composed of multipotent stem cells able to produce several, and in some cases, all the NC-derived lineages while other NCCs seem to be committed to a given type of derivatives (Henion and Weston, 1997; Luo et al., 2003). This led us to propose a hierarchical model of lineage diversification during avian NC ontogeny (Baroffio et al., 1991; Trentin et al., 2004). In mammals, a trunk NC progenitor for autonomic neurones, peripheral glial cells and myofibroblasts, has been characterised as a stem cell, on which the action of growth factors and regulatory genes has been deciphered at the single cell level (Shah et al., 1994, 1996; Hagedorn et al., 1999; Morrison et al., 2000). In quail NC cultures, the cytokine endothelin 3 (ET3) acts as a survival and mitogenic factor on committed melanocytic precursors and promotes proliferation and self-renewal of a common progenitor for melanocytes and glial cells (Lahav et al., 1996, 1998; Trentin et al., 2004). ET3 binds to G protein-coupled endothelin receptor B (ETRB) (Masaki, 2004) and ET3/ETRB signalling is required for early development of melanocytes and enteric nerve cells in the mouse (Baynash et al., 1994; Hosoda et al., 1994; Puffenberger et al., 1994; Shin et al., 1999; Lee et al., 2003). In avian NC, melanocytic precursors downregulate expression of *ETRB* and switch to another receptor, *ETRB2*, which later on is exclusively expressed by differentiated pigment cells (Lecoin et al., 1998). ET3 strongly induces expression of *ETRB2* by *ETRB*-expressing early-migrating NCCs, thus it can be considered as a specifying signal toward the melanocytic differentiation pathway (Lahav et al., 1998).

Consistent with a stepwise restriction model of NCC differentiation potentialities, post-migratory NCCs comprise mainly committed precursors that yield various types of differentiated cells. However, in vitro clonal analysis has revealed that NC-derived cells isolated from avian peripheral nervous system (PNS), skin, cardiac outflow tract and branchial arches, exhibit developmental capacities broader than expected (Duff et

al., 1991; Sextier-Sainte-Claire Deville et al., 1992, 1994; Ito and Sieber-Blum, 1993; Richardson and Sieber-Blum, 1993; Sieber-Blum, 2004). In rodents until late embryogenesis and even in adults, the dorsal root ganglia, sciatic nerves, enteric ganglia and the skin retain a resident pool of multipotent NC stem cells (Morrison et al., 1999; Hagedorn et al., 1999; Bixby et al., 2002; Kruger et al., 2002; Sieber-Blum et al., 2004; Fernandes et al., 2004). Those cells may be responsible for the wide plasticity of NC-derived cells, which was revealed when PNS ganglia taken from the quail, were back-transplanted into the NC migration pathway of younger chick host embryos. Following implantation, sensory and sympathetic ganglion cells either died (for post-mitotic neurones) or changed their fate, recovering migration and differentiation potentials normally found only in early NCCs (for references, Le Douarin and Kalcheim, 1999). The transplantation of chimeric sensory ganglia (wherein either neurones or non-neuronal cells harbour the quail nuclear marker) has proven the non-neuronal identity of the ganglion cells endowed with developmental plasticity (Ayer-Le Lievre and Le Douarin, 1982). However, direct evidence for the undifferentiated and stem cell state of the cells able to reprogram is currently lacking. The possibility therefore remains that NC-derived cells already engaged in cell differentiation might exhibit developmental plasticity, as reported recently for corneal stromal keratocytes (Lwigale et al., 2005). In that context, we previously reported lineage conversion by differentiated cells of NC origin. Thus, Schwann cells and pigment cells taken from embryonic sciatic nerves and dorsal epidermis, respectively, were able to convert into each other in vitro (Dupin et al., 2000; Dupin et al., 2003; Real et al., 2005). In both cases, the phenotypic transition was stimulated by the presence of ET3 and involved reversion of glial cells and melanocytes to a bipotent state. However, it was not determined whether these cells can be diverted to additional NC lineages and if the phenotype transition requires previous generation of dedifferentiated cells endowed with multipotency and self-renewal. These issues are important to understand how the stem cell phenotype may emerge and be retained in the NC and its derivatives. They also concern the role played by intercellular signals and cell proliferation in the stability of the differentiated state, both in development and in the adult.

Here we addressed these questions by investigating the cellular mechanisms involved in the phenotypic reprogramming of NC-derived pigment cells isolated from the skin of quail embryos up to hatching stage. Single pigment cells and their progeny were analysed in serially transplanted clonal cultures with respect to their developmental potential, mitotic activity and expression of lineage-specific markers. We provide evidence that single pigment cells can dedifferentiate and give rise to glial cells, myofibroblasts and multipotent NC-like progenitors capable of self-renewing during clonal propagation. Such reprogramming occurs as melanocytes divide and is highly favoured by ET3.

Materials and Methods

Pigment cell polyclonal and clonal cultures

Melanocytes from the dorsal skin of embryonic day 7.5 (E7.5) quails were obtained in 48-hour-cultures of dissociated epidermis as described (Dupin et al., 2000). Pigment cells were purified by micromanipulation and individually plated in culture plates (96 wells, Nunc) previously coated with human plasma fibronectin (Sigma, St Louis MO). Polyclonal cultures were prepared by seeding 20-25 cells per well and analysed at different time points (using the 2-day-epidermal cell cultures as d0-time point). Pigment cells were also isolated from quail skin at hatching (E16) stage, using the same procedure except for an additional incubation with collagenase/trypsin-EDTA (Sigma) (1:3 for 10 minutes at 37ºC). Clonal cultures of the pigment cells from E7.5 and hatching stages were verified for clonality 4 hours after plating and grown in culture medium ±100 nM human ET3 (Sigma) for 11 and 15 days, respectively. Control medium was as previously described for quail NC-derived cells (Dupin et al., 2000; Dupin et al., 2003; Trentin et al., 2004; Real et al., 2005).

In vitro subcloning experiments

Serial subcloning of the progeny of E7.5 melanocytes was performed essentially as described for NCCs (Trentin et al., 2004). Briefly, the cells in control and ET3-treated melanocyte clones were detached with trypsin-EDTA (Sigma) at culture day 11 (d11). From each clone, 64 cells were randomly selected by micromanipulation and replated individually in the same medium as the parental clone. The procedure was repeated until cell growth arrest (Fig. 5A). In each step, 3 to 4 distinct clones were used for subcloning and the other ones were analysed by immunocytochemistry.

In particular experiments, only the pigmented cells present in ET3-treated d11-primary clones were subcloned after selection under bright field microscopy.

In another series of experiments, we subcloned the immediate progeny of pigment cells at the 2- and 4-cell stages. The daughter and granddaughter cells grown in presence of ET3 were detached after 24 and 48 hours, respectively and re-plated individually in the same medium. The resulting siblings of clones were maintained until d11.

Isolation and clonal cultures of the HNK1-labelled cells present in pigment cell progeny

Polyclonal cultures of melanocytes derived from E7.5 epidermis were treated with ET3 and immunostained with HNK1 (Abo and Balch, 1981) at d11. Living cultures were incubated for 20 minutes at room temperature, first with undiluted HNK1 hybridoma supernatant and second, with Texas Red-conjugated anti-mouse IgM (1:75 in DMEM 3% FCS) (Southern Biotechnology Ass. Birmingham, Al). $HNK1^+$ cells were selected by micromanipulation under inverted fluorescence microscopy (X70 Olympus) and grown individually in ET3-supplemented medium until d11.

Immunocytochemistry and in situ hybridisation

Cell phenotypes in the cultures were analysed as previously described (Dupin et al., 2000; Dupin et al., 2003; Trentin et al., 2004) using the following markers: monoclonal antibody (Mab) against Schwann cell myelin protein (SMP) (Dulac et al., 1988) for glial cells, Melanocyte/melanoblast early marker (MelEM) Mab (Nataf et al., 1993) for unpigmented melanoblasts and pigmented cells, anti-alpha smooth muscle actin □SMA) (1A4, Sigma) for myofibroblasts (Skalli et al., 1986) and antibody against 200-kDa neurofilament protein (Sigma) and quail tyrosine hydroxylase Mab (Fauquet and Ziller, 1989), for neurones. Labelling with secondary antibodies (Southern Biotechnology Ass.) was performed according to Trentin et al. (2004).

Expression of the genes encoding chick transcription factors Sox10 (Cheng et al., 2000), FoxD3 (Dottori et al., 2001), Pax3 (Goulding et al., 1993) and Slug (Nieto et al., 1994) was assessed in d0, d3, d7 and d11-pigment cell polyclonal cultures by in situ hybridisation according to Lahav et al. (1998). RNA probes were labelled by incorporation of digoxigenin (dig)-UTP (In vitro transcription kit, Promega) and hybridisation was detected using anti-dig antibody conjugated with alkaline-phosphatase (Roche). The cultures were subsequently immunolabelled with HNK1, as described above.

Differences in clone numbers between ET3-treated and control cultures were analysed by χ^2 test (GraphPad, San Diego, CA) and considered to be statistically significant when $P<0.05$.

Results

Myofibroblastic and glial cells differentiate from cultures of isolated melanocytes

In order to characterise the ability of differentiated melanocytes to convert into alternative NCC phenotypes, we have grown in low-density cultures a pure population of pigment cells that were isolated from E7.5 quail epidermis cultures and selected individually under the microscope. These polyclonal cultures were analysed with NC-specific lineage markers after different times in absence or presence of ET3, a peptide that promotes avian melanocyte proliferation through binding to endothelin receptor B2 (ETRB2) (Lahav et al., 1998; Lecoin et al., 1998; Dupin et al., 2000).

In both ET3-treated and untreated cultures, pigmented melanocytes were the only cells detected one day after plating (d1) (Fig. 1A). They expressed MelEM antigen (Nataf et al., 1993) and *ETRB2* (Lecoin et al., 1998) (not shown), which both are specific for avian pigment cells in vivo, but they did not exhibit reactivity to the following non-melanocytic lineage markers: the □SMA myofibroblast marker (Skalli et al., 1986), the avian glial marker SMP (Dulac et al., 1988) and the neuronal markers, 200kDa neurofilament protein and tyrosine hydroxylase. During subsequent days of culture, pigment cells while dividing, exhibited progressively diluted pigment granules. At d3, a subset of $\square SMA^+$ cells was identified which showed a spindle-shape fibroblastic morphology (Fig. 1B). Later on, a number of $\square SMA^+$ cells adopted myofibroblastic morphology (Fig. 1D). SMP^+ glial cells appeared between d7 and d9 (Fig. 1C). Some of the glial and myofibroblastic cells still expressed the MelEM melanoblast marker. In both ET3-treated and untreated cultures, most of the melanocytic cells were pigmented after d9 and the neuronal markers (neurofilament proteins and tyrosine hydroxylase) were not detected at any time. D9 and d11-cultures thus contained three cell phenotypes that normally arise from the NC, i.e., melanocytes, myofibroblasts and glial cells. D11-cultures also presented some unpigmented cells in which none of the lineage markers could be identified (lin^- cells). It has to be noted that in both control and ET3-supplemented media, the onset of myofibroblastic and glial marker expression followed the same temporal sequence.

Dedifferentiated cells bearing early NC markers arise from cultured pigment cells

The presence of apparently undifferentiated (lin^-) cells suggested that cultured melanocytes have dedifferentiated to immature NC-like cells. We therefore looked for the presence of early NC markers, such as the transcription factors *Sox10*, *FoxD3*, *Pax3* and *Slug*, which are expressed from the earliest stages of avian NC development and, except for *Sox10*, are later down-regulated in differentiating melanocytes (Epstein et al., 1991; Nieto et al., 1994; Southard-Smith et al., 1998; Dottori et al., 2001; Kim et al., 2003). In addition, we looked for immunoreactivity to HNK1 epitope which, in birds, is carried by most of the migratory NCCs

and later in development, only by the glial and neuronal NC derivatives (Tucker et al., 1984; Nataf et al., 1993).

Consistent with their melanocytic phenotype, pigment cells from E7.5 epidermis did not express these early NC markers when transferred in vitro (d0-cultures), except for some slightly pigmented cells that contained low levels of *Sox10* transcripts (not shown). When melanocyte polyclonal cultures were further grown in control and ET3-supplemented medium, *Sox10* and *FoxD3* became expressed from d3 in many cells that exhibited reduced or no pigmentation (Fig. 2A, B). *Pax3* and *Slug* mRNAs were detected later, from d7 in presence of ET3, and from d11 in controls, mostly in unpigmented cells (Fig. 2C, D). All these genes were still expressed in d11-cultures (Fig. 2C-E). HNK1-labelled cells, identified from d5 onwards, were also present in ET3-treated and untreated d11-cultures (Fig. 2F). These cells accounted for about 5% of total cells and were composed mostly of lin^- cells, although about 10%, 5% and 20% were immunoreactive to SMP, □SMA or MelEM, respectively. In addition, most $HNK1^+$ cells expressed *Sox10* and *FoxD3* transcripts (Supplementary Fig. 1), further arguing that the majority of $HNK1^+$ cells are undifferentiated NC-like cells. Therefore, unpigmented dedifferentiated cells expressing early NC markers are generated in the progeny of pigment cells at early culture times and persist at least until d11, when they coexist with differentiated melanocytes, glial cells and myofibroblasts.

Single pigment cells generate multilineage clones

In order to investigate whether individual pigment cells can generate both glial and myofibroblastic cells, we analysed the clonal progeny of pigment cells obtained from E7.5 epidermis. The cloning efficiency was similar (about 80% of plated cells) in control and ET3-treated d11-cultures (Supplementary Fig. 2A). However, the cell proliferation rate was higher in presence of ET3 (Supplementary Fig. 2B), as previously reported (Dupin et al., 2000). Phenotypic analysis indicated that cells of the melanocytic lineage (i.e., pigmented and unpigmented $MelEM^+$ cells) were present in all clones, either alone (M clones) or together with other phenotypes. The heterogeneous clones were of three types: GMF clones containing glial and melanocytic cells together with myofibroblasts (Fig. 3A, B), GM clones with both glial cells and melanocytes (Fig. 3C, D) and MF clones with both melanocytic and myofibroblastic cells (not shown). Within these heterogeneous clones, most of the cells belonged to the melanocytic phenotype. The proportion of non-melanocytic cells in these clones was different in control and ET3-supplemented media. In the case of SMP^+ cells, they represented 2.0±0.7% of the total number of cells in control colonies (n=5), as compared to 0.5±0.3% in ET3-supplemented clones (n=5, P=0.0039). This difference results from the higher number of melanocytic cells generated in the presence of ET3. In addition to cells expressing phenotypic markers, all clones included a few lin^- cells (not shown).

Quantification of clone types (Fig. 3E) showed that, in control medium, 70% of pigment cells yielded M clones while the heterogeneous clones (i.e., sum of GMF, GM and MF clones; ∑h) accounted for 30% of total clones. In contrast, in presence of ET3, the proportion of heterogeneous clones reached 72% (Fig. 3E; ∑h, P=0.02) and the remaining clones were of the M type. Therefore, myofibroblasts and/or glial cells can be generated from single melanocytes that display multilineage developmental potentials.

Multipotent cells are generated during early cell divisions of cultured melanocytes

From the above results, it may be assumed that individual pigment cells behave as multipotent progenitors from which myofibroblastic and glial lineages segregate. However, the time when such segregation occurs remained unknown. Our finding that some □SMA-expressing cells are already present at d3 (Fig. 1B) raised the possibility that lineage-restricted precursors are generated during early pigment cell divisions. We thus compared the developmental potentials of paired daughter cells arising from the first pigment cell division in presence of ET3, after re-plating them separately in clonal cultures. The same experiment was performed after single pigment cells were allowed to develop until they yielded four (granddaughter) cells.

All d11-clone pairs (n=14) obtained from paired daughter cells grown in presence of ET3 were found to be of an identical (GMF, GM or M) phenotype. Fig. 4 shows one of these siblings comprising two GMF clones that displayed about the same total number of cells and distribution of differentiated cell types. Seven siblings of clones derived from granddaughter cells were also analysed (Table 1). In the siblings where the four clones derived from the same pigment cell had survived, 40% were composed of identical GMF clones. These data indicate that the corresponding founder pigment cells have undergone two symmetrical cell divisions giving rise to identical multipotent cells. In contrast, the remaining founder pigment cells yielded different types of granddaughter cells including both GMF cells and one or two more restricted (GM, MF or M) cells (Table 1). These findings thus suggest that the glial and myofibroblast lineages are not segregated in the immediate pigment cell progeny, which rather is composed of multipotent cells.

Multipotent cells arising from melanocytes are able to self-renew

To investigate whether the multipotent GMF, GM and MF cells produced by embryonic pigment cells during early cell divisions are able to self-renew (i.e., by definition, are generated in their own progeny), we performed successive subcloning of cells randomly taken from control and ET3-treated d11-primary clones (clones I) (Fig. 5A). Secondary (II), tertiary (III) and quaternary (IV) clones were analysed at d11 for cell growth and differentiation, as previously described for clones I (see Fig. 3E).

Between cloning I and II in control medium, we observed a decrease in the number of clone-forming cells (from 80% to 55% of plated cells) (Supplementary Fig. 2A) and in the total cell number per clone (from 50 to 4 cells per clone; Supplementary Fig. 2B). Further subcloning in control medium was precluded due to the very low cell proliferation rate found in clones II. By contrast, in presence of ET3, clones II exhibited more than 100 cells per clone (Supplementary Fig. 2B) and the progeny of single melanocytes could be propagated at high clonal efficiency until cloning step IV (Supplementary Fig. 2A). Cell phenotypes were analysed in clones II to IV to determine the different clone (and progenitor) types propagated in control and ET3-supplemented media (Fig. 5B). First, we found that clones II in both media included the GMF, GM (Fig. 6A, B), MF and M types already found in clones I. In addition, we identified new types of clones, either containing both glial cells and myofibroblasts (GF clones, Fig. 6C, D), or only glia (G clones), myofibroblasts (F clones) or lin^- cells (undefined U clones). In presence of ET3 as compared to controls, the frequency of the heterogeneous clones II (i.e., GMF+GM+GF+MF, ∑h) was highly enhanced due to significant increase in GM and GMF clones (∑h, P=0.03; Fig. 5B). Staining with HNK1 showed that dedifferentiated $HNK1^+$ cells were still present in the majority of ET3-treated clones II (57%, 29/51), whereas they were identified only in a small proportion of the untreated colonies (7%, 5/69).

As indicated previously, clones III and IV were only obtained in presence of ET3 (Fig. 5B). Clones III were of the same various types as ET3-treated clones II, wherein GM and M types predominated. Clones IV were only of the GM (Fig. 6E, F) and M types, in addition to rare U clones.

These data show that the multipotent GM, MF and GMF cells generated in pigment cell primary progeny have been propagated along subcloning and therefore these cells display the stem cell property of self-renewal. While limited to the second round of cloning in control medium, their ability to propagate was increased in presence of ET3.

The pigmented cells and the HNK1-expressing cells isolated from melanocyte progeny display multipotentiality

In the subcloning experiments described above, the self-renewal property of multipotent cells was inferred from analysing the serial clonal progeny of randomly selected cells from clones I. Consequently, the phenotype or "marker" identity of these stem-like cells could not be determined. In absence of known markers suitable to prospectively purify the multipotent cells in melanocyte cultures, we have examined the developmental potentials of defined subsets of pigment cell descendants that were identified at time of subcloning by either pigment content or HNK1 immunoreactivity.

Subcloning of the pigmented cells present in clones I treated with ET3 was performed after selection of the melanin-containing cells under microscopic control. In addition to M clones, the resulting clones II (n=50) comprised 66% of heterogeneous (GMF+GM+MF) clones. This distribution of colony types is the same as the one obtained for clones I derived from epidermal pigment cells (see Fig. 3E). Therefore, most of the pigment cells generated in ET3-treated melanocyte progeny behave as multipotent progenitors, similarly to the founder pigment cells freshly isolated from the skin.

We also examined the developmental potentials of $HNK1^+$ cells originating from cultured melanocytes (see Fig. 2F). Since their number in primary clones was very small, we choose to isolate the HNK1-expressing cells in living melanocyte polyclonal d11-cultures. Following selection and replating under microscopic control, single $HNK1^+$ cells generated various types of multilineage and unilineage clones in presence of ET3, among which the heterogeneous clones (of the GMF, GM, GF and GM types) accounted for 22% (Table 2). This proportion was lower but did not significantly differ from that previously obtained for the heterogeneous clones II originating from unselected cells (P=0.06; see Fig. 5B). Interestingly, we also determined that a large proportion of these clones (23/44, 52%) still contained $HNK1^+$ cells, thus showing that the $HNK1^+$ cells derived from cultured melanocytes are able to self-renew.

Taken together, these data show that the multipotent cells propagated in pigment cell primary progeny comprise immature $HNK1^+$ cells as well as pigmented melanocytes.

Role of ET3 in the induction and maintenance of cell multipotentiality by melanocyte progeny

The above data have shown that the emergence and self-renewal of multipotent progenitors derived from cultured melanocytes were promoted by ET3, which also stimulated their proliferation. To further analyse the role of ET3, we first investigated whether the multipotent GMF, GM and MF cells generated in clones I, required continuous treatment with ET3 in order to be maintained after subcloning. We also asked

whether the descendant cells of melanocytes grown in control medium could respond to a delayed exposure to ET3.

In the first experiment, the cells issued from the same ET3-treated clones I (n=2) were separated into two equivalent cell samples that were then subcloned, one in presence of ET3 and the other, in absence of this factor. Consistent with previous results of cloning II (Fig. 5B), the cells that were maintained in presence of ET3 for subcloning yielded mainly heterogeneous GMF, GM and MF clones (accounting for 67% of 28 clones analysed). The other cells, which were switched to ET3-deprived medium, generated almost exclusively M subclones of small size (92% of 24 clones analysed). Since in this last experiment, the clonal efficiency was as high as 75%, the almost absence of multilineage clones following ET3 removal is unlikely to result from impaired survival of the multipotent cells. Data rather argue that continuous exposure to ET3 is required to maintain the multipotentiality of the cells arising from ET3-treated melanocytes.

The next reverse experiment consisted in subcloning, either in absence or in presence of ET3, the two cell samples from the same clone I grown in control medium. The cells that remained in ET3-deprived medium yielded a majority of M subclones (not shown), as described for clones II previously obtained in control medium (see Fig. 5B). By contrast, those cells that were exposed to ET3 following subcloning, started to divide actively and generated heterogeneous GMF and GM d11-clones only (100% of 15 clones analysed), which contained a high total cell number (more than 1000 cells) similar to that found previously in ET3-treated clones I (Supplementary Fig. 2B). These findings therefore suggest that cells issued from untreated d11-colonies can respond to delayed treatment with ET3 by acquiring multipotentiality and increased proliferation rate, as did the epidermal pigment cells exposed in vitro to ET3 from d0.

Single pigment cells from hatching stage can also reprogram into multipotent progenitors

As the above experiments have shown the reprogramming capacity of differentiated melanocytes that were isolated at mid-incubation (E7.5) stage, when the skin has not fully matured, we asked whether the pigment cells taken from fully differentiated skin of hatching quails were still able to reprogram along non-melanocytic NC phenotypes. For this purpose, pigmented cells were isolated at E16 (i.e., one day before hatching) and clonally cultured in absence and presence of ET3, as described for E7.5 pigment cells. About 50% of the pigment cells were clonogenic in both media and they gave rise to GMF, GM, MF and M progeny types (Table 3). Consistent with data obtained with pigment cells isolated from younger embryos, treatment with ET3 increased the total cell number per clone (not shown) and the proportion of heterogeneous clones (∑h; ET3 versus controls, P=0.02; Table 3). Therefore, the potential of differentiated melanocytes to change their phenotype and generate multipotent progenitors is still present at the end of embryonic development.

Discussion

In the present work, we have provided evidence that, when clonally cultured in vitro, pigment cells are able to give rise to cells that are both multipotent and endowed with self-renewal capacity. Differentiated melanocytes yielded a clonal progeny that included myofibroblastic, glial and melanocytic cells. Moreover, while dividing, the pigment cells underwent dedifferentiation to immature cells that re-expressed NC markers characteristic of earlier developmental stages. The progenitors generated by cultured melanocytes display developmental potentials similar to those of intermediate precursors previously identified in the early migratory NC (Trentin et al., 2004). Thus, when withdrawn from their normal in vivo environment and isolated in culture, a certain proportion of melanocytes fail to maintain the differentiated phenotype and, as they divide, regain an earlier phenotype. The presence of ET3 strongly favours this phenomenon without being strictly necessary for it to take place.

Single melanocytes can generate a multiphenotypic NC-like progeny

Differentiated melanocytes isolated from the skin of E7.5 and hatching quails gave rise in vitro to multiple NCC types. The transition of pigment cells to myofibroblasts and glial cells occurred sequentially as the cells divided, both in presence and absence of ET3 (Fig. 1). Using clonal cultures, we demonstrated that single pigment cells behave as multipotent progenitors, yielding a subset of glial cells and/or myofibroblasts, in addition to large numbers of melanocytes. This capacity to yield multilineage (GMF, GM and MF) clonal progeny was extended to the large majority of pigment cells in presence of ET3 (up to 90%; Fig. 3E and Table 3).

Progenitors of the GMF and GM types have been previously identified in clonal cultures of quail NCCs (Lahav et al., 1998; Trentin et al., 2004) and Schwann cells (Real et al., 2005). Although endowed with the same developmental potentials as these progenitors, the GMF and GM cells derived from melanocytes exhibited a strong bias towards melanocyte differentiation, since melanocytes were the predominant cell type in mixed clones. Likewise, the same type of progenitors arising from single Schwann cells, generated mainly glial cells (Real et al., 2005). A particular property of reprogrammed pigment cells is the ability to generate

MF clones that were neither found in NCC nor Schwann cell cultures.

According to previous clonal analysis of quail trunk NCCs, melanocytes arise from several types of precursors, i.e., from the GM and GMF progenitors, as well as from multipotent cells endowed with neurogenic potential (i.e., GMN and GMNF progenitors) (Le Douarin et al., 2004; Trentin et al., 2004). In the present conditions, the melanocytes have never given rise to clones containing differentiated neuronal cells. Whether the multipotent cells derived from melanocytes lack neurogenic capacity or if they require specific cues to yield differentiated neurones, remains to be investigated.

Cultured melanocytes undergo dedifferentiation

Besides differentiated phenotypes (myofibroblasts, glial cells and melanocytes), a diversity of cells exhibiting immature characteristics was generated in the progeny of embryonic melanocytes. A major subpopulation of melanocyte-derived cells lost pigment, yet preserving expression of early melanocytic markers MelEM and/or *ETRB2*, the avian receptor for ET3 present in melanocytic NCCs (Lecoin et al., 1998). Other cells in the cultures were devoid of all phenotypic markers analysed (lin^- cells).

Simultaneously with loss of melanocytic traits, the majority of the cells derived from pigment founder cells acquired expression of *Sox10*, *FoxD3*, *Pax3* and *Slug* transcription factor genes (Fig. 2). All, except for *Sox10*, were not expressed by differentiated pigment cells at time of their isolation from the quail skin. These transcription factors have been shown to play a role in early NC development and to be down-regulated during melanocyte differentiation (Epstein et al., 1991; Nieto et al., 1994; Southard-Smith et al., 1998; Dottori et al., 2001; Kim et al., 2003). Re-expression of these genes can thus be interpreted as a manifestation of the dedifferentiation of melanocytes. The ability of FoxD3 to repress melanogenesis in avian NCCs (Kos et al., 2001) suggests that FoxD3 may favour the conversion of melanocytes into alternative phenotypes.

In addition, the HNK1 epitope became expressed by a small cell subpopulation derived from cultured pigment cells (Fig. 2). In vivo, HNK1 is present in most migratory NCCs and later, is retained only by PNS glia and neurones, being excluded from melanocytes (Tucker et al., 1984; Nataf et al., 1993). In our experiments, we verified independently that the majority of $HNK1^+$ cells expressed *Sox10* and/or *FoxD3* and none of the lineage markers tested (Supplementary Fig. 1). The cells in melanocyte progeny which accumulated these features (i.e., $HNK1^+$ $Sox10^+$ $FoxD3^+$ lin^- cells), may be considered as having recapitulated the molecular identity of early NCCs.

A dedifferentiation step, in which cells reacquire markers of early stages of development, has already been reported for other examples of phenotype conversion (Fischer and Reh, 2001; Henry, 2003; Tanaka, 2003).

Pigment cells yield NC-like progenitors displaying heterogeneous phenotypes

The finding that isolated pigment cells gave rise to multiple NCC types might be explained by a direct conversion of dividing melanocytes into myofibroblastic and glial cells. Alternatively, pigment cells might need to produce intermediate multipotent cells in order to generate alternative phenotypes. The results of our in vitro subcloning experiments strongly support the latter possibility that multipotent cells are generated and persist in the progeny of melanocytes (Fig. 5).

The comparison between the potentialities of daughter and granddaughter cells arising from single pigment cells has revealed that multipotent cells are produced during the first, symmetrical, pigment cell division while lineage-restricted cells arise only later, through asymmetrical cell divisions (Fig. 4). Therefore, although myofibroblasts differentiate early in melanocyte cultures, they are not derived from a fate-restricted precursor that would have directly segregated from the initial pigment cell division. By subcloning cells derived from single melanocytes cultured for a longer time (d11), we have shown that the multipotent progenitors generated in early pigment cell divisions are maintained later in pigment cell progeny and coexist with differentiated cells. Based on their ability to both self-renew and generate differentiated NC-derived cell types, the progenitors arising from pigment cells can be considered as endowed with stem cell properties. The GMF and GM progenitors in melanocyte progeny exhibited self-renewal ability in response to ET3 similar to that already described for the same progenitors in the early NC (Trentin et al., 2004). In both NC and melanocyte cultures, only GM cells were capable of long-term serial propagation upon continuous exposure to ET3.

The multipotent progenitors produced in pigment cell cultures were identified retrospectively by the subcloning experiments; consequently their identity (with respect to marker expression) remained to be determined. In previous studies, NC stem cells isolated from early NC and NC derivatives in rodents have been characterised by expression of low-affinity p75 neurotrophin receptor and no differentiation marker (Stemple and Anderson, 1992; Hagedorn et al., 1999; Morrison et al., 1999; Bixby et al., 2002). In our study, the large proportion of dedifferentiated cells that expressed one or more of the early NC markers (HNK1, *Sox10*, *FoxD3*, *Pax3* and *Slug*) could correspond to the NC-like multipotent cells present at high frequency in melanocyte progeny. To address this possibility, we took advantage of HNK1 as a surface marker to

prospectively isolate HNK1$^+$ cells from pigment cell cultures and analyse their developmental potentials. These experiments have shown that a subset of HNK1$^+$ cells is multipotent while others yield a variety of colonies containing only one cell type (Table 2). Moreover, HNK1$^+$ cells could be propagated along subcloning. Therefore, although HNK1 cannot be considered as a general marker for melanocyte-derived progenitor cells, it led to identify a subpopulation of these cells as dedifferentiated NC-like cells.

Following isolation and clonal cultures, the pigment cells that are produced in d11-primary progeny of epidermal melanocytes was also shown to be able to generate multiphenotypic clones, of the same types and at a similarly high frequency as did their founder pigment cells. The reprogramming capacity is thus present not only in melanocytes freshly isolated from the epidermis; it is also retained by pigment cells that have developed in vitro.

Therefore, the multipotent progenitors identified in the present experiments form a heterogeneous population of cells including both pigmented cells and HNK1$^+$ cells (Fig 7). Given that all the multipotent cells represented about 70% of the progeny derived from ET3-treated pigment cells, it is likely that other multipotent progenitors belong to unpigmented, HNK1$^-$ cells; however, their precise phenotype identity remains unknown in the absence of markers available to isolate them.

Heterogeneity of the stem cell population has already been described for the CNS, where cells with different phenotypes and of various developmental stages share the same differentiation potentialities and stem cell properties (Doetsch et al., 1999; Seri et al., 2001; Gotz, 2003; Merkle et al., 2004).

Is ET3 critical for pigment cell reprogramming?

We have shown that ET3 has multiple effects on the progeny of cultured pigment cells, stimulating cell proliferation and regulating stem cell properties of multipotent progenitors (Fig 7). However, reprogramming of pigment cells took place, though at a lower rate, in cultures devoid of ET3. Treatment with ET3 increased the mitotic activity and replicative lifespan of single pigment cells and their descendants, thus allowing serial propagation of pigment cell progeny along several rounds of subcloning, as we have reported previously for NCCs (Lahav et al., 1996; Trentin et al., 2004). Melanocytes as well as NCCs in culture undergo replicative senescence, a process also observed in most somatic cells (Hayflick, 1965).

ET3 favoured the generation of GM, MF and GMF multipotent cells in primary clones of skin pigment cells obtained from both early and late developmental stages. The continuous treatment with ET3 specifically ensured long-term amplification of the GM progenitors while permitting propagation of MF and GMF cells at low frequency for two passages only (Fig. 5B). These results are consistent with previous findings that GM progenitors of the quail NC are particularly dependent upon ET3 for growth and self-renewal in vitro (Lahav et al., 1998; Trentin et al., 2004). Moreover, a delayed exposure to ET3 triggered extended proliferation potential and acquisition of multipotentiality by "naïve" (i.e., unexposed to ET3) pigment cell primary progeny. ET3 is also critical for the maintenance of multipotent cells in pigment cell progeny. Indeed, the multipotent cells arising from melanocytes exposed to ET3 exhibited, following ET3 removal, reduced proliferation and strong tendency to lose multipotentiality, recovering the initial restricted fate of their founder cells. These results support the idea that "epigenetic marks" of the melanocytic lineage have not been totally erased following reprogramming to NC-like multipotent progenitors.

Conclusions

In the epidermis, the melanocytes interact closely with the keratinocytes forming structural and functional cellular units that tightly control the proliferation and regulate the function of melanocytes (for references, Slominski et al., 2004). The interactions with the cellular and growth factor microenvironment probably reinforce the identity and impair the dedifferentiation of skin melanocytes. However, as shown in the present work, when they are removed from their in vivo "niche" and isolated in vitro, the differentiated pigment cells are able to reprogram their phenotype and generate progenitors expressing NC-like stem cell properties. Such reprogramming has not been so far described in vivo; however, it might take place after local injury or in pathological conditions. The melanocytic phenotype, at least until late embryonic stages, is therefore unstable, and even differentiated pigment cells taken from the mature skin of hatching quails can be diverted to follow multiple NCC fates.

Aknowledgments

This work was supported by Centre National de la Recherche Scientifique and grant from Institut National de la Santé et de la Recherche Médicale (N° 4CS13H). We thank S. Gournet and M. Fromaget for illustrations and M. Scaglia for bibliography. We also thank P. Scotting, M. Nieto and M. Goulding for generously providing chick Sox10, Slug, FoxD3 and Pax3 plasmids, respectively. C.R. was recipient of doctoral fellowship from Fundação para Ciência e Tecnologia, Portugal (SFRH/BD/990/2000).

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Table 1. Developmental potentials of the granddaughter cells issued from single pigment cells.

Clones from granddaughter cells				Number of sibling
GMF	GMF	GMF	GMF	2
GMF	GMF	GMF	GM	1
GMF	GMF	GM	GM	1
GMF	GMF	MF	M	1
GM	GM	GM	†	1
GMF	GMF	GMF	†	1

The four granddaughter cells of single pigment cells cultured in presence of ET3 were replated individually in the same medium. At d11, the clones were classified after immunocytochemical detection of glial cells, melanocytes and myofibroblasts (see Fig. 3C) in order to determine the developmental potentials of granddaughter cells (n=28). The siblings were found to comprise GMF progenitors only (2/7), or GMF and more restricted (GM, MF or M) cells (3/7). In two other cases, one of the granddaughter cells did not survive (†) following subcloning.

Table 2. Dedifferentiated HNK1$^+$ cells isolated from pigment cell progeny are able to multilineage differentiation in clonal culture.

Multilineage clones (%)	Single lineage clones (%)			
GMF+GF+GM+MF	M	G	F	U
22 ± 11	44 ± 22	11 ± 7	3 ± 1	20 ± 16

Living cells in d11-melanocyte polyclonal cultures grown in presence of ET3 were immunolabelled for HNK1 surface marker. Individual positive cells were subcloned in the same culture medium by selection under fluorescence microscopy. About 82% (169/206) of plated HNK1$^+$ cells were clonogenic. The clones were classified according to the presence of melanocytes (M), glial cells (G) and myofibroblasts (F) in d11-cultures. U, unidentified clones with none of these cell types. Data represent the mean percentage of clones ± s.e.m. in three independent experiments.

Table 3. Single pigment cells isolated from the skin of E16 quails display multiple developmental potentials

	Cloning efficiency	Types of clones (%)				
		GMF	GM	MF	∑h	M
Control	44 ± 15	10 ± 7	22 ± 9	19 ± 4	52 ± 12	48 ± 12
ET3	50 ± 6	41 ± 7	10 ± 5	39 ± 11	90 ± 4*	10 ± 4*

Pigment cells were isolated by micromanipulation from dissociated cells of E16 quail skin and clonally plated in absence and presence of ET3. Clonal efficiency is given by the percentage ± s.e.m of plated cells that yielded a colony. At d15, the clones were analysed by immunocytochemistry and classified according to the presence of melanocytes, glial cells and myofibroblasts (see Figs. 3, 5). Data show the mean percentage of clones ± s.e.m. for four independent experiments in control (62 clones) and ET3-supplemented (192 clones) media. ∑h total of heterogeneous clones (GMF+GM+MF). *P=0.024 relative to control.

Figure and legends

Fig. 1. Myofibroblasts and glial cells differentiate in melanocyte cultures.
Polyclonal cultures (20-25 pigment cells per well) were performed in presence of ET3 and analysed with lineage-specific markers. (A, bright field) At d1 pigment cells divide and maintain melanin granules; (B, C, D) epifluorescence: □SMA-expressing cells are detected from d3 (B); SMP^+ glial cells are present at d9 (C); d11-cultures comprise □SMA^+ cells of myofibroblastic morphology (D). Bar= 70 µm.

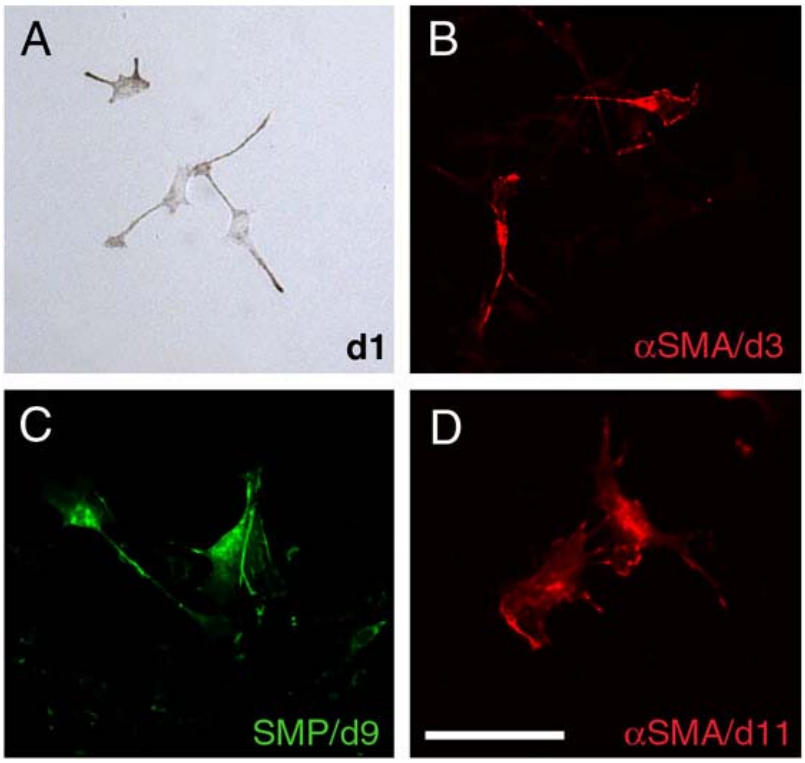


Fig. 2. Expression of NC markers is up-regulated in melanocyte cultures.
Pigment cell polyclonal cultures exposed to ET3 were treated by in situ hybridisation with *Sox10*, *FoxD3*, *Pax3* and *Slug* RNA probes (A-E, bright field) and HNK1 immunocytochemistry (F, epifluorescence). (A) *Sox10* and (B) *FoxD3* genes are expressed from d3; (C) *Pax3*, (D) *Slug* and (F) HNK1 starts to be expressed later and is present at d11, as *Sox10* (E). Bar= 70 µm.

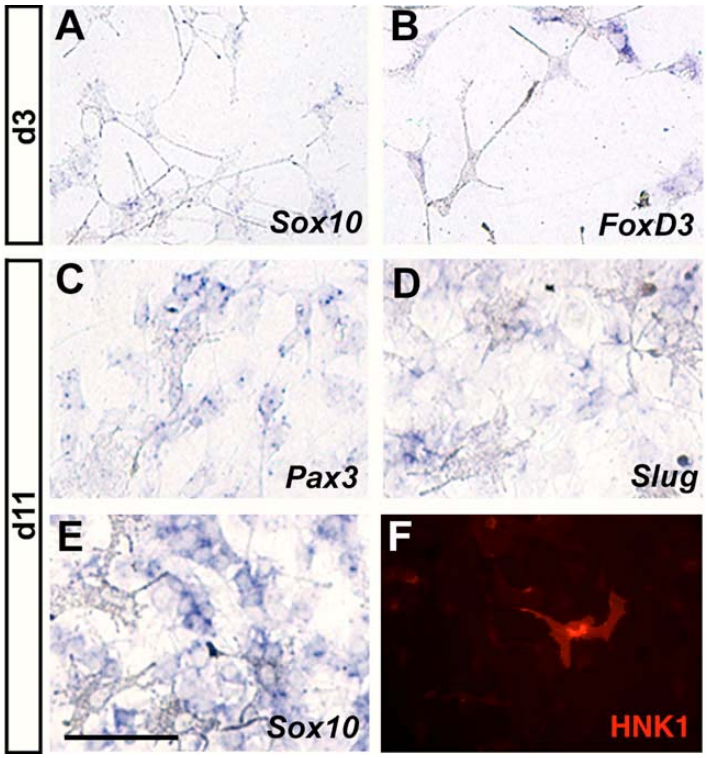


Fig. 3. Pigment cells generate multilineage clonal progeny.

Melanocyte clonal cultures grown in control and ET3-supplemented media were analysed at d11 and classified as GMF, GM, MF and M clones according to the presence of SMP^+ glial cells, $\square SMA^+$ myofibroblasts and $MelEM^+$ melanocytic cells. (A-D) epifluorescence micrographs of ET3-treated clones: (A, B, same field) GMF clone containing glia (A), myofibroblasts (B) and melanocytes (not shown); (C, D) GM clone containing glial (C) and melanocytic cells (D). Bar=70 µm. (E) Quantification of the various types of pigment cell-derived clones as given by the mean percentage of clones ±s.e.m. Data are taken from three independent experiments including 172 and 169 clones in presence of ET3 (grey) and in controls (black), respectively. ∑h is the total (GMF+GM+MF) of heterogeneous clones. Statistically significant differences between control and treated cultures are indicated (*P=0.02).

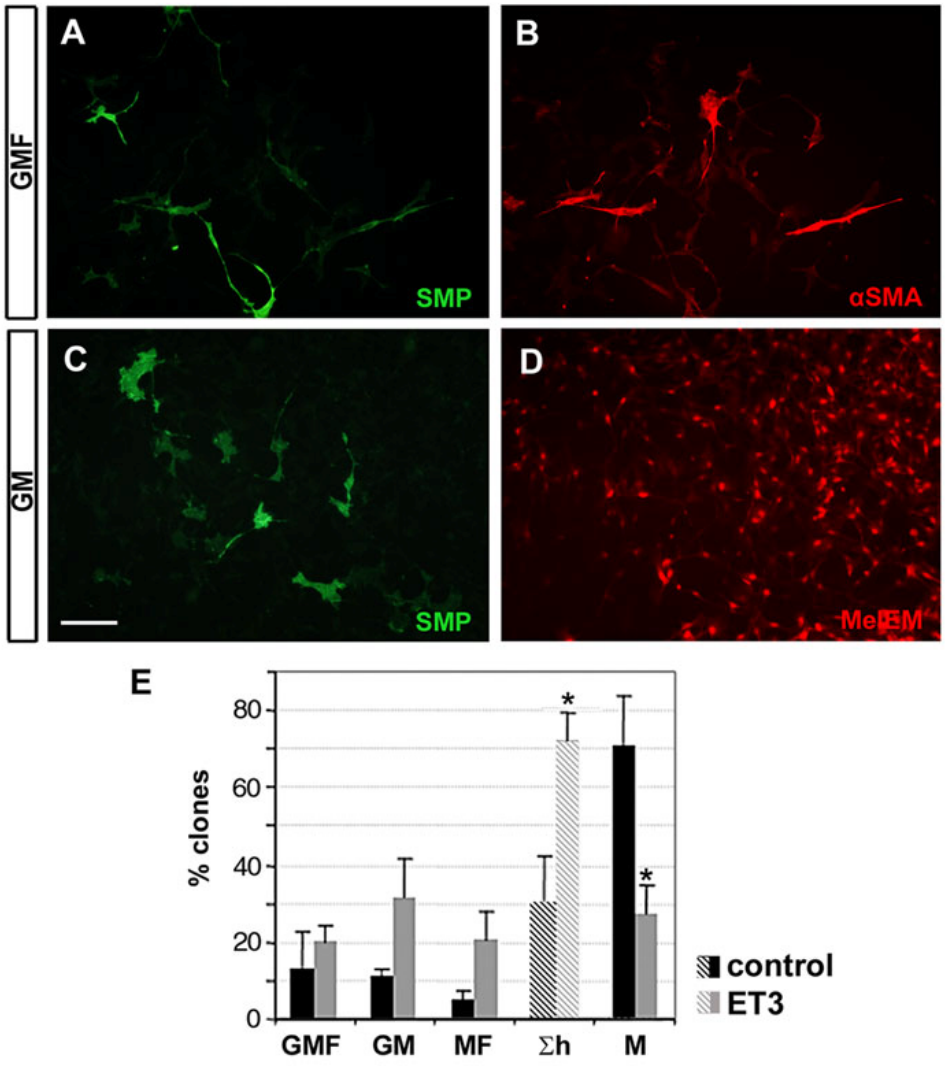


Fig. 4. Paired daughter cells arising from single pigment cells generate identical GMF clones.

After the first division of individual melanocytes in presence of ET3, the daughter cells were subcloned in the same medium and their progeny analysed with lineage-specific markers at d11. (A, D) bright field and (B, C, E, F) epifluorescence: (A-C, same field) GMF1 and (D-F, same field) GMF2 paired clones contain pigmented melanocytes (A, D), myofibroblasts (B, E, arrows) and glia (C, F). Bar= 150 μm.

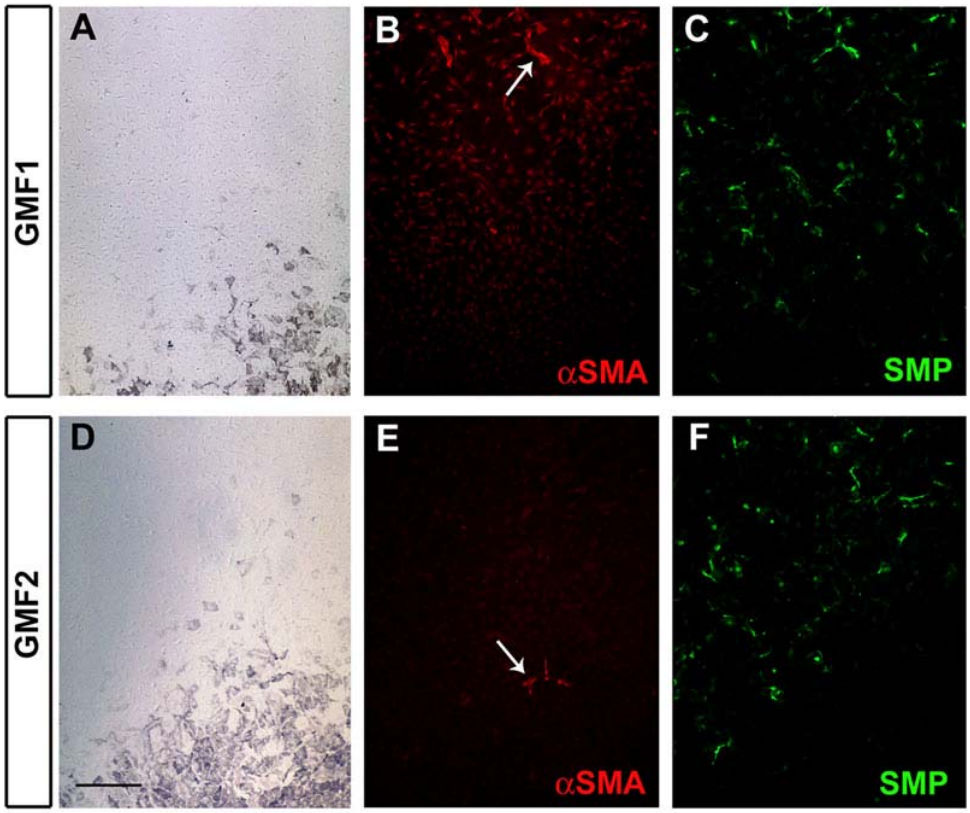


Fig. 5. Subcloning of pigment cell clonal progeny shows the self-renewal capacity of multipotent cells.

(A) Schematic illustrating the subcloning procedure. Epidermal pigment cells plated at d0 yielded clones I, which at d11, were subcultured as single cells in order to generate clones II. This procedure was repeated to obtain clones III and IV. (B) Quantification of the different clone types after subcloning of melanocyte primary colonies. At each step (II, III and IV), clones were classified according to the presence of melanocytic, glial and myofibroblastic cells, as for clones I (see Fig. 3E). Some small unidentified (U) clones contained none of these cell phenotypes. Data are shown as mean percentage of clones ±s.e.m. The total number of clones was 197 clones II in control medium and, in presence of ET3, 341 clones II, 354 clones III and 127 clones IV. ∑h, total of heterogeneous clones. Statistics between control and treated cultures are indicated (* *P*=0,03).

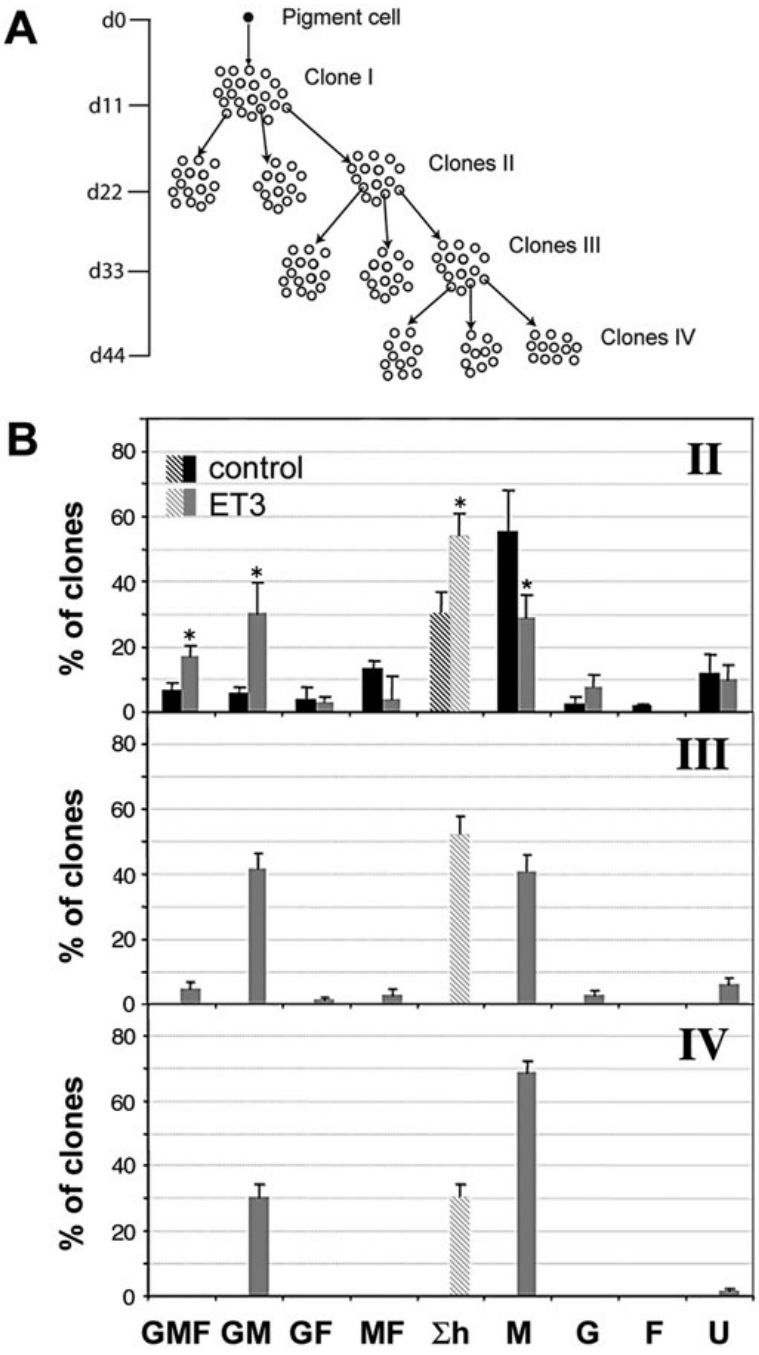


Fig. 6. Propagation of multilineage progenitors derived from single melanocytes in presence of ET3.

The clonal progeny of melanocytes was propagated along successive subloning steps II to IV in presence of ET3 (see Fig. 5). (A, B, same field) GM clone II containing glia (A) and melanocytic cells (B); (C, D) GF clone II with glial cells (C) and myofibroblasts (D); (E, F, same field) GM clone IV containing glial (E) and melanocytic cells (F). Bar= 70 µm.

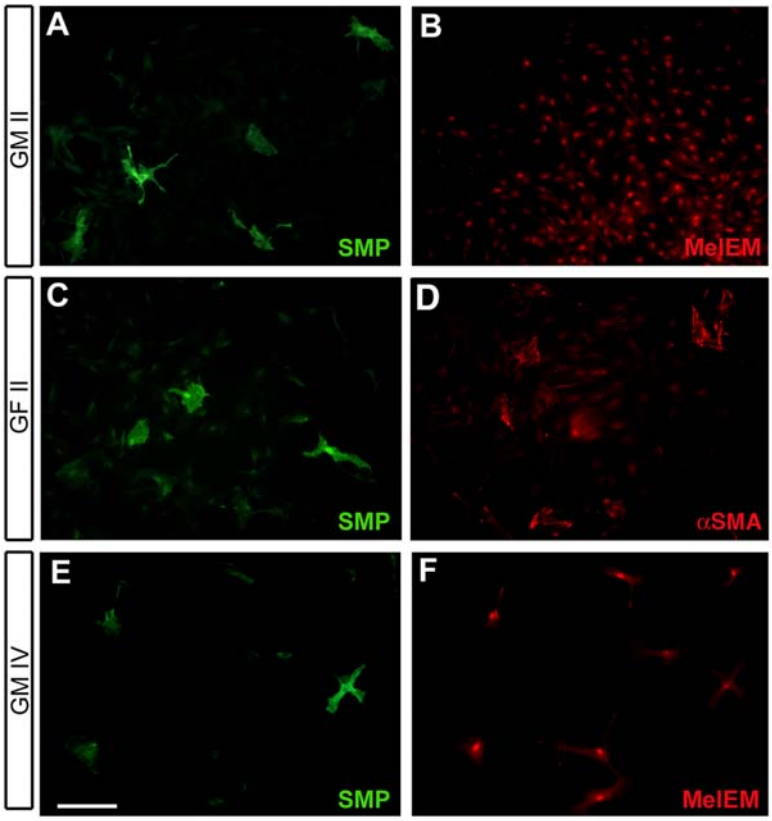
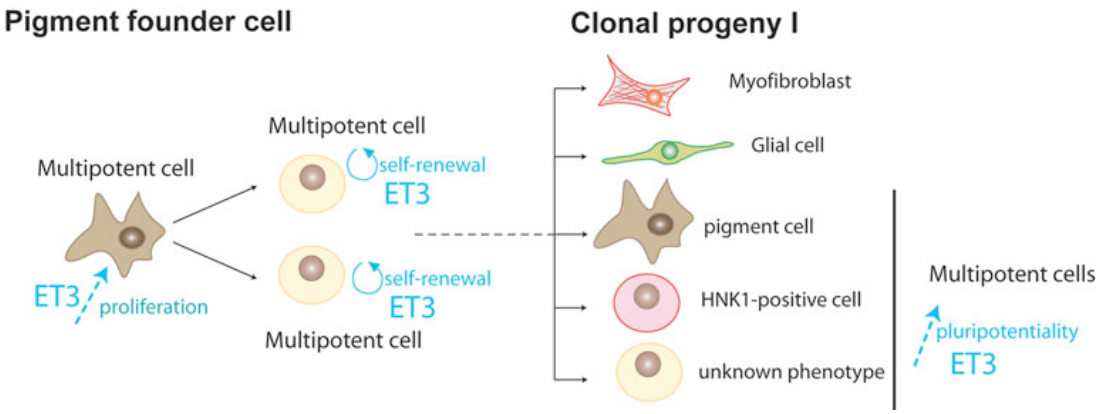


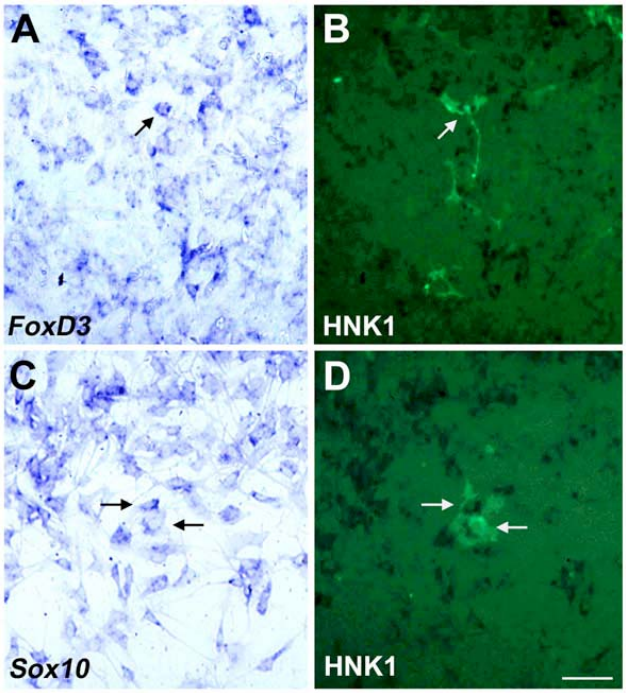
Fig. 7. Model of melanocyte reprogramming

Pigment cells removed from the epidermal microenvironment and transferred to in vitro cultures can reprogram to multipotent self-renewing progenitors. They generate multipotent daughter cells in the first symmetrical division and, later on, undergo asymmetrical cell divisions to yield more restricted cells. D11-clonal progeny of pigment cells comprises, in addition to melanocytes, myofibroblasts, glial cells and multipotent progenitors able to self-renew. These progenitors are heterogeneous with respect to their differentiation state, including pigment cells and dedifferentiated HNK1^{+} cells (and likely other, unpigmented cells of unknown phenotype). ET3 favours the reprogramming process, promoting proliferation of the melanocytes and maintaining multipotentiality and self-renewal of the NC-like progenitors.



Supplementary Fig. 1. Co-expression of early NC markers in melanocyte progeny.

(A, B, same field) Cells that co-express *FoxD3* gene (A) and HNK1 immunoreactivity (B) are present in d11 ET3-treated melanocyte cultures. (C, D, same field) At d7, ET3-treated melanocyte cultures show $HNK1^+$ cells (D) expressing *Sox 10* transcripts (C). Bar= 100 µm.



Supplementary Fig. 2. Serial subcloning of melanocyte progeny in presence and absence of ET3.
(A) Clonal efficiency (% of clone-forming cells) after successive cloning steps I to IV. (B) Quantification of cell growth in serial clonal cultures, given by the mean total cell number per clone at indicated time points.