Human Melanoblasts in Culture: Expression of BRN2 and Synergistic Regulation by Fibroblast Growth Factor-2, Stem Cell Factor, and Endothelin-3

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The BRN2 transcription factor (POU3F2, N-Oct-3) has been implicated in development of the melanocytic lineage and in melanoma. Using a low calcium medium supplemented with stem cell factor, fibroblast growth factor-2, endothelin-3 and cholera toxin, we have established and partially characterised human melanocyte precursor cells, which are unpigmented, contain immature melanosomes and lack L-dihydroxyphenylalanine reactivity. Melanoblast cultures expressed high levels of BRN2 compared to melanocytes, which decreased to a level similar to that of melanocytes when cultured in medium that contained phorbol ester but increased to a level similar to that of melanocytes when cultured in medium containing menadione but which lacked endothelin-3, stem cell factor and fibroblast growth factor-2. This decrease in BRN2 accompanied a positive L-dihydroxyphenylalanine reaction and induction of melanosome maturation consistent with melanoblast differentiation seen during development. Culture of primary melanocytes in low calcium medium supplemented with stem cell factor, fibroblast growth factor-2 and endothelin-3 caused an increase in BRN2 protein levels with a concomitant change to a melanoblast-like morphology. Synergism between any two of these growth factors was required for BRN2 protein induction, whereas all three factors were required to alter melanocyte morphology and for maximal BRN2 protein expression. These findings implicate BRN2 as an early marker of melanoblasts that may contribute to the hierarchy of melanocytic gene control. Key words: melanoma/POU/melanocyte/tyrosinase. J Invest Dermatol 121:1150–1159, 2003

The POU domain DNA-binding transcription factors are developmental regulators of multiple cell lineages and represent a conserved family of proteins throughout metazoan evolution (Ryan and Rosenfeld, 1997; Dailey and Basilio, 2001). The Class III factor BRN2 (also known as POU3F2 and N-Oct-3) has been implicated in the development of several neural and glial cell lineages, including neurons and astrocytes (Fujii and Hamada, 1993; Schreiber et al, 1994), as well as the neural-crest-derived melanocytic lineage, owing to its expression in murine melanoblasts (Eisen et al, 1995). BRN2 also appears to be important for development of malignant melanoma because its expression in human melanoma cell lines is much higher than in primary melanocytes (Cox et al, 1988; Thomson et al, 1993; Eisen et al, 1995), and melanoma cell lines expressing antisense BRN2 lose the ability to form tumors in mice (Thomson et al, 1995). Additionally, BRN2 ablated melanoma cells revert to a less mature cell type lacking many markers of differentiated melanocytes such as tyrosinase (TYR)-related protein (TYRP) pigmentation genes and the microphthalmia associated transcription factor (Thomson et al, 1995), implicating BRN2 in sustaining the melanocytic phenotype. Interestingly, expression of BRN2 can be modulated in human melanoma cell lines, with differentiating agents decreasing expression and depigmentation agents increasing expression (Sturm et al, 1991), implying a role for BRN2 in maintaining the undifferentiated melanocytic phenotype.

Melanoblasts are the neural-crest-derived precursor of the melanocytes. Their study is valuable for the analysis of basic mechanisms in cell differentiation, for comparison with poorly differentiated cells from melanoma, and for the molecular analysis of the many known genetic disorders of melanocyte development. Melanoblasts have been defined as unpigmented cells that lack functional TYR, the critical enzyme of melanin synthesis (Hirote, 1992; Sviderskaya et al, 1995) containing only immature melanosomes (Kawa et al, 2000). However, others have defined melanoblasts as cells able to synthesize melanosomes (Jimbow et al, 1999) which mature through a four stage process, with Stage I–II melanosomes unable to synthesize melanin and hence considered immature, whereas Stage III–IV contain melanin (Marks and Seabra, 2001).

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Expression of the melanogenic enzymes TYR, TYRP1, and dopachrome tautomerase is useful in identifying melanoblasts. Of these, dopachrome tautomerase is the earliest melanoblast marker in murine embryos, being detected at 10 d postcoitum, whereas Tyr and Tyrp1 are not expressed until 14.5 d postcoitum and pigment production is not visible until 16.5 d postcoitum (Steel et al., 1992). In human embryos, melanoblasts have been detected by expression of another melanosomal protein SILV (determined by HMB-45 immunoreactivity) in the epidermis after 7 wk of gestation, with premelanosomes detectable at 10 wk (reviewed by Reedy et al., 1998).

Recently, Nishimura et al. (2002) demonstrated that murine melanoblasts reside in the lower permanent portion of the hair follicle and act as stem cells to supply melanocytes that provide melanin to the growing hair. Additionally, migration of melanoblasts from one hair follicle through the epidermis to follicles lacking melanoblasts can occur, where they assume the stem cell role in the new follicle. In hairless epidermis, melanoblasts differentiate to melanocytes which provide the surrounding keratinocytes with melanosomes for protection from ultraviolet radiation (Herlyn et al., 2000; Seiberg, 2001).

Cultures of murine melanoblasts have been described previously (Sviderskaya et al., 1995; Kawa et al., 2000; Sviderskaya et al., 2001) and are capable of differentiation in vitro to melanocytes. Several peptide growth factors are known to be required for melanocytic development in mouse and for the culture of both human and mouse melanocytes (Bohm et al., 1995; Meullmann and Halaban, 1998; Reedy et al., 1998; Goding, 2000; Halaban, 2000). Conditions for melanoblast culture typically include these growth factors in the medium. Stem cell factor (SCF) acts as a chemotactic factor for migration of murine melanoblasts into hair follicles (Jordan and Jackson, 2000) and can mediate interactions with the environment by altering integrin expression (Scott et al., 1994). SCF enhances murine melanoblast survival and proliferation in culture and can inhibit pigmentation (Reid et al., 1995; Sviderskaya et al., 2001). Endothelin-3 (EDN3) causes the differentiation of chick neural crest cultures to melanocytic and glial precursor cells (Lahav et al., 1996; Lahav et al., 1998); however, proliferation of immortal murine melanoblasts is not affected by removal of EDN3 (Sviderskaya et al., 2001). Interestingly, murine melanoblast cultures from piebald mice, which carry mutations in the EDN3 receptor Ednr, have impaired growth and differentiation (Sviderskaya et al., 1998).

Fibroblast growth factor-2 (FGF2) is a mitogen for melanocytes (Halaban et al., 1988a, b) and like SCF is required for murine melanoblast proliferation (Sviderskaya et al., 2001). FGF2 may also be involved in melanoma progression, because adenosivally delivered FGF2 conferred melanoma-like growth characteristics to human melanocytes (Nesbit et al., 1999). In combination with ultraviolet B it also induced lentiginous melanoma in skin reconstruction models (Berkling et al., 2001). The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) is routinely used as a mitogen in melanocyte cultures to prolong survival (Arita et al., 2000; Halaban, 2000). Importantly, FGF2, SCF, EDN3, and TPA have been classified as “potent synergistic mitogens” for human melanocyte growth owing to the convergence of signaling initiated by these growth factors on the mitogen-activated protein kinase pathway and activation of cAMP-responsive element-binding protein (Bohm et al., 1995; Halaban, 2000).

Conditions for the culture of primary human melanoblasts have yet to be described. Here, we report the establishment and partial characterization of primary human melanoblast cultures and demonstrate molecular and morphologic changes in response to different media conditions. Furthermore, we show that human melanoblasts express the BRN2 protein at high levels comparable to that seen in metastatic melanoma cell lines and in contrast to the low levels expressed in melanocytes. Reports describing the exquisite sensitivity of the levels of POU proteins in dimerization which subsequently determine stem cell fate (Niwa et al., 2000) and adult tissue specific gene expression (Ryan and Rosenfeld, 1997) implicate the BRN2 molecule as a possible essential control mechanism in the melanocytic differentiation pathway. Synergistic regulation of BRN2 levels by peptide growth factors within the melanocytic lineage may enable cell fate to be determined by an analogous dimerization mechanism (Smit et al., 2000). For this reason, we have examined the response of BRN2 to peptide growth factors in human melanoblast and melanocyte cell cultures.

This project complies with the provisions contained in the National Statement on Ethical Conduct in Research Involving Humans and complies with the regulations governing experimentation on humans.

MATERIALS AND METHODS

Mammalian cell culture Throughout this article, “melanoblasts” refers to melanocytes established from neonatal foreskin tissue in melanoblast growth medium and “MB:MC” refers to melanocytes grown in melanocyte growth medium for 1 wk before assay. Likewise, “melanocytes” refers to cells established from foreskin tissue in melanocyte medium and “MC:MB” refers to melanoblasts grown in melanoblast growth medium. Both melanoblasts and melanocytes were obtained from neonatal foreskin tissue, and melanocytes cultured as described with 10 ng per mL TPA and 0.6 µg per mL chola toxin (Smith et al., 2001) after release of epidermal sheets from the dermis by Disperse II (Roche, Basel, Switzerland) treatment. For melanocytes, trypsin was neutralized by addition of RPMI 1640 medium plus 10% fetal bovine serum (FBS; CSL Biosciences, Victoria, Australia) or for melanoblasts MCBD 153 (Sigma Chemical Co., St. Louis, MO) medium containing 10% chelated FBS and 2% FBS. Melanoblasts were plated out in MCBD 153 medium containing 10% chelated FBS, 2% FBS, 2 mM glutamine, 1.66 µg per L cholera toxin, 10 ng per mL SCF (Sigma), 100 nM EDN3, and 2.5 ng per mL FGF2 (Nesbit et al., 1999) at 37°C in 5% CO2/5%O2. Chelated FBS was prepared by mixing 15 g of Chexlab-100 (Sigma) per 500 mL of FBS for 1.5 h at 4°C with gentle stirring. Culture medium for melanoma cell lines was 10% FBS in RPMI 1640. The MM96L e8 LC cell line was established by phenotypically ablating BRN2 expression using BRN2 antisense RNA (Thomson et al., 1995). All culture media contained FBS of the same batch and contained penicillin (50 U/mL) and streptomycin (50 µg/mL). Primary murine neural crest cell lines were as described in Murphy et al. (1991). Fresh cultures were established according to Reid et al. (1996). Cultures that were Kit-positive and unpigmented were cultured in the presence of SCF (Reid et al., 1995, 1996) and contained approximately 10% Kit-positive cells. Pigmented cultures that were Kit-positive contained at least 68% pigmented cells and were cultured in the presence of SCF, EDN3, and 2-MSH (Reid et al., 1996). Fresh neural crest cultures were harvested 2 wk after initiation of culture.

Transmission electron microscopy Cells were harvested and washed in PBS, before resuspension of cell pellets in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Samples were postfixed in potassium-ferrocyanide-reduced osmium tetroxide in phosphate buffer (1% osmium and 0.25% ferricyanide) and stained en bloc in 2% uranyl acetate.

Blocks were dehydrated in ascending concentrations of acetone and subsequently infiltrated and embedded in Spurr’s resin. Sections were contrasted with lead citrate and viewed on a Hitachi H600 transmission electron microscope at 100 kV.

1-Dihydroxyphenylalanine reactivity Cells were grown on glass coverslips overnight at 37°C. 1-Dihydroxyphenylalanine (DOPA; Sigma Chemical Co.) was dissolved at 250 mM in 0.5 M HCl and then added to the growth medium to a final concentration of 1, 2, or 5 mM, with the medium pH after assay of approximately 7.5. Cells were cultured with DOPA for 3 h, washed 1 x in PBS for 2 min, fixed in freshly diluted Fast Green Concentrate (Sigma Chemical Co.) for 5 min, washed in PBS, and mounted in a medium onto ethanol-cleaned slides with Crystal/Mount (Biomeda Corp, Foster City, CA). Cells were photographed using an Olympus BH2 inverted microscope, and photographs were scanned to convert to digital images.

Western blotting Western blotting was performed essentially as described previously after resolution of 10 µg of total protein in SDS–PAGE gels (Leonard et al., 2002). Membranes were blocked for 1 h at room
demonstrated that a medium containing a low concentration of calcium (MCDB 153; 0.03 mM Ca\(^{2+}\)) was better than ones containing higher levels (RPMI 1640; 0.42 mM Ca\(^{2+}\)) as this inhibited growth of any contaminating fibroblasts. The concentration of EDN3 was best at 100 nM (range tested 1–100 nM) although there was not much increase in growth between 10 and 100 nM concentrations. Additions of FGF2 at 2.5 ng per mL and SCF at 50 to 100 ng per mL gave the best increase in growth, but culture with acceptable growth rates occurred at 10 ng per mL SCF and this was used routinely.

**Melanoblast morphology and marker expression**

Primary human melanoblasts typically had a triangular or bipolar morphology (Fig. 1A, panel f), similar to that reported for murine melanoblasts (Sviderskaya et al., 1995; Kawao et al., 2000). Like murine melanoblasts, they were unpigmented and unreactive to DOPA (Fig. 1A, panel f), indicating lack of active TYR and consistent with the cells’ having predominantly Stage I and II melanosomes as seen by transmission electron microscopy (Fig 1B, panel f). When melanoblasts were cultured in melanocyte medium (MB:MC cells), the cells changed subtly to be predominantly more bipolar in morphology, typical of primary melanocytes, and became DOPA reactive to a level comparable to that of melanocyte cultures (Fig 1A, panels 2 and 3). HeLa cells (Fig 1A, panel 4) are not melanocytic and therefore are DOPA-negative and were used as a negative control. Consistent with a positive DOPA reaction, the melanosomes in MB:MC cells had matured to Stage III and IV melanosomes (Fig 1B, panel 2) and resembled those of primary melanocytes (Fig 1B, panel 3). Additionally, melanoblast cells became pigmented after 1 wk in melanocyte medium, as seen in the cell pellets (Fig 1C).

A number of pigment markers were examined by immunohistochemistry in melanoblasts, MB:MC cells, and primary melanocytes (Fig 2.4). It was found that melanoblasts express TYR, although at lower levels than melanocytes (Fig 2A, panels 1–3 and 4–6). Localization in melanoblasts was primarily perinuclear, whereas TYR in MB:MC cells and melanosomes was dispersed throughout the cytoplasm. Immunoblot analysis showed that melanoblasts and MB:MC cells contained amounts of TYR protein similar to mature melanocytes (Fig 2B, lanes 1–3), suggesting that the DOPA reactivity in MB:MC cells occurred through protein activation or maturation, rather than de novo synthesis. The human melanoma cell line MM96L expressed lower amounts of TYR, consistent with cells’ having predominantly Stage I and II melanosomes as seen by transmission electron microscopy (Fig 1B, panels 10–12). MM96L expressed lower amounts of TYRP1 by Western blot analysis, and as expected, TYRP1 was not detected in MM96L c8 LC cell extracts (Fig 2B, lanes 4 and 5 respectively).

The HMB45 monoclonal antibody has been previously used to detect melanocytes in human skin (Holbrook et al., 1989). The antibody recognizes the product of the silver locus, SILV (Adema et al., 1994; Kawakami et al., 1994), mutation of which causes progressive graying of hair owing to loss of follicular melanocytes (Kwon et al., 1991, 1995). SILV has been implicated as having several roles in melanin synthesis, including melanosome biogenesis (Berson et al., 2001) and melanin stabilization and/or polymerization (Chakraborty et al., 1996; Lee et al., 1996). Immunohistochemical analysis of SILV expression in melanoblasts showed a perinuclear localization (Fig 2A, panels 10–12),
similar to TYR and STYRP1. In MB:MC cells, however, localization of the SILV extended throughout the cytoplasm. This distribution differed from that of melanocytes, where SILV localization was perinuclear.

Recently another melanocytic protein, MART-1, has been shown to have a distinct localization from melanocytic enzymes and to decrease in level upon melanosome maturation (De Maziere et al., 2002), implying a role in melanosome biogenesis. In melanoblasts, immunohistochemical analysis of MART-1 (Fig 2A, panels 13–15) revealed a perinuclear localization where present but not all cells were positive. Nevertheless, in both melanocytes and MB:MC cells, MART-1 localization was highly specific and presented as regularly spaced foci along the length of the dendrites. MART-1 was detectable by immunoblotting (Fig 2B) in melanoblasts and MB:MC cells at similar levels, which was approximately twice that of primary melanocytes, whereas MM96L and BRN2-ablated MM96L c8 LC cells had no detectable MART-1.
Numerous transcription factors have been implicated in melanoblast medium sustained high levels of BRN2 (MM96L extracts (lane 3) compared to a level more similar to that of primary melanocytes (lane 8). For each lane, BRN2 (N-Oct-3) DNA-binding activity was compared to Oct-1, and then this ratio was normalized to melanoblast (MB) or melanocyte (MC) cell, or melanocyte cultures, indicating that specific reactivity had occurred with other antibodies tested.

BRN2 expression by primary human melanoblasts

Numerous transcription factors have been implicated in melanocyte development; nevertheless, given the absence of melanocyte-specific proteins in BRN2-ablated melanoma cell lines (Thomson et al., 1995), we focused on the BRN2 transcription factor. Expression was analyzed by immunoblotting using anti-BRN2C polyclonal antibody (Smith et al., 1998). BRN2 was detected in melanoblast extracts at a level similar to that of the metastatic melanoma cell line MM96L (Fig. 3A, compare lanes 1 and 9), but not in primary fibroblasts and HeLa cells (lanes 7 and 8). Nevertheless, in MB:MC cells, expression of BRN2 decreased to a level more similar to that of primary melanocytes (Fig. 3A, compare lanes 2 and 4), whereas melanoblasts maintained in melanoblast medium sustained high levels of BRN2 (Fig. 3A, lane 3).

Because POU domain transcription factors bind both wild-type and divergent octamer sequences and are able to form homodimers on some sequences (Khee et al., 1998; Smit et al., 2000), EMSA were performed using probes containing BRN2-binding sites (Fig. 3B). The position of N-Oct-3 and N-Oct-5 (BRN2 products) DNA-binding activities were identified in MM96L extracts (Fig. 3B, lanes B–E) compared to the BRN2-ablated MM96L c8 LC (Fig. 3B, lanes 16–18). All extracts contained the ubiquitous Oct-1-binding activity, and no binding to the mutant octamer probe dpm8 was seen, consistent with previous results (Thomson et al., 1995). Results showed that melanoblasts had a typical melanocytic octamer DNA-binding profile, having Oct-1 as well as N-Oct-3 and trace amounts of N-Oct-5-binding activities (Fig. 3B, lanes 1–3) to H2B wild-type and OA25 divergent octamer sequences, with no specific binding to dpm8. Relative to melanocytes (Fig. 3B, lanes 7–9), melanoblasts had more N-Oct-5-binding activity to the H2B and OA25 probes. For MB:MC cells (Fig. 3B, lanes 4–6), however, no clear difference in the N-Oct-3 activity from melanocytes was seen for either probe.

Regulation of BRN2 expression in primary human melanocyte cultures

To determine the effects of medium, melanocytes were cultured in melanoblast medium (MC:MB cells). After 1 wk, western blot analysis showed an increase in BRN2 to levels similar to that of melanoblasts (Fig. 3A, compare lanes 6 and 8), whereas continued melanocyte cultures retained lower levels of BRN2 (Fig. 3A, lane 5). Furthermore, MC:MB cells acquired a morphology more akin to that of melanoblast concomitant with a reduced DOPA reaction (Fig. 3C) and had a reduced ability to synthesize pigment as seen by a less-pigmented cell pellet after centrifugation (Fig. 3D). When primary melanocytes were cultured in MCDB 153 (melanoblast) medium lacking SCF, FGF2, and EDN3, but supplemented with TPA, the cells remained bipolar but did not continue to grow (unpublished data). Taken together, these results imply BRN2...
upregulation by growth factor(s) in the melanoblast medium or repression by factor(s) in the melanocyte medium. Interestingly, despite dramatically increased BRN2 protein levels in MC:MB cells to a level similar to that of melanoblasts (Fig 3A), there was only a slight increase in N-Oct-3-binding activity relative to melanocytes (Fig 3B, lanes 10–12). To determine whether protein localization was responsible for the slight increase in N-Oct-3 DNA-binding activity in MC:MB cells, immunohistochemical analysis of BRN2 was performed (Fig 3E). Melanoblast, MC:MB cells, and MM96L cultures (Fig 3E, panels 1, 4, and 5, respectively) all showed strong nuclear reactivity for BRN2, whereas in MB:MC cells and melanocytes (Fig 3E, panels 2 and 3), BRN2 was undetectable by immunohistochemistry and showed only background staining, similar to BRN2 ablated MM96L c8 LC cells (Fig 3E, panel 6). Hence the ability of BRN2 to bind DNA in vitro is not as efficient in MC:MB cells compared to melanoblasts.

To determine which growth factor(s) were responsible for increased BRN2 in MC:MB cells, melanocytes were cultured in MCDB 153 supplemented with SCF, FGF2, or EDN3 separately, or in combination, either with or without TPA for 3 d and then harvested for western blot analysis (Fig 4A). Compared to routine melanocyte cultures, melanocytes grown for 3 d in MCDB 153 supplemented with SCF, EDN3, and FGF2 had a dramatically increased level of BRN2 (3.5-fold, Fig 4A, lane 6), with further upregulation when TPA was added (5.6-fold, Fig 4A, lane 7). Concomitant with this was a morphologic change to a polydendritic phenotype (Fig 4B, compare panel 1 (melanocyte medium) to cell morphology of selected examples of melanocytes grown with varying growth factor supplements as indicated in A, inset, lane number from A. (C) EMSA analysis of selected examples from A. The level of BRN2 (N-Oct-3) DNA-binding activity was compared to Oct-1 for each lane, with this ratio being normalized to that of melanocytes (lanes 1–3). FP, free probe.

Figure 4. Regulation of BRN2 in melanocytic cells. (A) Western blot analysis of melanocytes cultured in RPMI 1640 or MCDB 153 supplemented with FGF2, SCF, EDN3, or TPA as indicated. For each lane, the level of BRN2 was compared to IFA expression levels and the BRN2:IFA ratio was normalized to melanocytes cultured in melanocyte growth medium (lane 1). (B) Phase contrast photomicrographs for comparison melanocyte morphology (panel 1) to cell morphology of selected examples of melanocytes grown with varying growth factor supplements as indicated in A; inset, lane number from A. (C) EMSA analysis of selected examples from A. The level of BRN2 (N-Oct-3) DNA-binding activity was compared to Oct-1 for each lane, with this ratio being normalized to that of melanocytes (lanes 1–3). FP, free probe.
MCDB 153. When melanocytes were grown in MCDB 153 supplemented with only one growth factor, BRN2 levels did not increase and actually decreased in some cases (Fig. 4A, lanes 8–15), irrespective of TPA. Interestingly, when RPMI 1640 was supplemented with FGF2 alone, BRN2 levels increased in the presence, but not absence of TPA (Fig. 4A, compare lanes 2 and 3). For melanocytes grown in MCDB 153, addition of two growth factors (SCF and EDN3, FGF2 and EDN3, or FGF2 and SCF) resulted in increased BRN2 (Fig. 4A, lanes 14 and 15), whereas melanocytes cultured with FGF2 and EDN3 (Fig. 4A, lanes 16 and 17) or FGF2 and SCF (Fig. 4A, lanes 18 and 19) expressed lower levels of BRN2, similar to each other. It was noted that irrespective of the growth factor combination used, addition of TPA to the culture medium increased BRN2 for each combination with the exception of FGF2 and SCF. Increased BRN2 levels were also usually seen on EDN3 addition.

The morphology of selected cultures assayed for total BRN2 protein levels are shown in Fig. 4B. When melanocytes (Fig. 4B, panel 1) were cultured in MCDB 153 supplemented with one or two of SCF, EDN3, or FGF2 (Fig. 4B, panels 8, 10, 12, 14, 16, and 18), the cells remained bipolar. When all three growth factors were present in RPMI 1640 (Fig. 4B, panel 4) or in MCDB 153, with or without TPA (Fig. 4B, panels 6 and 7, respectively) an increase in the proportion of polydendritic cells in the culture was consistently observed. Hence, despite the presence of two growth factors in MCDB 153 resulting in a similar BRN2 increase similar to that obtained with all three factors, the change to a polydendritic morphology required SCF, FGF2, and EDN3.

Additionally, primary melanocytes were cultured for 3 d in melanoblast medium supplemented with two of FGF2, SCF, and EDN3, or all three factors, and then harvested for EMSA analysis (Fig. 4C) to support the findings by western blot analysis. When cultured with FGF2, SCF, and EDN3, there was an increase in BRN2 DNA binding to both the H2B and OA25 probes (Fig. 4C, compare lanes 4 and 5 with lanes 1 and 2). Interestingly, this increase is greater than the BRN2 increase observed after 7 d of culture (MCM:MB cells, Fig. 3B). When melanocytes were cultured in melanoblast medium supplemented with any two of FGF2, SCF, or EDN3 (Fig. 3C, lanes 7–15), there was a slight increase in N-Oct-3-binding activity to wild-type and divergent octamer sequences compared to melanocytes, with no binding to mutant octamer sequences.

Because MCDB 153 and RPMI 1640 differ greatly in the concentration of CaCl2, CaCl2 was added to MCDB 153 such that the concentration in MCDB 153 and RPMI 1640 was the same. Subsequently, melanoblasts were cultured in CaCl2-supplemented MCDB 153 with TPA alone or SCF, EDN3, and FGF2 and then assayed for BRN2 by western blot analysis. Results showed that in CaCl2-supplemented MCDB 153, the addition of SCF, EDN3, and FGF2 maintained high levels of BRN2 and the polydendritic melanoblast morphology, whereas addition of TPA (and absence of peptide growth factors) resulted in decreased BRN2 protein levels and a bipolar morphology (unpublished data) similar to MBMC cells. Additionally, supplementation of RPMI 1640 with SCF, EDN3, and FGF2 also increased BRN2 protein levels in melanocytes (Fig. 4A, lanes 4 and 5). Hence, the presence of peptide growth factors and not the differences in Ca2+ between the media is responsible for the differing BRN2 levels.

N-Oct-3 activity in murine neural crest To determine whether primary neural crest cells express BRN2, retrovirally transformed murine cell lines established from isolated trunk neural tubes of 9 d postcoitum embryos (Murphy et al., 1991) as well as mouse brain and MM96E melanoma cell nuclear extracts were assayed by EMSA for octamer DNA-binding activity. Oct-1 DNA-binding activity was detected in all extracts examined (Fig. 5A). Strong N-Oct-3 and N-Oct-5 activity was seen in the MM96E melanoma cell line extract (Fig. 5A, lane 3), whereas mouse brain had lower N-Oct-3 activity (Fig. 5A, lane 8). Of the neural crest cultures examined, NC 14.3.3G, NC 14.4.9D, NC 14.4.8, and NC 14.4.6E had detectable N-Oct-3-binding activity.
(Fig 5A, lanes 5, 11, 13, and 15 respectively). Two of these cell lines, NC 14.49D and NC 14.46E, have been classified as “differen-
tiated” cell lines, whereas NC 14.4.8 are considered “partially differentiating” cells and NC 14.3.3G as an “undifferentiated”
nervous crest-like cell type (Murphy et al, 1991).

Further to this, Reid et al (1995) demonstrated that Kit-positive
in neural crest cultures were precursors of melanocytes and that
addition of TPA resulted in cell pigmentation. Hence, we
examined primary neural crest cultures of Kit-negative, Kit-
positive, unpigmented and Kit-positive, pigmented cells using
mouse brain and A2058 melanoma cell extracts as controls for
N-Oct-3 DNA-binding activity. The A2058 melanoma cells
(Fig 5B, lanes 1–3) contained the Oct-1, N-Oct-3, and N-Oct-5
DNA-binding activities. The N-Oct-5 complex was detected in
all three neural crest cultures, whereas N-Oct-3 was not
apparent (Fig 5B, lanes 4–12). The N-Oct-5 protein has been
speculated to arise from proteolytic clipping of the N-Oct-3
protein (Aranasoski et al, 1997; Smith et al, 1998) and its
presence in less differentiated primary melanoma cell lines prompted
speculation that it may play a role in the differentiation process
during melanocyte development. Inclusion of an anti-BRN2C
antibody resulted in a supershift of the N-Oct-5 complex, indicating
that the protein complex detected contained a BRN2
gene product.

DISCUSSION
Melanoblasts are a neural-crest-derived cell type defined as spe-
cified melanocyte precursors which are unable to synthesize
melanin (Hirobe, 1992; Sviderskaya et al, 1995). Here, we have
described conditions for culturing human melanoblasts. We have
shown that they are unpigmented cells containing immature mel-
anosomes that are unreactive to DOPA (Fig 1). This is despite hu-
man melanoblasts expressing TYR and TYRP1 protein, unlike
murine melanoblasts in which Tyr was detected in only a minority
of cells by immunohistochemistry (Sviderskaya et al, 1995;
Kawa et al, 2000). Examination of the POU domain transcription
factor BRN2, implicated in differentiation of the melanocytic
lineage (Eisen et al, 1995; Thomson et al, 1995), demonstrated that
melanoblasts expressed high levels of BRN2 protein and N-Oct-
3 DNA-binding activity, similar to metastatic melanoma cell
lines and at a much higher level than melanocytes (Fig 3). Pre-
sumably, the lower relative fold increase in N-Oct-3 activity
compared to the increase in total BRN2 protein is due to a num-
ber of factors including normalization to different proteins and
inherent differences between western blot analysis and DNA-
binding assays. We have shown that BRN2 localization is not
the reason for these differences (Fig 3E). Nevertheless, in all ex-
periments, changes in BRN2 protein levels followed the same
qualitative trends as N-Oct-3 DNA-binding activity.

Upon change of growth factor supplementation, we were able
to induce primary human melanoblasts to become DOPA-posi-
tive and furthermore acquire the ability to synthesize melanin
owing to melanosome maturation as determined by transmission
electron microscopy (MB:MC cells, Fig 1). Concomitant with
this was a change in cell morphology to one resembling that of
melanocytes (Fig 1A) and a decrease in BRN2 to levels similar to
those of melanocytes (Fig 3A). Given the similarities between
MB:MC cells and melanocytes, and the definition of a melano-
blast (Sviderskaya et al, 1995), we consider MB:MC cells to be
melanocytes and that melanoblasts have been induced to differ-
entiate in vitro by the change in culture conditions.

Further to this, culture of melanocytes in melanoblast medium
(MC:MB cells) produced a decrease in DOPA reactivity and mel-
anin synthesis, a change in morphology, and an increase in
BRN2 protein and DNA-binding activities (Fig 3). Hence,
MC:MB cells are very similar to melanoblasts, and we inter-
preted this as a dedifferentiation of melanocytes in response to
medium changes. It is interesting to note that avian melanocytes
cultured with EDN3 have been reported to revert to a bipotent
progenitor cell capable of redifferentiation to melanocytes (Dupin
et al, 2000). Furthermore, avian Schwann cells cultured with
EDN3 revert to a bipotent glial-melanocyte precursor cell popu-
lation able to differentiate into melanocytes (Dupin et al, 2003).
It was not reported, however, whether the dedifferentiation of avian
melanocytes and glial cells seen with EDN3 resulted in an in-
crease in BRN2, similar to the dedifferentiation of melanocytes
seen here.

To elucidate which growth factors were responsible for the in-
crease in BRN2 protein, melanocytes were grown with several
combinations of growth factors and then assayed for BRN2 pro-
tein. This revealed a possible synergistic mechanism controlling
BRN2, requiring at least two of SCF, FGF2, and EDN3 to in-
crease BRN2 protein levels in MCDB 153, irrespective of the
presence of TPA. Endothelin-1 and SCF can act synergistically
to induce primary human melanoblasts to become DOPA-posi-
tive and furthermore acquire the ability to synthesize melanin
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in vivo (Collarini et al, 1992; Jaegle et al, 1996) and the embryonic
POU domain factor Oct-3/4 (POU5F1) is ex-
pressed by ES cells (Niwa et al, 2000; Reubinoff et al, 2000), but
are downregulated as the cells differentiate. Similar to BRN2
in melanoma, Oct-3/4 is upregulated again in cancer cell lines
(Monk and Holding, 2001). Interestingly, the neural-crest-derived
peripheral nervous system glial cell, the Schwann cell, expresses
the cell-type-specific transcription factors SOX10 and PAX3 (also
expressed by melanocytes) in addition to BRN2 and grows in
vitro as a dendritic cell similar to melanocytes (reviewed in
Jessen and Mirsky, 1998), consistent with the existence of a melanocyte–
Schwann cell duality during early development (Sawamoto et al, 1999).
NDP seen here.

BRN2/N-Oct-3 DNA-binding activity was also detected in
murine neural crest cultures (Fig 5). The NC 14.3.3G cell line
had high levels of N-Oct-3 and has been classified as an “undif-
ferentiated” neural crest-like cell type, because it expresses no
markers specific for terminally differentiated neurons or glia
(Murphy et al, 1991), suggesting that BRN2 is expressed early in
neural crest cell segregation. Interestingly, the NC 14.49D cell
line which was classified as a “differentiated” cell type, but was
considered bipotent owing to expression of both Schwann cell
and neuronal markers (Murphy et al, 1991), has N-Oct-3 activity
(Fig 5). Additionally, NC 14.49D has a strong mitogenic response
to FGF2, and a dendritic morphology similar to melanocytes and
Schwann cells (Murphy et al, 1991), and hence NC 14.49D may
represent a bipotent glia–melanocyte progenitor cell (Le Douarin
and Kalcheim, 1999). N-Oct-3 activity has also been detected in
neural-crest-derived carcinoma cell lines, such as small cell lung

carcinomas, Merkel cell carcinomas, astrocytomas, glioblastomas,
and Ewing's sarcomas (Schreiber et al., 1992; Schreiber et al., 1994; Thomson et al., 1994; Leonard et al., 2002) suggesting BRN2 involvement in the development of multiple neural crest lineages.

In conclusion, we have shown that human cultured melanoblasts express BRN2 at high levels comparable to melanoma cell lines and that this level decreased on differentiation to melanocytes. This supports the involvement of BRN2 in maintenance of the melanoblast phenotype and a reciprocal impairment of melanocytes maturation. The flexible DNA recognition and homodimerization properties of BRN2 in melanocytic cells (Rhee et al., 1998; Smit et al., 2000) make the levels of this protein critical and may allow it to act as both an activator and a repressor of melanocytic gene expression patterns mediating cell fate transitions.

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