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A cloned, immortal line of murine melanoblasts inducible to differentiate to melanocytes

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SUMMARY

Cultures of differentiated melanocytes can readily be grown from the dissociated epidermis of neonatal mice, and immortal cell lines often develop from these. However, the first cells that grow and transiently dominate the cultures, while similar to melanocytes, are unpigmented. These have been shown to be precursors of melanocytes and may be termed melanoblasts. Under our previous standard culture conditions, involving the use of keratinocyte feeder cells, foetal calf serum, the phorbol ester 12-*O*-tetradecanoyl phorbol acetate (TPA) and cholera toxin, all the melanoblasts spontaneously differentiated to pigmented melanocytes within about 3 weeks. We now describe some factors affecting the proliferation and differentiation of diploid murine melanoblasts in the presence of serum. Murine stem cell factor/steel factor (SCF), basic fibroblast growth factor (bFGF) and murine leukaemia inhibitory factor/differentiation-inhibiting activity (LIF/DIA) all increased melanoblast numbers. SCF and LIF also slightly inhibited melanoblast differentiation, while cholera toxin and TPA promoted differentiation. Using some of these findings, and by regular replacement of keratinocyte or fibroblastoid feeder cells, we have established a clonal line of immortal murine melanoblasts, 'melb-a'. These cells express tyrosinase-related protein-2 but not, in general, tyrosinase. They can be induced to differentiate irreversibly to functional melanocytes (also proliferative and immortal) by plating in the absence of feeder cells. Thus a new immortal melanocyte line, 'melan-a2', has also been produced.

Key words: melanocyte, differentiation, LIF, SCF, mouse, cell culture

INTRODUCTION

The murine melanocyte lineage makes an attractive model system for the study of developmental genetics. Nearly 70 distinct genetic loci are known to affect mouse coat colour (Silvers, 1979; Green, 1989; Bennett, 1993). About 25 of these loci act on melanocyte **development**, while others contribute to mature melanocyte **function**, either specifically or via a systemic effect (Bennett, 1993). Methods for the long-term culture of mammalian melanocytes were introduced by Eisinger and Marko (1982) using human cells; these were extended and exploited by numerous groups (see e.g. Halaban et al., 1987; Herlyn et al., 1988; Abdel-Malek et al., 1993; Tang et al., 1994). Application of similar methods to murine cells led to the isolation of immortal lines of wild-type and mutant murine melanocytes, which have proved invaluable in the molecular characterization of those loci and mutations that act primarily on melanocyte function, like the *albino*, *brown* and *silver* loci (Halaban et al., 1988; Yamamoto et al., 1989; Jackson and Bennett, 1990; Bennett et al., 1990; Zhou et al., 1994). For example the *albino* locus was shown to encode tyrosinase, the principal enzyme of melanin pigment synthesis. Mutant phenotypes (such as a brown colour) tend to be retained stably by such cell lines.

To date, however, there have been no reliable methods for the long-term culture and immortalization of melanoblasts, the precursors of melanocytes, although such methods should similarly facilitate the cellular and molecular analysis of the developmental loci and their mutant phenotypes. Melanoblasts are also of interest in relation to cancer research and the phenomenon of unpigmented melanoma cells. At present, melanoblasts are not well-defined as a cell type. Integumental melanocytes originate by migration of unpigmented cells from the dorsal neural crest, through the embryonic dermis, to the epidermis and hair follicles (reviewed by Bennett, 1993; Hirobe, 1994). These migratory neural crest cells are said to become first melanoblasts, then melanocytes. The nearest thing to a consensus definition of a melanoblast is perhaps 'a cell that serves at all stages of the life cycle as the precursor of the melanocyte' (Fitzpatrick et al., 1979). However, what line should be drawn between the precursor and the mature cell? The same authors define a melanocyte as 'a cell capable of synthesizing tyrosinase', but also as 'a cell in which melanin derived by tyrosinase activity is synthesized' (Fitzpatrick et al., 1979). Yet intermediate cells can be found that make tyrosinase but no melanin (e.g. Bennett et al., 1985; Ito et al., 1993). Melanin (here black or brown granules) has the great advantage, as a marker, that it is visible in living cells. In the

present study therefore we chose to examine those melanocyte precursors that were unpigmented, whether or not they contained tyrosinase, and provisionally to call them melanoblasts (as did Hirobe, 1994). Definitions could be modified if any more fundamental distinction emerged.

Previous workers have described short-term cultures of murine neural crest cells (Ito and Takeuchi, 1984; Huszar et al., 1991; Ito et al., 1993; Morrison-Graham and Weston, 1993), and murine melanoblasts from embryonic skin (Mayer, 1982). Hirobe (1991, 1992, 1994) has steadily improved conditions for maintenance and proliferation (for a few weeks) of murine melanoblasts cultured from neonatal skin, using a serum-free medium. Since, however, few cell types can be maintained indefinitely without serum, we decided to investigate whether serum-based culture protocols would permit further extension of melanoblast culture lifespans to allow spontanous immortalization as previously seen with melanocytes.

An immortal melanoblast line, '**melb-a**' (**mel**ano**b**lasts of genotype **a**/a), was indeed obtained. Although clonal, it contains unpigmented cells both with and without tyrosinase. We now describe some of the factors that affected the proliferation and differentiation of primary diploid neonatal melanoblasts in the presence of foetal calf serum (FCS), and those that permitted long-term growth. Mitogens for primary melanoblasts included SCF, bFGF and LIF/DIA; it is interesting that these three factors are also mitogenic for primordial germ cells (Matsui et al., 1992; Resnick et al., 1992), and for pluripotent haemopoietic stem cells (Flanagan and Leder, 1990; Gabbianelli et al., 1990; Hilton, 1992). Factors we retained for long-term cultures were the use of immortal keratinocytes treated with mitomycin C (feeder cells) and TPA. When plated in the same medium without keratinocyte feeder cells, melb-a cells differentiated rapidly into black melanocytes, which remained proliferative and immortal.

MATERIALS AND METHODS

Materials

Tissue culture plastics (Nunc) and FCS were from Gibco Europe (Uxbridge, UK). 12-*O*-tetradecanoyl phorbol acetate (TPA), cholera toxin (CT), CT subunit B, mitomycin C, triiodothyronine (sodium salt), aminoguanidine, hydrocortisone acetate and L- and D-dihydroxyphenylalanine (dopa) were from Sigma Chemical Co. (Poole, UK). bFGF (bovine) was supplied by Peninsula Laboratories Europe (St. Helens, UK). Recombinant murine SCF and recombinant murine LIF were generously provided by Drs Doug Williams (Immunex, Washington, USA) and John Heath (Oxford University, UK) respectively. Platelet-derived growth factor (PDGF)-AA and -BB homodimers were kindly donated by Professor Carl-Henrik Heldin (Ludwig Institute, Uppsala, Sweden). All protein factor stock solutions were prepared in phosphate-buffered saline with bovine serum albumin (1 mg/ml) as carrier, and stored at −70°C.

Medium

The basic culture medium (growth medium) was RPMI 1640 supplemented with penicillin, streptomycin, glutamine (2 mM) and FCS (5%, except where specified). Other supplements were included as specified. Incubation was with 10% CO_2 at 37°C, which gives a suitably low pH of 7.0 to 7.1 without the need for modification of the bicarbonate level. We are now using this routinely as a basic medium for melanocyte and melanoblast primary cultures and lines.

Feeder cells

Keratinocyte feeder cells were from the immortal XB2 murine keratinocyte line, provided by Dr Jim Rheinwald (Rheinwald and Green, 1975), and were prepared as described before (Bennett et al., 1989) except that mitomycin C treatment was for 3 hours instead of 2 hours. Feeder cells were plated usually 1 day, and no more than 3 days, before use. Where specified, feeder cells were prepared in the same way from line SC1 (**S**kin **C**ells **1**), an immortal fibroblastoid line developed by us from the same neonatal murine skin culture as the melb-a melanoblast line (see Results). SC1 cell stocks were grown in RPMI 1640 growth medium with 10% FCS.

Primary cultures of melanoblasts

Primary cultures were made from trunk skin of C57BL/6J (black, *a/a*) mice up to 24 hours old, more or less as described previously for melanocyte cultures (Bennett et al., 1989; Spanakis et al., 1992). In short, the skin was split with trypsin, epidermal sheets were pooled and dissociated briefly with trypsin and EDTA, and the resulting cell suspension was plated on to XB2 feeder cells. Generally 12 ml of culture (six 3-cm or three 5-cm dishes) were prepared per mouse. Differences from the cited methods were that RPMI 1640 rather than MEM medium was used, TPA (200 nM except where stated) was added from the start of culture instead of day 4, and cholera toxin (previously 1 nM) was present at 200 pM except where stated, also from the start of culture. bFGF, SCF and all other supplements where specified were added from 1 day after the start of primary cultures.

Serial passage of melanoblasts

During and after establishment, the cells were grown in RPMI 1640 growth medium. Other supplements were added at varying concentrations (see Results); ultimately the melb-a line was grown with FCS (5%) and TPA (20 nM) only, and was routinely plated at 2.5×10^4 cells/ml on XB2 feeder cells. During establishment, once melanoblasts were separated from other cells, new feeder cells were added after 2 weeks if a culture was not passaged by then. Melanoblasts, including melb-a cells, were passaged when well below confluency (up to about 8×10^4 cells/ml) to avoid spontaneous differentiation. The subculture procedure was as described for immortal murine melanocytes (Bennett et al., 1989).

Dopa histochemistry for tyrosinase activity

Cultures were fixed briefly (5 minutes) in Dulbecco's phosphate-buffered saline lacking $CaCl_2$ and $MgCl_2$ (PBSA), containing formaldehyde (4%, w/v). They were rinsed in PBSA adjusted to reach pH 6.8 at 37°C, and incubated in the dark in the same buffer at 37°C (White et al., 1983) containing either L-dopa or D-dopa (5 mM). This solution was changed at 45-minute intervals and incubations were stopped at various times by rinsing and postfixation with formaldehyde. Specific staining (for tyrosinase) was defined as a brown to black reaction developing after a given time in the presence of L-dopa but not D-dopa (White et al., 1983).

Proliferation and differentiation assays

Primary cultures for growth/differentiation experiments were plated in 3-cm dishes and were grown in the media and for the times specified. Media were renewed twice weekly. To ensure that feeder cells and other cells such as fibroblasts (not common) were excluded, counts were done on cells attached to plates and retaining their specific forms. The plates were rinsed in PBSA, fixed in formaldehyde (4% w/v in PBSA) for at least 30 minutes, rinsed in distilled water and air-dried. For counting, plates were re-coded randomly and counted 'blind'. They were rehydrated in distilled water and viewed using an inverted microscope with a 10× objective and eyepiece graticule. Only melanin pigment was visible by bright-field optics, enabling the counting of pigmented melanocytes, while total melanocytic cells (melanocytes + melanoblasts) were scored using

phase contrast. Melanoblasts were distinguished from other nonpigmented cells such as keratinocytes by their distinctive morphology (much like small melanocytes; see Results). At least 15 fields (about 150-500 cells) were counted for each dish, and dishes were in triplicate for each treatment.

Growth of immortal melanoblasts was assessed by triplicate haemocytometer counts, on cells trypsinised and resuspended in the same volume of medium as used for culture (2 ml per 3-cm dish). Cell suspensions were sometimes fixed by addition of 1% (v/v) glacial acetic acid, and stored at 4°C before counting.

Immunocytochemistry

The αPEP8 polyclonal rabbit antiserum against tyrosinase-related protein-2 (Tsukamoto et al., 1992) was kindly provided by Dr Vincent Hearing (NCI-NIH, Bethesda, MD, USA). Other reagents were from Sigma Chemical Co. (Poole, UK). Cultures on 3 cm dishes were fixed for 1 minute with 1:1 methanol-acetone and rinsed in complete PBS (PBSA with $CaCl_2$ and $MgCl_2$). All PBS rinses were for at least 35 minutes. An area was outlined with melted wax and incubated with the following solutions in PBS: 10% goat serum, for 20 minutes; αPEP8 serum (1/800) or normal rabbit serum (1/500), overnight at 4°C; PBS rinse; alkaline phosphatase-conjugated goat anti-rabbit IgG serum (1/100) for 2 hours at room temperature; PBS rinse. Bound alkaline phosphatase was visualized according to the method of Warburton et al. (1982); in brief the reagents were naphthol AS-B1 phosphate and Fast Red (TR salt) in veronal acetate buffer (pH 9.2) with levamisole to inhibit endogenous alkaline phosphatase, giving a red product. The reaction was for 6.5 minutes for αPEP8 or 14 minutes for normal rabbit serum. Coverslips were added with an aqueous mountant before photography.

Clonal growth in suspension

This was measured as described by Wakeling et al. (1992). The culture medium was Ham's F10 medium with 18 mM bicarbonate (pH 6.9), 5% FCS and TPA (20 nM) throughout. Each assay was done in triplicate. Medium (4 ml) containing 0.6% v/v agarose (Seaplaque) was allowed to set in each 5-cm culture dish. This was overlaid with 2 ml of medium with 0.3% agarose and 1000 cells (prepared as for subculture), and incubated. Medium without agarose (2 ml) was added on day 7 and replaced on day 14. Colonies visible with a dissecting microscope were counted after 21 days.

Photography

Unmounted, fixed cultures were rehydrated as for cell counting, and photographed with an Olympus IMT-2 inverted microscope, using a 10× planachromat objective and Technical Pan film (Kodak). After immunohistochemistry, mounted cultures were photographed using EPT 160T colour slide film (Kodak).

RESULTS

Factors affecting proliferation and differentiation of diploid melanoblasts in primary cultures

In our previous standard medium containing TPA (200 nM) and cholera toxin (200 pM), cultures from neonatal skin would develop almost entirely into pigmented melanocytes within about 2-3 weeks, the keratinocytes being killed by the TPA. Initially however few or no pigmented cells were present, and unpigmented melanoblasts constituted the major growing cell population present after about 1 week. These were very small cells, bipolar, tripolar, polygonal or dendritic and with a small dark nucleus as seen by phase-contrast optics (Bennett et al., 1989; Hirobe, 1991, 1992, 1994), as shown in Fig. 1. The majority of such cells are positive for the specific dopa-pre-melanin histochemical reaction (e.g. Hirobe, 1992), and forms intermediate between these melanoblasts and mature melanocytes (e.g. faintly pigmented) are readily found. We experimented with various supplements in attempts to reduce the rate of differentiation of melanoblasts. We present several factors that had some effect. In the following, 'total cell number' or 'cell yield' refers only to melanocytes plus melanoblasts, although there were few feeder cells or other cells present anyway by the time of assay.

Basic fibroblast growth factor (bFGF) and cholera toxin (CT)

bFGF was reported to sustain proliferation of murine melanoblasts in a serum-free medium in the presence but not the absence of dibutyryl cAMP, and to inhibit their differentiation (Hirobe, 1992). We tested its effects in our medium containing 5% FCS and TPA, with or without CT (a potent stimulator of cAMP biosynthesis). Typical results after 2 weeks are illustrated in Fig. 2. Even in such a mitogenic medium the cell yield was increased about 1.5 fold after growth with exogenous bFGF (20-50 pM), in the presence or absence of CT. In other experiments no significant additional growth was obtained with bFGF at 200 pM compared to 50 pM (not shown). In general, bFGF did not significantly affect differentiation (proportion of melanoblasts relative to melanocytes) in our experiments. bFGF (20 pM) was subsequently included in the medium for long-term but non-established melanoblast cultures, and for some tests of other potential mitogens.

The effect of CT (200 pM) on total cell number in these primary cultures was substantial (4- to 5-fold increases with or without bFGF), and consistent in all experiments. Fig. 3 shows the dose-dependent increase in cell yield with CT levels between 2 pM and 200 pM. However, the absolute number of melanoblasts at 2 weeks was virtually independent of CT concentration (not shown), resulting in a dose-dependent reduction of the proportion of melanoblasts in the presence of CT (Fig. 3). These data might indicate that CT had no effect on differentiation and was only promoting growth of melanocytes. However, microscopic observation suggested rather that CT was also stimulating both proliferation and differentiation of melanoblasts. Thus CT was retained in long-term, non-established cultures at (usually) 50 pM, to promote growth without excessive differentiation.

Stem cell factor

SCF, also known as steel factor (SLF), mast-cell growth factor (MGF) and Kit ligand (KL), is important in melanoblast development according to genetic evidence (Flanagan et al., 1991; Funasaka et al., 1992; reviewed by Bennett, 1993), and has been reported to stimulate proliferation of diploid human melanocytes (Funasaka et al., 1992). The effects of addition of recombinant murine SCF to this culture system are shown in Fig. 4. Significant stimulations of net growth in the presence of SCF were seen over 2 weeks; total cell number was increased modestly by up to 40-50% but melanoblast number increased by up to 2 fold (with SCF at 100 ng/ml) in the presence or absence of CT. The proportion of melanoblasts was thus higher after growth with SCF (especially where CT was absent), in other words the overall degree of differentiation of the cultures was lower. The percentage of melanoblasts was 20-23% with SCF and no CT, compared to 3.5% (and fewer

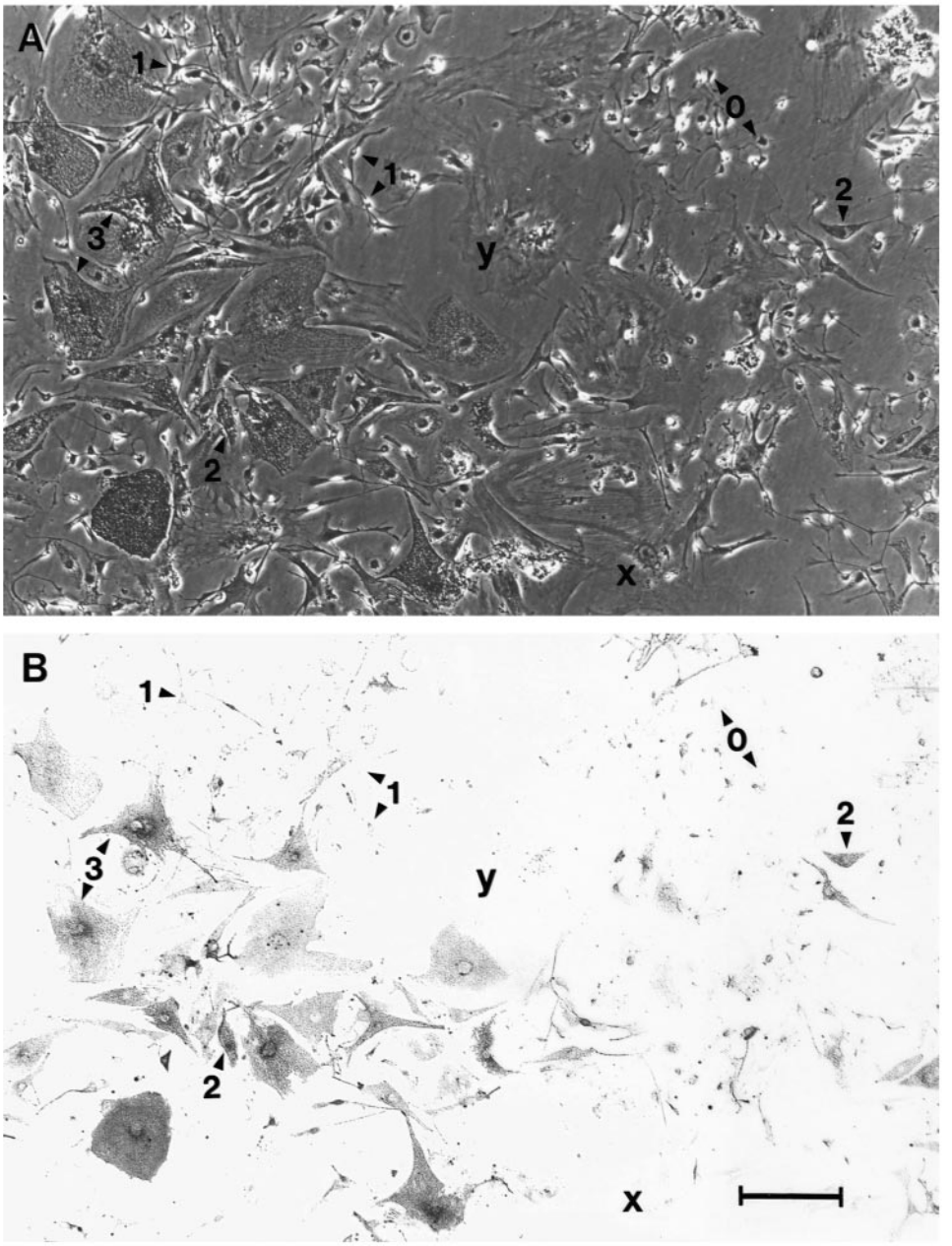

Fig. 1. Appearance of melanoblasts and other cells in fixed primary cultures from neonatal skin. The same region is viewed by (A) phase contrast optics to show all cells, and (B) bright-field optics to show melanin pigment. This culture was plated as usual (see Methods) and grown for 14 days with TPA (200 nM), CT (200 pM) and SCF (100 ng/ml). A spectrum of degrees of pigmentation can be seen, from no visible pigment (0) to well-pigmented (2). All cells with any pigment are scored as melanocytes in counting assays. Very large melanocytes (3) become predominant in senescent cultures (not shown) and are presumably senescent cells. Melanoblasts (0) tend to be very small and bipolar or dendritic, resembling the smallest melanocytes. Surviving keratinocytes (normal or feeder cells) can be recognised by their filamentous cytoplasm, lack of melanin (or melanin in a perinuclear ring), and flat nucleus with visible heterochromatin (x). Some keratinocytes appear to lose their nuclei (y), possibly because of differentiation. Scale bar, 150 μm.

in total) with CT and no SCF. However, the levels of the factor required for these favourable effects were too high to indicate routine inclusion in cultures.

Leukaemia inhibitory factor/differentiation-inhibiting activity

From genetic evidence, two embryonic precursor cell lineages besides melanoblasts, namely primordial germ cells and pluripotent bone marrow stem cells, have been found to be dependent on SCF and its receptor Kit for normal development (reviewed by Funasaka et al., 1992; Bennett, 1993). Similarly bFGF is a growth factor for primordial germ cells (Matsui et al., 1992; Resnick et al., 1992) and pluripotent bone marrow cells (Gabbianelli et al., 1990) as well as for melanoblasts (Hirobe, 1992). A third factor, LIF (also known as DIA and HILDA) stimulates the growth of both primordial germ cells (Matsui et al., 1992; Resnick et al., 1992) and pluripotent bone marrow cells, among others, (Hilton, 1992), and inhibits the differentiation of the primordial germ cells (Resnick et al., 1992). It recently became possible to grow long-term cultures of primordial germ cells by supplementation with SCF, bFGF and LIF in combination (Matsui et al., 1992; Resnick et al., 1992). We therefore decided to test the effects of recombinant murine LIF on melanoblasts. The results are shown in Fig. 5. Even in the presence of FCS, CT and TPA, cell numbers were significantly increased with LIF at concentrations of 20 and 50 ng/ml (about 1-2.5 nM); thus LIF appears to be a new growth factor for the melanocytic lineage. The number of melanoblasts after 2 weeks with LIF at 50 ng/ml was higher than in control cultures by about 60%, but there were only about 30% more melanocytes, so that there was a modest increase in the proportion of melanoblasts. However, as with SCF, the active

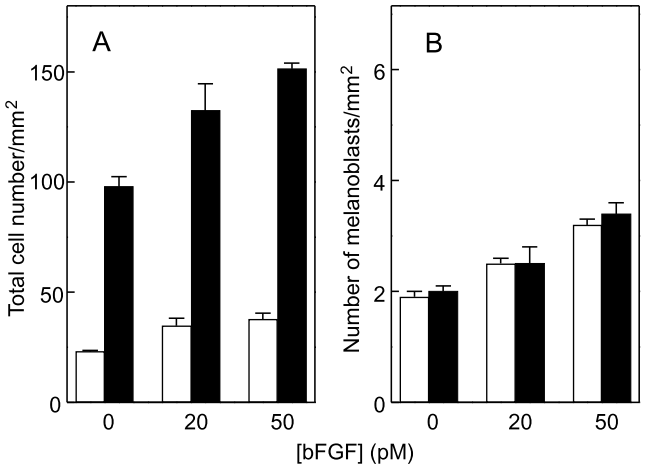

Fig. 2. Effects of bFGF on growth and differentiation of primary melanoblast/melanocyte cultures in the presence of FCS (5%), TPA (200 nM) and feeder cells. Primary cultures were prepared as usual (Materials and Methods), and grown for 2 weeks with the stated concentrations of bFGF, and either with (■) or without (□)CT (200 pM). Medium was changed twice weekly with the same supplements. Counts of melanoblasts and melanocytes were made (see Materials and Methods); means and s.e.m. are shown.

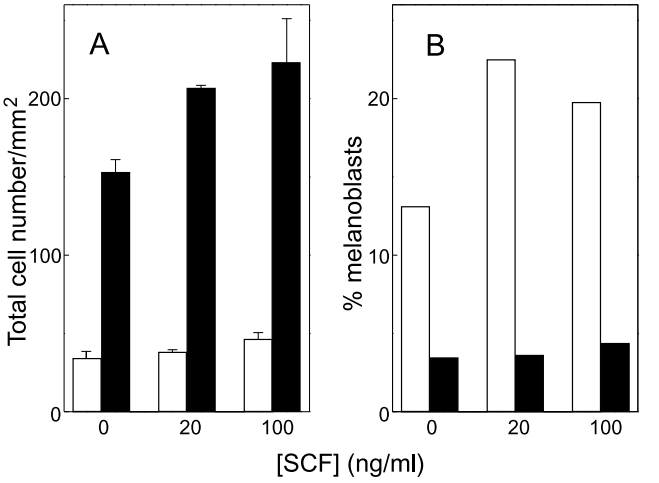

Fig. 4. Effects of SCF on primary melanoblast/melanocyte cultures after 2 weeks. Cells were grown with FCS, TPA, feeder cells and either with (■) or without (□) CT (200 pM). Other details are as in Fig. 2. The s.e. of the quotient (% melanoblasts) was not calculated, but the increases in melanoblast numbers with SCF (100 ng/ml) were significant by Student's *t*-test both in the presence ($P<0.001$) and absence ($P<0.01$) of CT, while the increase with SCF at 20 ng/ml was significant only in the absence of CT ($P<0.05$).

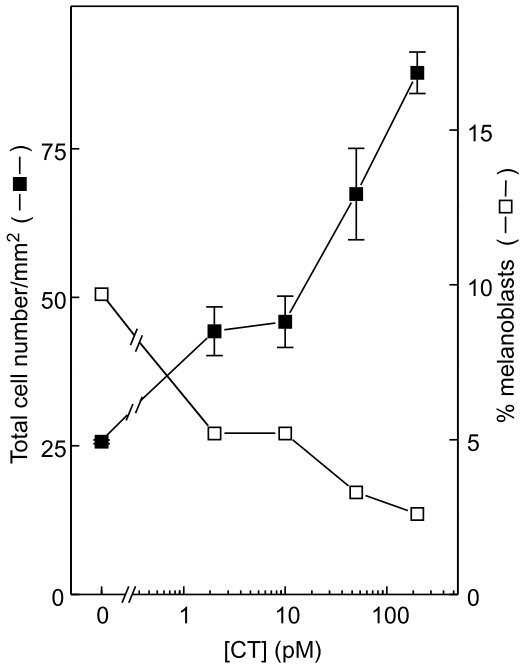

Fig. 3. Effects of CT on primary melanoblast/melanocyte cultures after 2 weeks. Cells were grown with FCS, TPA, feeder cells and bFGF (20 pM). Other details are as in Fig. 2.

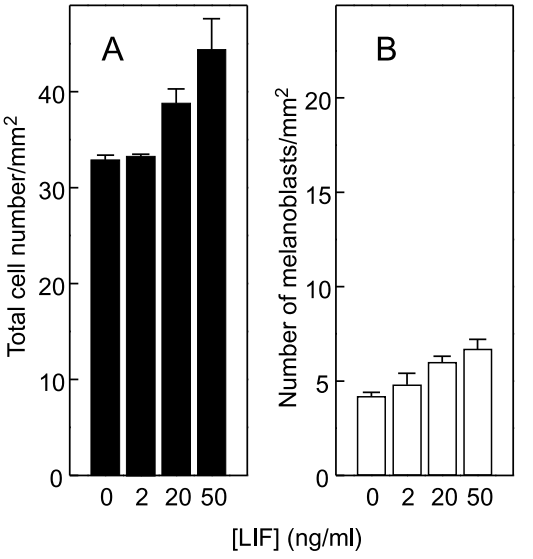

Fig. 5. Effects of LIF on primary melanoblast/melanocyte cultures. Cells were grown with FCS, TPA, CT (200 pM) and feeder cells. Other details are as in Fig. 2. The number rather than percentage of melanoblasts is shown, the effects of LIF on the number being greater, and significant by Student's *t*-test for the concentrations 20 and 50 ng/ml ($P<0.01$).

levels of LIF under our conditions were unsuitable for routine inclusion in cultures.

In a preliminary experiment, the triple combination of bFGF (20 pM), LIF (20 ng/ml) and SCF (20 ng/ml) did not have the dramatic effects on melanoblasts that were seen with primordial germ cells, but produced similar increases in the numbers and proportions of melanoblasts to those seen with LIF alone or SCF alone (data not shown).

TPA

TPA is a principal mitogen in our culture system for murine melanocytes; even immortal melanocyte lines generally cease to grow when it is omitted (Bennett et al., 1987, 1989). We

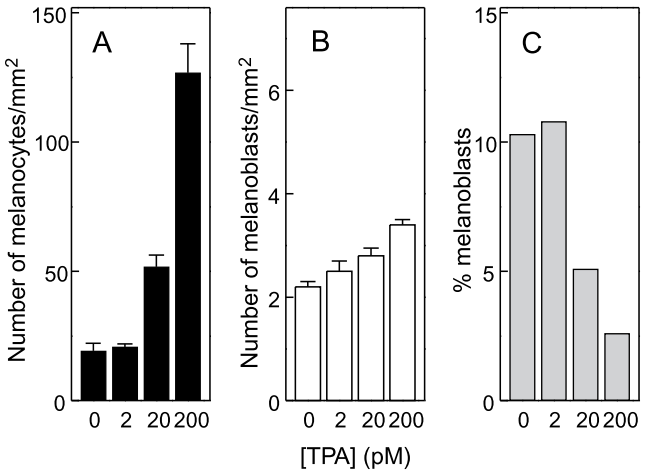

Fig. 6. Effects of TPA on primary melanoblast/melanocyte cultures. Cells were grown with FCS, CT (200 pM), bFGF (20 pM) and feeder cells. Other details are as in Fig. 2. Both number and percentage of melanoblasts are shown, to illustrate the opposing trends.

tested, for the first time, the effect of TPA on growth and differentiation of diploid melanoblasts in this system. As shown in Fig. 6, there was only a small increase in the number of melanoblasts with increasing concentration of TPA, but a marked increase in the number and proportion of melanocytes. This may have been because the mitogenic effect of TPA on melanoblasts (in the presence of bFGF and CT) was much smaller than that on melanocytes, or because there was a marked mitogenic effect on melanoblasts but it was accompanied by stimulation of differentiation to melanocytes. Microscopic examination of longer-term cultures indicated that melanoblast growth was sustained better when some TPA was present, and this was supported by quantitative experiments on immortal melanoblasts as discussed below. Thus for long-term melanoblast cultures we added TPA at 20 nM rather than 200 nM as used for melanocytes.

Other factors tested on primary cultures

A number of other tested factors had no significant beneficial effects on primary cultures of melanoblasts; these will be mentioned briefly for reference. Quantitative data are available on request. **Triiodothyronine** and **hydrocortisone** are common ingredients of keratinocyte media, but had little effect on melanoblasts (with or without CT) when added at 100 pM and at 100 nM-1 μM respectively. **PDGF-AA and -BB** pure homodimers were tested because of the coat-colour mutation *patch (Ph)*, in which melanoblast development is deficient and a DNA segment including the PDGF receptor A gene is deleted (references in Bennett, 1993). However, neither homodimer affected melanoblast numbers or differentiation in our cultures at concentrations up to 5 ng/ml and in the presence of CT. It remains possible that the *Ph* deletion impinges on the *Kit* promoter region. **Cholera toxin subunit B** was reported to be as effective a mitogen as intact CT on human diploid melanocytes (Herlyn et al., 1988), but had no clear effect on melanoblasts at 200 pM-5 nM; a mitogenic effect detected at 20 nM could be attributed to the 1% contaminating intact CT present (supplier's data), being indistinguishable from that seen with CT at 200 pM. **Aminoguanidine** was reported to prevent 'crisis' (promote immortalization) in some embryonic cells in culture (Parchment et al., 1990), but did not do so in our cultures (they senesced as usual by about 4 weeks), and it appeared, by visual inspection, to increase the proportion of pigmented cells (not quantitated).

Immortalization of melanoblasts: the melb-a line

Initial supplements were FCS (5%), TPA (50 nM for 4 days, then 20 nM); CT (50 pM), bFGF (20 pM) and the following reagents used in Hirobe's method (1992): insulin (10 μg/ml), ethanolamine (EA) (1 μM), phosphoethanolamine (PEA) (1 μM) and amphotericin B (250 ng/ml). Insulin and amphotericin B were omitted after about 2 months, without obvious effect. The cells were subcultured after about 2 weeks on to fresh feeder cells, and more feeder cells were added 2 weeks later. At about 6 weeks (total), although the bulk of the cultures had senesced, growing melanoblast-like cells were observed in association with growing colonies of fibroblast-like cells. Some dishes were subcultured, without feeder cells because the fibroblast-like cells appeared to be promoting melanoblast growth. The concentration of FCS was varied between 2.5% and 10% to test whether selective growth of melanoblasts over fibroblastoid cells could be obtained thus. Cells of both types grew for 4 more passages over 3 weeks, but fibroblastoid cells began to predominate. Some mixed cultures were frozen, and others were grown in RPMI 1640 medium and 10% FCS only, to select the fibroblast-like cells. The resulting line of immortal fibroblastoid cells was designated **SC1** (**S**kin **C**ells 1). Some SC1 cells were treated with mitomycin C to make feeder cells (see Methods). A sample of the mixed culture was now thawed and plated on SC1 feeder cells in medium with 5% FCS and TPA, CT, bFGF, EA and PEA as above. This was treated for 3 days with antibiotic G418 (75 μM), known to allow selective survival of melanocytes and kill fibroblasts (Halaban et al., 1987). This was successful: melanoblasts too proved to be resistant to G418, while the growing fibroblastoid cells were all lost. At the next passage some cultures were plated very sparsely in 9-cm dishes containing either SC1 or XB2 feeder cells. Clonal colonies of melanoblasts were obtained with both types of feeders, and several were subcultured by ring-cloning. The best-growing clonal line was one initiated and passaged on XB2 feeder cells and was designated **melb-a**. Shortly after cloning, CT, PEA and EA were omitted from the medium without adverse effect. It took about 5 months in all from the primary culture to freezing of cloned melb-a stocks. This line has been grown to passage 22 since cloning (passage 28 since primary culture).

The appearance of melb-a cells after growth on XB2 feeder cells is shown in Fig. 7A,B. When plated in the same medium in the absence of XB2 cells, most cells in the culture spontaneously differentiated to black melanocytes within about 1-2 weeks (Fig. 7C,D), showing that the cells were indeed melanocyte precursor cells. Surprisingly, this differentiation was accompanied by faster proliferation; in a typical experiment 22.1×10^4 cells/ml were obtained 16 days after plating with feeder cells (crude doubling time 5.0 days) and 45.3×10^4 cells/ml without (crude doubling time 3.8 days), plating being at 2.4×10^4 cells/ml. Thus the inhibition of differentiation by feeder cells is not through stimulation of growth. The differentiation is expected to become irreversible, since we have fre-

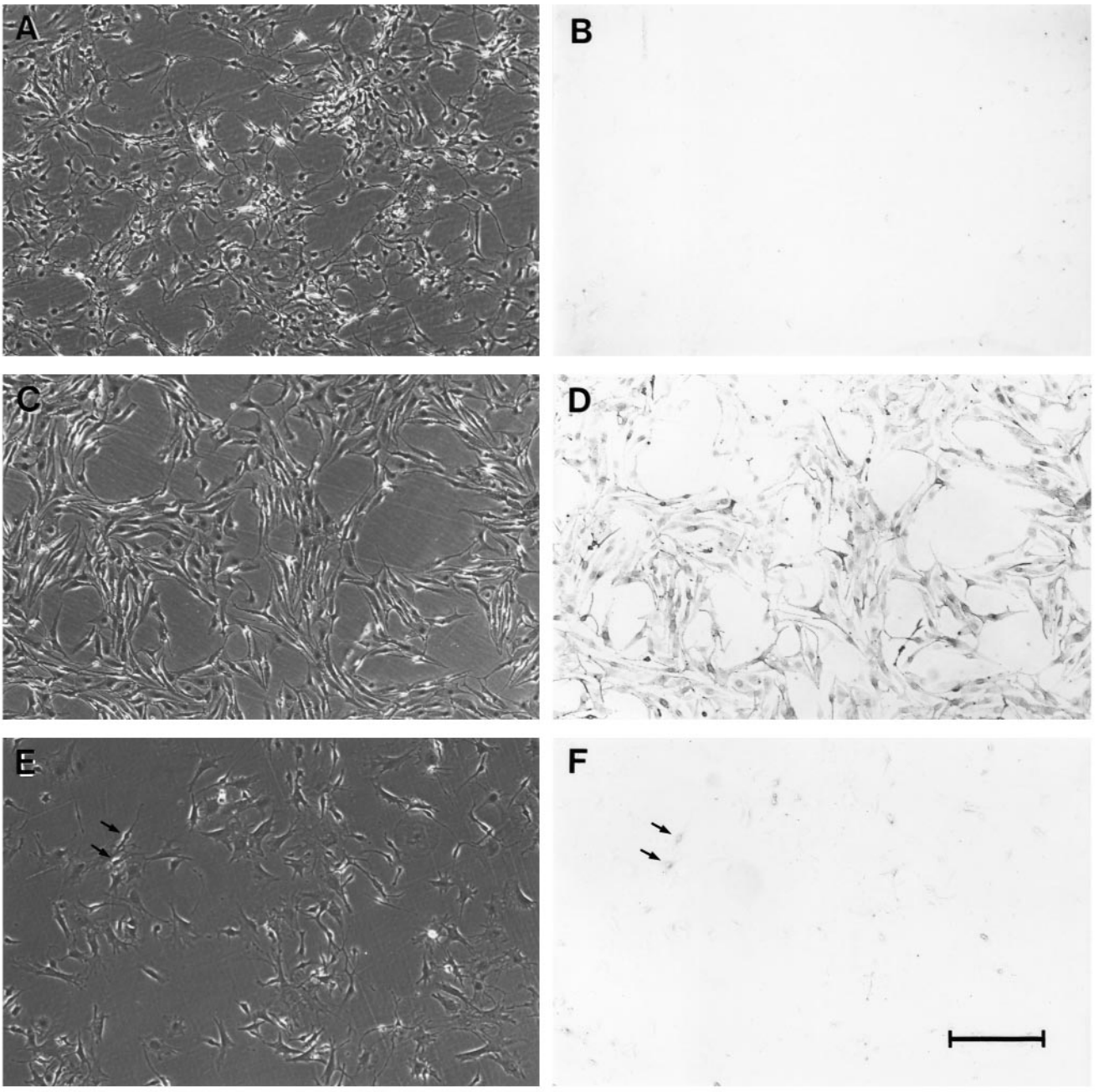

Fig. 7. Appearance of cloned melb-a immortal melanoblasts and derived melanocytes. The same regions are shown by phase-contrast (A,C,E) and bright-field (B,D,F) optics. Photographic parameters such as light levels were matched for comparability. (A,B) melb-a cells grown under standard conditions (with feeder cells and 20 nM TPA) for 5 days after plating at 5×10^4 cells/ml. Virtually no pigmented cells are seen. (C,D) melb-a cells plated in the same medium but without feeder cells, for 9 days including one subculture. Virtually all cells have differentiated to pigmented melanocytes. The cells appear somewhat larger, flatter and more aligned than in (A). (E, F): melb-a cells plated at 3×10^4 cells/ml on feeder cells, grown for 3 days and stained histochemically with L-dopa (90 minutes; see Methods) for tyrosinase. The larger, flat, epithelioid cells are XB2 feeder cells (not dead by day 3). A minority of melb-a cells show a specific reaction (arrows). A few rounded cells in (F) show some false contrast (not true stain). Scale bar =250 μm throughout.

quently grown immortal melanocytes on XB2 cells without seeing any melanoblasts. However, when melanocytes derived from the melb-a line were plated back on to XB2 cells after only 4 passages, unpigmented cells did emerge again within 1 passage. We do not know whether this was due to dedifferentiation or selective growth of residual melanoblasts. Parallel cultures remained stably pigmented in the absence of XB2 cells; some plates were subcultured with additional TPA (200 nM) and CT (200 pM) and were expanded to yield a new immortal line of black (*a/a*) melanocytes, designated **melan-a2**.

Melanocyte/melanoblast markers

In relation to alternative definitions of melanoblasts (see Intro-

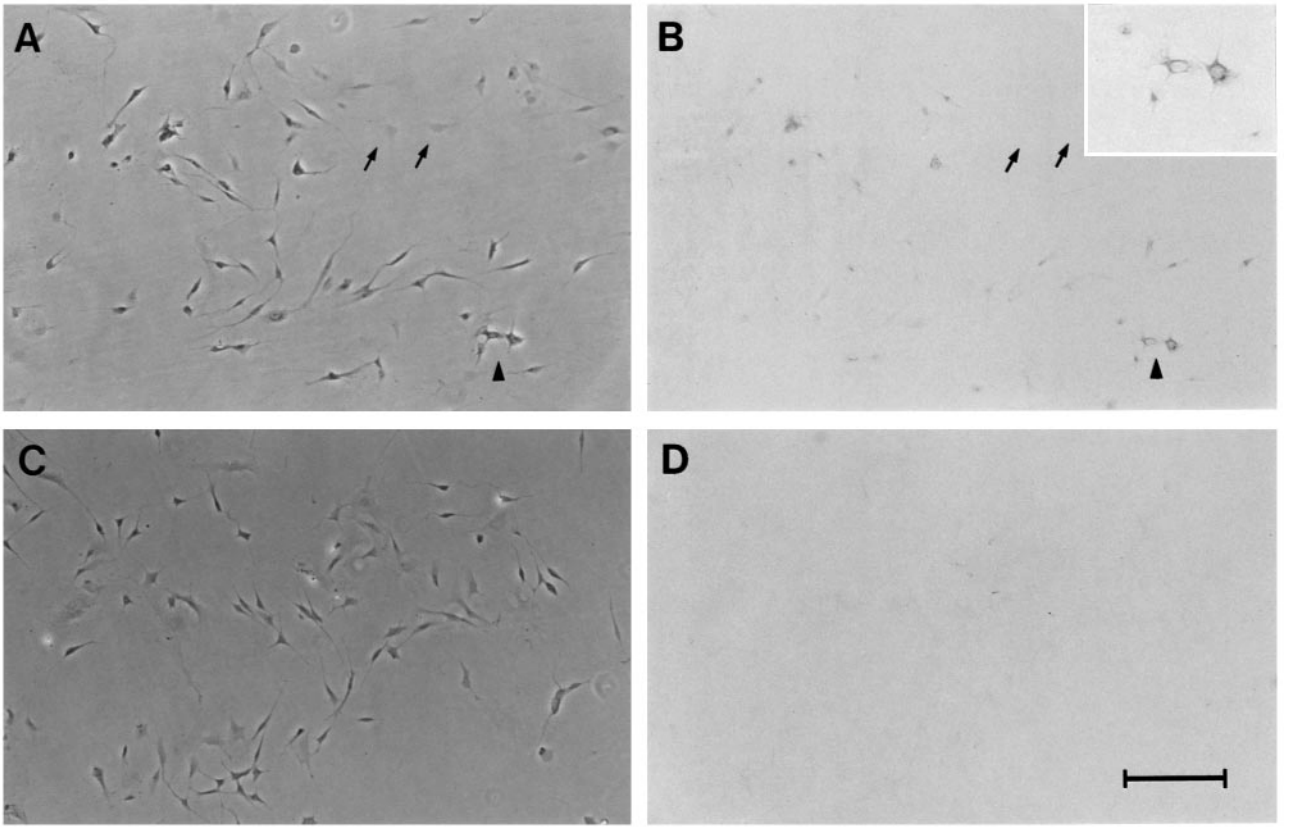

Fig. 8. Immunohistochemical staining for TRP-2. Cultures of melb-a cells at passage 28 were grown under standard conditions (see Fig. 7) for 5 days. They were fixed and stained (see Methods) using either the αPEP8 (anti-TRP-2) antiserum (A,B) or normal rabbit serum (C,D). A few cells in B (arrowhead) are shown at 2× higher magnification in the inset. Most or all cells showed specific staining, although the colour was faint in some cells and some apparently enucleate cells (possibly feeder cells) were unstained (arrows). The staining pattern (B, inset) was generally at one side of the nucleus in the smallest, bipolar cells, or throughout the cytoplasm in larger cells. Scale bar, 150 μm.

duction), it was of interest to use the dopa histochemical test for the melanogenic enzyme tyrosinase (see Methods). This (Fig. 7E,F) demonstrated that while some melb-a cells were synthesizing tyrosinase (presumably in an inactive or sequestered form, since no melanin is present), most (approx. 80%) of the cells present had no detectable tyrosinase. The melb-a line thus consists largely of immortal melanoblasts whether or not these are defined as making tyrosinase.

The melanogenic enzyme dopachrome tautomerase or tyrosinase-related protein-2 (TRP-2), is expressed earlier in development than tyrosinase; its expression can be detected by in situ hybridization (Steel et al., 1992) or the αPEP8 antibody (Pavan and Tilghman, 1994) in embryonic murine melanoblasts in vivo. We used this antibody in immunocytochemical staining of melb-a cells (Fig. 8). All or virtually all of the melb-a cells, but not feeder cells, showed specific staining for TRP-2, either at one side of the nucleus in small, bipolar cells, or throughout the cytoplasm in larger cells. Staining appeared granular at higher magnification (not shown), suggesting a location in GERL and in some form of immature melanosomes (melanosomes are produced from GERL).

Growth requirements of immortal melanoblasts

Once adequate stocks were available, we tested which of the components used during the isolation of the line were required for its growth. The experiment shown was done with the uncloned melanoblast stock from which melb-a was derived, which might be more representative than one clone. As shown in Fig. 9, the only important supplement appeared to be TPA. No significant change in cell number was seen after omission of EA, PEA, and bFGF, and the reduction in number on omission of CT was small. We have subsequently obtained immortal melanoblast lines of other genotypes without EA or PEA (unpublished data) suggesting that the levels of EA and PEA in 5% FCS are sufficient for melanoblasts. Other growth requirements are likely to be diminished by immortalization (see Discussion). The melb-a line is currently cultured in growth medium with FCS and TPA (20 nM) only. An experiment with varying concentrations of TPA (Fig. 10) showed that melb-a cells did not grow without TPA, and growth was more rapid at higher concentrations of TPA up to 1 μM; however the cultures with these higher concentrations also became pigmented (determined visually). Further work is in progress to determine whether this pigmentation results from higher cell densities, in which case it can be prevented by earlier subculture, or is independent of density.

Growth in suspension

Melanoma cells are often unpigmented in culture; we decided

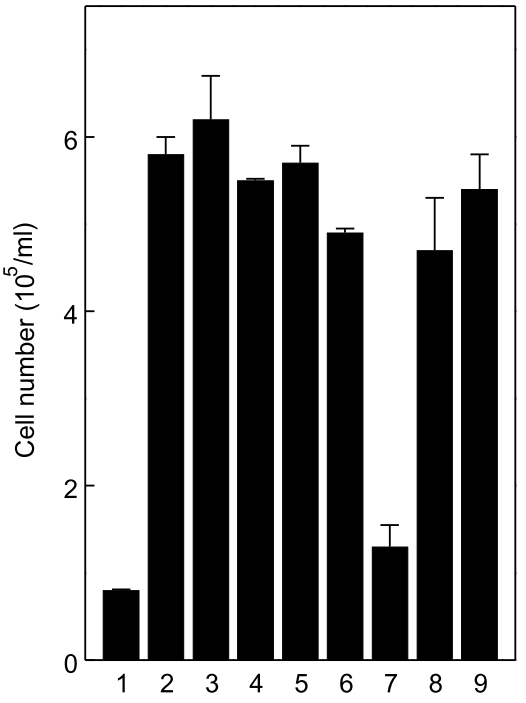

Fig. 9. Growth of uncloned immortal melanoblasts in the presence of various supplements. Cultures were plated on XB2 feeder cells at 4×10^4 cells/ml, in RPMI growth medium with 5% FCS and other supplements as specified, from the following five: TPA (20 nM), CT (50 nM), bFGF (20 pM), EA (1 μM) and PEA (1 μM). Media were changed twice weekly. Triplicate haemocytometer counts were made after 13 days (Materials and Methods). Means and s.e.m. are shown. Supplements: (1) none; (2) all five; (3) all except EA; (4) all except PEA; (5) all except bFGF; (6) all except CT; (7) all except TPA; (8) TPA only; (9) TPA and CT. Only treatments 1, 6 and 7 show significant differences in cell number from treatment 2, by Student's *t*-test.

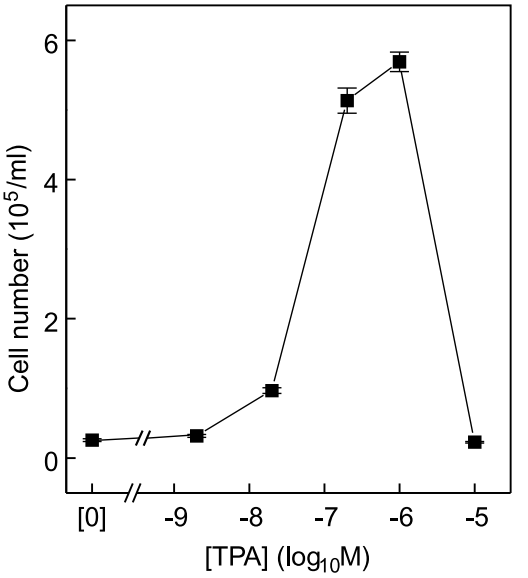

Fig. 10. Growth of cloned melb-a cells in the presence of TPA at different concentrations. Cell were plated at 2.4×10^4 cells/ml on XB2 feeder cells, and harvested after 17 days. Media were changed twice weekly. Means and s.e.m. of triplicate haemocytometer counts are shown.

to test whether immortal melanoblasts resembled malignant or transformed cells in being able to grow in suspension in semisolid agarose. Growth in agarose was assayed (see Methods) in three separate experiments, but no growing colonies at all were observed. B16 murine melanoma cells form many colonies in the same assay in our hands (e.g. Wakeling et al., 1992). Thus melb-a cells lack at least two properties of malignant melanoma cells (see Bennett, 1993): growth without TPA and growth in suspension.

DISCUSSION

Melanoblasts

What are melanoblasts, and is there only one kind? The isolation of immortal melanocytic precursors should help to answer this question. We do not yet know how many discrete changes in cell type (if they are discrete) are needed to convert a neural crest cell into a melanocyte (Stemple and Anderson, 1993), although clonal analysis in culture has greatly assisted the definition of successive precursor cell types in various cell lineages including early avian and murine neural crest cells (Sieber-Blum and Cohen, 1980; Baroffio et al., 1988; Ito et al., 1993; Stemple and Anderson, 1993). Some neural crest cells are pluripotent, and some appear to be bipotent (forming for example melanocytes and Schwann cells only) (Ito et al., 1993). Others form clones containing only melanocytes (unipotency), indicating that commitment to the melanocytic lineage can occur early in neural crest migration. We do not know whether these early committed precursor cells are of essentially the same type as the postnatal cells we describe here. Nor do we know yet whether the detectability of tyrosinase defines a significant step. All, or nearly all, cloned melb-a cells contain the melanogenic enzyme and early lineage marker TRP-2; but they include unpigmented cells both with and without tyrosinase, indicating either that there is no significant difference, or that 'two types of melanoblast' can grow together in a clonal culture. Conversely, clonal analysis has shown a difference in state of commitment between well-pigmented human melanocytes, which form clones only of pigmented cells, and unpigmented but tyrosinase-positive cells (then called 'premelanocytes'), whose progeny include both pigmented and unpigmented cells (Bennett et al., 1985). At present it seems economical to define 'melanoblasts' as any **unipotent precursors of melanocytes,** where melanocytes are cells that make melanin via tyrosinase. Further marker studies should help to define any subclasses in future.

Growth and differentiation of melanoblasts in primary culture

We found three protein factors that stimulated the net growth of primary melanoblasts even in the presence of 5% FCS, TPA, CT and keratinocyte feeder cells, namely bFGF, SCF and LIF. It is possible that these factors may be acting at least partly indirectly, by modulating activities of the feeder cells, although others have reported direct effects of bFGF and SCF upon melanocytes or melanoblasts in the absence of feeder cells.

bFGF has already been reported to stimulate proliferation of murine melanoblasts (Hirobe, 1992), as well as human melanocytes (Halaban et al., 1987; Herlyn et al., 1988), both in serum-free medium. Without serum, bFGF was found to inhibit melanoblast differentiation (Hirobe, 1992); however under our conditions no inhibition was observed. Keratinocytes can produce bFGF (Halaban et al., 1987), so some endogenous bFGF may have been present in our cultures, but not very much, since exogenous bFGF at even 20 pM had significant effects here. Concentrations that were active in the absence of serum were similar (optimal at 2.5 ng/ml or approx. 160 pM; Hirobe, 1992). Without serum, bFGF was not mitogenic except when dibutyryl cAMP was present (Halaban et al., 1987; Hirobe, 1992). In our medium bFGF was mitogenic even without the cAMP agonist CT.

Something else in our cultures (perhaps serum ACTH; see Hunt et al., 1994) may be increasing endogenous cAMP levels. Even if this is so, the cAMP levels are likely to be well below optimal levels for melanoblast/melanocyte proliferation, as judged by the large stimulatory effect of CT. We ascertained that the action of CT was not occurring through its B-subunit. The promotion of proliferation and pigmentation by CT probably involve the cAMP pathway (although CT can activate enzymes besides adenylate cyclase; Neer and Clapham, 1988), since these are well-known effects of cAMP agonists in pigment cells (e.g. Herlyn et al., 1988), including melanoblasts (Hirobe, 1992). By contrast **immortal** melb-a melanoblasts, like at least 5 lines of immortal murine melanocytes (Bennett et al., 1987, 1989; Spanakis et al., 1992), have little mitogenic response to CT. These findings raise the possibility that this loss of response may be required for immortalization of (non-tumorigenic) pigment cells, unlike the dependence on TPA, which is consistently retained.

SCF (synonyms SLF, MGF, KL) is produced by cells in both secreted and transmembrane forms, by alternative splicing (Flanagan et al., 1991). Melanocyte development is severely impaired by a germline mutation (*Steel-Dickie*) that affects only the transmembrane form of SCF (ibid.), in other words this form appears to be particularly important for melanoblasts. However, soluble SCF has been shown to stimulate thymidine incorporation and net growth in human diploid melanocytes (Funasaka et al., 1992), and we show here that it increases net growth of murine melanoblast cultures at concentrations of 20-100 ng/ml, or about 0.7-3.3 nM (higher than required for bFGF). High concentrations may be needed because (1) soluble SCF may be less active than transmembrane SCF for melanoblasts, or less stable, and/or (2) subsaturating amounts of SCF may already be present in our cultures, especially since we have detected SCF mRNA in both immortal melanocytes and XB2 keratinocytes by northern blotting (D. J. Easty, D. C. B. and others, unpublished data). Two studies have suggested that the primary effect of SCF on melanoblasts is as a survival factor (Steel et al., 1992; Morrison-Graham and Weston, 1993). Our data (on net cell population growth) do not exclude this, since melanoblast/melanocyte death is very hard to assess in growing cultures containing dying feeder cells. Increased melanoblast survival may also play a part in other cell-number increases described here.

LIF/DIA is shown here for the first time to promote (population) growth of cells of the melanocyte lineage. LIF is already known to have a wide range of biological activities (Hilton, 1992; Resnick et al., 1992), including promotion of differentiation and survival of another product of mouse neural crest, sensory neurons (Murphy et al., 1991). Its original 'differentiation inhibiting activity' was on early embryonic stem cells (ES cells). Here it increased melanoblast numbers more than melanocyte numbers, suggesting either inhibition of differentiation or differential mitogenesis. This can be tested in future using purer (immortal) melanoblast cultures. Again rather high concentrations were required for responses. Again there may be various reasons including instability or presence of endogenous LIF.

Keratinocyte or fibroblastoid feeder cells seem to be crucial for maintenance of the immortal melanoblasts without differentiation. We have now used similar methods to isolate other lines of melanoblasts of other genotypes, using either XB2 or SC1 cells (i.e. SC1 cells are not essential), taking typically 4-5 months per line. These lines are still being characterized, but at least three show the same response of differentiation on removal from feeder cells (unpublished work). The mechanism of this interesting effect will be addressed by future studies. The XB2 line originated from embryonal carcinoma cells (Rheinwald and Green, 1975), so we cannot be certain that they are typical keratinocytes. Preliminary work indicates that medium conditioned by XB2 cells can have a similar effect, suggesting that it is not mediated primarily by extracellular matrix or by cell-bound factors. The mechanism may involve either removal of something from the initial medium, or secretion of something. As already mentioned, bFGF and SCF appear to be among substances produced by keratinocytes, while LIF is secreted by some embryonic fibroblasts, especially in the presence of bFGF (Resnick et al., 1992).

Future uses of melanoblast lines

One primary aim in isolating immortal melanoblasts was for the cellular and molecular study of melanoblasts carrying germline mutations affecting their development (see Introduction). However the existence of these lines opens a number of other research avenues. These include molecular studies of melanocyte differentiation and its control; these have previously depended largely on the use of melanoma lines like B16 and S91 (Cloudman), in which differentiation may well be abnormal. (Immortal cells are not completely normal either, but from experience with melanocytes they tend to be a good deal more normal than malignant or transformed cells). Secondly these lines can be compared by clonal and molecular analyses with cells isolated from earlier stages of development, to help define the step(s) in differentiation and commitment between pluripotent neural crest cells and melanocytes. Lastly the cells will form a valuable normal analogue for comparison with unpigmented melanoma cells, for elucidation of those cellular properties associated with cellular immaturity as opposed to malignancy.

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REFERENCES

Abdel-Malek, Z., Swope, V., Collins, C., Boissy, R., Zhao, H. and Nordlund, J. (1993). Contribution of melanogenic proteins to the heterogeneous pigmentation of human melanocytes. *J. Cell Sci.* **106**, 1323-1331.

Baroffio, A., Dupin, E. and Le Douarin, N. M. (1988). Clone-forming ability and differentiation potential of migratory neural crest cells. *Proc. Natl. Acad. Sci. USA* **85**, 5325-5329.

Bennett, D. C. (1993). Genetics, development and malignancy of melanocytes. *Int. Rev. Cytol.* **146**, 191-260.

Bennett, D. C., Bridges, K. and McKay, I. A. (1985). Clonal separation of mature melanocytes from premelanocytes in a diploid human cell strain: spontaneous and induced pigmentation of premelanocytes. *J. Cell Sci.* **77**, 167-183.

Bennett, D. C., Cooper, P. J. and Hart, I. R. (1987). A line of non-tumorigenic mouse melanocytes, syngeneic with the B16 melanoma and requiring a tumour promoter for growth. *Int. J. Cancer* **39**, 414-418.

Bennett, D. C., Cooper, P. J., Dexter, T. J., Devlin, L. M., Heasman, J. and Nester, B. (1989). Cloned mouse melanocyte lines carrying the germline mutations albino and brown: complementation in culture. *Development* **105**, 379-385.

Bennett, D. C., Huszar, D., Laipis, P. J., Jaenisch, R. and Jackson, I. J. (1990). Phenotypic rescue of mutant brown melanocytes by a retrovirus carrying a wild-type tyrosinase-related protein gene. *Development* **110**, 471-475.

Eisinger, M. and Marko, O. (1982). Selective proliferation of normal human melanocytes in the presence of phorbol ester and cholera toxin. *Proc. Natl. Acad. Sci. USA* **79**, 2018-2022.

Fitzpatrick, T. B., Szabó, G., Seiji, M. and Quevedo, W. C. (1979). Biology of the melanin pigmentary system. In *Dermatology in General Medicine*, 2nd edn (ed. T. B. Fitzpatrick, A. Z. Eisen, K. Wolff, I. M. Freedberg and K. F. Austen), pp. 131-163. New York: McGraw Hill.

Flanagan, J. G. and Leder, P. (1990). The *kit* ligand: a cell surface molecule altered in steel mutant fibroblasts. *Cell* **63**, 185-194.

Flanagan, J. G., Chan, D. C. and Leder, P. (1991). Transmembrane form of the *kit* ligand growth factor is determined by alternative splicing and is missing in the Sl^d mutant. *Cell* **64**, 1025-1035.

Funasaka, Y., Boulton, T., Cobb, M., Yarden, Y., Fan, B., Lyman, S. D., Williams, D. E., Anderson, D. M., Zakut, R., Mishima, Y. and Halaban, R. (1992). c-Kit-kinase induces a cascade of protein tyrosine phosphorylation in normal human melanocytes in response to mast cell growth factor and stimulates mitogen-activated protein kinase but is down-regulated in melanomas. *Mol. Biol. Cell* **3**, 197-209.

Gabbianelli, M., Sargiacomo, M., Pelosi, E., Testa, U., Isacchi, G. and Peschle, C. (1990). 'Pure' human hematopoietic progenitors: permissive action of basic fibroblast growth factor. *Science* **249**, 1561-1564.

Green, M. C. (1989). Catalog of mutant genes and polymorphic loci. In *Genetic Variants and Strains of the Laboratory Mouse*, 2nd edn (ed. M. F. Lyon and A. G. Searle), pp. 12-403. New York: Oxford University Press.

Halaban, R., Ghosh, S. and Baird, A. (1987). bFGF is the putative natural growth factor for human melanocytes. *In Vitro Cell Dev. Biol.* **23**, 47-52.

Halaban, R., Moellmann, G., Tamura, A., Kwon, B. S., Kuklinska, E., Pomerantz, S. and Lerner, A. B. (1988). Tyrosinases of murine melanocytes with mutations at the *albino* locus. *Proc. Natl. Acad. Sci. USA* **85**, 7241-7245.

Herlyn, M., Mancianti, M. R., Jambrosic, J., Bolen, J. B. and Koprowski, H. (1988). Regulatory factors that determine growth and phenotype of normal human melanocytes. *Exp. Cell Res.* **179**, 322-331.

Hilton, D. J. (1992). LIF: lots of interesting functions. *Trends Biochem. Sci.* **17**, 72-76.

Hirobe, T. (1991). Selective growth and serial passage of mouse melanocytes from neonatal epidermis in a medium supplemented with bovine pituitary extract. *J. Exp. Zool.* **257**, 184-194.

Hirobe, T. (1992). Basic fibroblast growth factor stimulates the sustained proliferation of mouse epidermal melanoblasts in a serum-free medium in the presence of dibutyryl cyclic AMP and keratinocytes. *Development* **114**, 435-445.

Hirobe, T. (1994). Keratinocytes are involved in regulating the developmental changes in the proliferative activity of mouse epidermal melanoblasts in serum-free culture. *Dev. Biol.* **161**, 59-69.

Hunt, G., Todd, C., Kyne, S. and Thody, A. J. (1994). ACTH stimulates melanogenesis in cultured human melanocytes. *J. Endocrinol.* **140**, R1-R3.

Huszar, D., Sharpe, A. and Jaenisch, R. (1991). Migration and proliferation of cultured neural crest cells in W mutant neural crest chimeras. *Development* **112**, 131-141.

Ito, K. and Takeuchi, T. (1984). The differentiation *in vitro* of the neural crest cells of the mouse embryo. *J. Exp. Embryol. Morphol.* **84**, 49-62.

Ito, K., Morita, T. and Sieber-Blum, M. (1993). *In vitro* clonal analysis of mouse neural crest development. *Dev. Biol.* **157**, 517-525.

Jackson, I. J. and Bennett, D. C. (1990). Identification of the albino mutation of mouse tyrosinase by analysis of an *in vitro* revertant. *Proc. Natl. Acad. Sci. USA* **87**, 7010-7014.

Matsui, Y., Zsebo, K. and Hogan, B. L. M. (1992). Derivation of pluripotent embryonic stem cells from murine primordial germ cells in culture. *Cell* **70**, 841-847.

Mayer, T. C. (1982). The control of embryonic pigment cell proliferation in culture by cyclic AMP. *Dev. Biol.* **94**, 509-514.

Morrison-Graham, K. and Weston, J. A. (1993). Transient steel factor dependence by neural crest-derived melanocyte precursors. *Dev. Biol.* **159**, 346-352.

Murphy, M., Reid, K., Hilton, D. J. and Bartlett, P. F. (1991). Generation of sensory neurons is stimulated by leukemia inhibitory factor. *Proc. Natl. Acad. Sci. USA* **88**, 3498-3501.

Neer, E. J. and Clapham, D. E. (1988). Roles of G protein subunits in transmembrane signalling. *Nature* **333**, 129-134.

Parchment, R. E., Lewellyn, D., Swartzendruber, D. and Pierce, G. B. (1990). Serum amine oxidase activity contributes to crisis in mouse embryo cell lines. *Proc. Natl. Acad. Sci. USA* **87**, 4340-4344.

Pavan, W. J. and Tilghman, S. M. (1994). Piebald lethal (s^l acts early to disrupt the development of neural crest-derived melanocytes. *Proc. Natl. Acad. Sci. USA* **91**, 7159-7163.

Resnick, J. L., Bixler, L. S., Cheng, L. and Donovan, P. J. (1992). Longterm proliferation of mouse primordial germ cells in culture. *Nature* **359**, 550-551.

Rheinwald, J. G. and Green, H. (1975). Formation of a keratinizing epithelium in culture by a cloned cell line derived from a teratoma. *Cell* **6**, 317-330.

Sieber-Blum, M. and Cohen, A. M. (1980). Clonal analysis of quail neural crest cells: they are pluripotent and differentiate in the absence of noncrest cells. *Dev. Biol.* **80**, 96-106.

Silvers, W. K. (1979). *The Coat Colors of Mice*. New York: Springer-Verlag.

Spanakis, E., Lamina, P. and Bennett, D. C. (1992). Effects of the developmental colour mutations silver and recessive spotting on proliferation of diploid and immortal mouse melanocytes in culture. *Development* **114**, 675-680.

Steel, K. P., Davidson, D. R. and Jackson, I. J. (1992). TRP-2/DT, a new early melanoblast marker, shows that steel growth factor (c-kit ligand) is a survival factor. *Development* **115**, 1111-1119.

Stemple, D. L. and Anderson, D. J. (1993). Lineage diversification of the neural crest: *In vitro* investigations. *Dev. Biol.* **159**, 12-23.

Tang, A., Eller, M. S., Hara, M., Yaar, M., Hirohashi, S. and Gilchrest, B. A. (1994). E-cadherin is the major mediator of human melanocyte adhesion to keratinocytes in vitro. *J. Cell Sci.* **107**, 983-992.

Tsukamoto, K., Jackson, I., Urabe, K., Montague, P. and Hearing, V. (1992). A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed dopachrome tautomerase. *EMBO J.* **11**, 519-526.

Wakeling, W. F., Greetham, J., Devlin, L. M. and Bennett, D. C. (1992). Suppression of properties associated with malignancy in murine melanoma-melanocyte hybrid cells. *Br. J. Cancer* **65**, 529-537.

Warburton, M. J., Mitchell, D., Ormerod, E. J. and Rudland, P. S. (1982). Distribution of myoepithelial cells and basement membrane proteins in the resting, pregnant, lactating, and involuting rat mammary gland. *J. Histochem. Cytochem.* **30**, 667-676.

White, R., Hu, F. and Roman, N. A. (1983). False dopa reaction in studies of mammalian tyrosinase: some characteristics and precautions. *Stain Technol.* **58**, 13-19.

Yamamoto, H., Takeuchi, S., Kudo, T., Sato, C. and Takeuchi, T. (1989). Melanin production in cultured albino melanocytes transfected with mouse tyrosinase cDNA. *Jpn. J. Genet.* **64**, 121-135.

Zhou, B.-K., Kobayashi, T., Donatien, P. D., Bennett, D. C., Hearing, V. J. and Orlow, S. J. (1994). Identification of a melanosomal matrix protein encoded by the murine *si* (silver) locus using 'organelle scanning'. *Proc. Natl. Acad. Sci. USA* **91**, 7076-7080.

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