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Functional neurons and melanocytes induced from immortal lines of postnatal neural crest-like stem cells

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ABSTRACT **Stem cells, that is, cells that can both reproduce themselves and differentiate into functional cell types, attract much interest as potential aids to healing and disease therapy. Embryonic neural crest is pluripotent and generates the peripheral nervous system, melanocytes, and some connective tissues. Neural-crest-related stem cells have been reported previously in postnatal skin: committed melanocytic stem cells in the hair follicle, and pluripotent cell types from the hair follicle and papilla that can produce various sets of lineages. Here we describe novel pluripotent neural crest-like stem cells from neonatal mouse epidermis, with different potencies, isolated as 3 independent immortal lines. Using alternative regulatory factors, they could be converted to large numbers of either Schwann precursor cells, pigmented melanocytes, chondrocytes, or functional sensory neurons showing voltage-gated sodium channels. Some of the neurons displayed abundant active TRPV1 and TRPA1 receptors. Such functional neurons have previously been obtained in culture only with difficulty, by explantation. The system was also used to generate comparative gene expression data for the stem cells, melanocytes, and melanoblasts that sufficiently explain the lack of pigment in melanoblasts and provide a rationale for some genes expressed apparently ectopically in melanomas, such as ephrin receptors.—Sviderskaya, E. V., Easty, D. J., Lawrence, M. A., Sánchez, D. P., Negulyaev, Y. A., Patel, R. H., Anand, P., Korchev, Y. E., Bennett, D. C. Functional neurons and melanocytes induced from immortal lines of postnatal neural crest-like stem cells. *FASEB J.* 23, 3179-3192 (2009). www.fasebj.org**

Key Words: determination · differentiation · melanoma · chondrocyte · Schwann cell

POSTNATAL STEM CELLS ARE ATTRACTING wide interest in relation to their possible potential for repair by autologous transplantation in humans following injury. They are also valuable for more basic studies, for example, those on cell lineage determination. There are still few experimental systems in which determination can be studied in mass cell cultures, in other words, where a pluripotent cell type can be either grown stably as a cell line or induced to differentiate into alternative lineages. One potential model from vertebrates is the neural crest. This group of pluripotent stem cells migrates from the dorsal neural tube, through the embryo, to produce restricted precursors and then neurons, melanocytes, peripheral glial (Schwann and satellite) cells, endoneurial fibroblasts, various endocrine cells, and diverse mesenchymal derivatives in the head (1–4).

There have been many studies of embryonic neural crest stem cells explanted in primary culture, generally from chick, quail, or mouse (refs. 5–8 and literature reviewed in refs. 2, 9). These cells have not, however, been propagated for long periods. Mizoguchi and colleagues established cell lines from mouse neural crest, but the immortal cells were melanoblasts or melanocytes rather than pluripotent cells (10). Normal, primary, neural-crest cell cultures are small in scale, laborious to prepare, and short term. These studies, together with analyses of gene mutations in mice, have nonetheless produced much information

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on extracellular factors able to direct differentiation into particular lineages (reviewed in refs. 2, 9, 11, 12). Likewise, various transcription factors have been implicated in controlling specification of the vertebrate neural crest and in the differentiation or survival of its derived lineages. These include Pax3, Sox10, Sox4, Snail, Snai2/Slug, Brn2/Pou3f2, Mitf, Foxd3, Tfap2 (AP2), and others, as discussed in several reviews (13–15).

There is evidence for retention of pluripotent neural crest-like stem cells (NCLSCs) and intermediate precursors in various mammalian tissues, both before and after birth, including fetal dorsal root ganglia (16), nerve (3), and adult gut (17). Several groups have identified neural crest-like cells in postnatal skin appendages. Sieber-Blum *et al.* (18) obtained cells from the bulge region of adult mouse whisker follicles that could generate neurons, melanocytes, Schwann cells, and chondrocytes. Neural crest-like cells designated SKPs (skin-derived precursors) were found in adult mouse dermal hair and whisker papillae; these could produce mesenchymal cells, neurons, and glial cells (19). Yu *et al.* (20) derived stem cells from human scalp hair follicle epithelium that could form melanocytes, neurons, and smooth muscle cells. Wong *et al.* (21) reported a difference between head and trunk: neural crest stem cells from head hair/whisker follicles produced cells including mesenchymal derivatives and neurons, but those from follicles of the trunk yielded only melanocytes and glia. However, in none of these reports were the stem cells isolated as pure populations or permanent lines. Here we describe 3 independent stem cell lines with potencies differing from those of the stem cell types previously described. They form an attractive experimental system to study both determination and neurophysiology, since they can be obtained without sacrificing animals, and in bulk. The cells can produce high yields of pre-Schwann cells, chondrocytes, functional melanocytes, or functional sensory neurons. We present what we believe to be the first physiological study of mammalian neurons derived in culture from stem cells; there was also an early study by Bader *et al.* (22) using avian neural crest cells. The system has also been used to generate gene expression data sufficient to explain the lack of pigment in melanoblasts, and that highlight resemblances of melanoma cells to melanoblasts and stem cells, for example, in ephrin receptor expression.

MATERIALS AND METHODS

Materials

Tissue culture plastics (Nunc), FCS, and newborn calf serum (NBCS) were obtained from Life Technologies Europe (Uxbridge, UK). Fibroblast growth factor 2 (FGF2) and neuregulin 1-β1 (NRG1-β1) were obtained from R&D Systems Europe (Abingdon, UK). Bone morphogenetic protein 2 (BMP-2) was obtained from PeproTech EC (London, UK). Other peptide factors, TPA, mitomycin C, and 5-azacytidine were obtained from Sigma-Aldrich (Gillingham, UK). NDP-MSH is an analog of αMSH that is resistant to degradation by serum enzymes. Protein factor stock solutions were prepared in PBSA (Dulbecco's PBS lacking $CaCl_2$ and $MgCl_2$) with bovine serum albumin (1 mg/ml) as carrier, and stored at −70°C.

Feeder cells

Growth-inactivated feeder cells were either XB2 murine keratinocytes (23), or SC1 dermal fibroblastoid cells (24). XB2 stocks were grown in DME medium with 10% FCS, and SC1 cells in RPMI 1640 medium with 5 or 10% FCS. Feeder cells were prepared by treatment with mitomycin C (6 μg/ml for 3 h), followed by washing, resuspension, and freezing as described previously (25). Feeder cells were thawed and plated usually 1 d before use.

Establishment and passage of NCLSCs

Primary cultures from mouse trunk skin were made as for melanoblast/melanocyte cultures (24). The skin was from neonatal *misty* (*m/m*) mice. [The *misty* mutation impairs proliferation of melanocytes and increases their pigmentation but has no detectable effect on melanoblasts (26), so it seems unlikely that this mutation is relevant to the presence in skin of the cell type described here.] In short, trypsin-separated epidermal sheets were dissociated with trypsin and EDTA, and the resulting cell suspension was plated onto keratinocyte feeder cells. The basic growth medium was RPMI 1640 with penicillin, streptomycin, and glutamine (2 mM). Incubation was with 10% v/v CO_2 at 37°C throughout. Initial supplements in these cultures were FCS (5%), TPA (200 nM), cholera toxin (CT) (200 pM), FGF2 (20 pM), ethanolamine (1 μM), and phosphoethanolamine (1 μM) as used in a melanoblast growth medium (27). Fresh XB2 cells were added at 3 wk, and after this the medium was supplemented with TPA (200 nM) and CT (50 pM) only. From passage 1, cultures were plated onto SC1 fibroblastoid feeder cells (24). New feeder cells were added every 2–3 wk. Cells were subcultured by rinsing gently in PBSA and then in 250 μg/ml trypsin and 200 μg/ml EDTA in PBSA; they were incubated until completely detached, and resuspended and diluted in growth medium as required. At one point cultures were treated with antibiotic G418 (75 μM, 3 d) to kill fibroblasts; this drug can select for melanocytes and melanoblasts (24, 28) and appeared effective also in selecting for NCLSCs.

NCLSC lines, once established, were routinely grown in RPMI 1640 medium with 10% NBCS and 2 nM TPA. For experiments, various supplements were added, at the following concentrations unless specified: FCS (10%), NBCS (10%), TPA (2 nM), NDP-MSH (1 nM), CT (1 nM), 5-azacytidine (300 pM), ascorbic acid 2-phosphate (50 μg/ml), TGFβ1 (120 pM), NRG1-β1 (10 nM), brain-derived neurotrophic factor (BDNF) (25 ng/ml), nerve growth factor (NGF) (50 ng/ml), neurotrophin 3 (NT3) (25 ng/ml), BMP-2 (10 ng/ml), and FGF2 (40 pM).

Other cell lines

Mouse melanoblast lines melb-a (nonagouti, *a/a*) and melb-s (piebald, *Ednrb^s/ Ednrb^s*) were grown in RPMI 1640 medium with 10% FCS and 20 nM TPA as described previously (melanoblast medium) (24, 26); SC1 feeder cells were used for melb-a cells and omitted for the final 2 wk. Mouse melanocyte lines melan-a, melan-a2, melan-c (albino, *Tyr^c/Tyr^c*), melan-s (*Ednrb^s/Ednrb^s*), melan-m1 (misty, *m/m*), melan-p1 (pink-eyed dilution, *p*-null), melan-rs (recessive spotting, *rs/rs*), and melb-p3 (*p*-null) melanoblasts (24, 29–31) were grown in RPMI 1640 medium with FCS (10%), TPA (200 nM), and CT (200 pM)

(melanocyte medium). Melan-s1 and melan-a2 are melanocyte lines derived by differentiation from melanoblast lines melb-s1 and melb-a, respectively (26). B16-F1 and K1735 murine melanoma cells, originally obtained from I. J. Fidler (M. D. Anderson Cancer Center, Houston, TX, USA), were grown in RPMI 1640 with FCS (10%).

Digital image acquisition

Unstained cultures for photography were usually fixed in 4% formaldehyde in PBSA, rinsed in distilled water, and air dried; they were photographed in water. TIFF images were obtained at room temperature through an Olympus IMT-2 inverted microscope and ×10 objective (numerical aperture 0.30; Olympus, Tokyo, Japan), using a Pixelink PL-A662 camera and Pixelink A6xx-Capture software (Pixelink, Ottawa, ON, Canada). Immunostained cells were mounted under coverslips in Aquamount (BDH, Poole, UK) and (except where stated,) photographed using a Zeiss Axioplan microscope with an ×40 objective (numerical aperture 0.75) and Axiovision 4.6 digital acquisition software (Carl Zeiss, Oberkochen, Germany). Montages were prepared in Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Cell proliferation assays

Triplicate cultures for growth/differentiation experiments were plated at the specified density in 3-cm dishes (2 ml/dish) and cultured as specified. Media were renewed twice weekly. Cells were harvested separately from each plate by trypsinization. Triplicate cell counts from each suspension were made by hemocytometer.

To coat culture dishes with gelatin, the dish bases were covered with a sterile gelatin solution (10 μg/ml in water) and left to stand for 5 min. The solution was removed and the dishes were rinsed in PBSA before plating cultures.

Immunocytochemistry and histochemistry

αPEP8 and αPEP13 rabbit polyclonal antisera, against murine dopachrome tautomerase and Si/gp87, respectively, were kindly provided by Dr. Vince Hearing (U.S. National Institutes of Health, Bethesda, MD, USA). Rabbit monoclonal anti-substance P, rabbit anti-neurofilament 200 (IgG fraction), mouse monoclonal anti-GAP43 (clone GAP-7B10), and Alcian blue were obtained from Sigma-Aldrich. Rabbit polyclonal anti-GFAP and anti-nestin and FITC-conjugated goat anti-rabbit IgG were obtained from Abcam (Cambridge, UK).

Procedures for immunocytochemistry with these antibodies were as described previously (24), except that the secondary antibody conjugate for anti-GAP43, GFAP, and substance P was alkaline phosphatase from EnVision System-AP (Dako, Cambridge, UK) and was used according to the manufacturer's instructions; the secondary antibody for nestin was goat anti-rabbit IgG-FITC (1:200). Nuclei were counterstained where specified with DAPI (1 μg/ml). Primary antibodies were diluted as follows: substance P, 1:40,000; GAP43 and GFAP, 1:10,000; nestin, 1:250; others 1:1000. For neurofilament and TRPV1 staining, mixed neurofilament antibodies (NA 1297; Affiniti Research Products, Exeter, UK; 1:5000) and rabbit anti-TRPV1 antisera (GSK, Harlow, UK; 1:10,000 for GSK-C22 and 1:400 for GSK no. 2) were used. The specificity of GSK-C22 has been reported previously (32), while GSK no. 2 against a different epitope can be preabsorbed using homologous peptide antigen on tissue sections. Cells were washed in PBS, fixed in 4% w/v paraformaldehyde (30 min), permeabilized in Triton X-100 (0.2% in PBS) (30 min), and dehydrated with graded alcohols. Endogenous peroxide was blocked with 0.3% v/v H_2O_2 in methanol (30 min). Cells were rehydrated, and primary antibodies in PBS with BSA (0.5 mg/ml) and sodium azide (1 mg/ml) were applied overnight, then visualized by a nickel-enhanced avidin-biotin-immunoperoxidase method (33). Nuclei were counterstained with neutral red (0.5 mg/ml), and cells were photographed with an Olympus BX50 photomicroscope.

Northern blot analysis

Poly(A)-enriched RNA was isolated from cell lines and from normal mouse liver and kidney, and separated by agarose gel electrophoresis (5 μg/lane); Northern blot analysis was performed as described previously (31, 34). Probe cDNA sequences were kindly provided by the following: Colin Goding (Oxford Ludwig Institute, Oxford, UK) (Brn2, Mitf, Pax3, Snai2), Ian Jackson (MRC Human Genetics Unit, Edinburgh, UK) (Tyr, Tyrp1, Dct), Mike Clemens (St. George's, University of London, London, UK) (Gapd), Roger Cone (Vollum Institute for Advanced Biomedical Research, Portland, OR, USA) (Mc1r), Christine Farr (Genetics, University of Cambridge, Cambridge, UK) (Sox4), Ed Geissler (Harvard Medical School, Boston, MA, USA) (Kit), Byoung Kwon (University of Ulsan, Ulsan, South Korea) (Si), Rick Lindberg (Amgen, Thousand Oaks, CA, USA) (Efna1), Toshi Shioda (Massachusetts General Hospital Cancer Center, Boston, MA USA) (Cited1), Richard Spritz (University of Colorado Health Sciences Center, Denver, CO, USA) (P), Jim Weston (University of Oregon, Portland, OR, USA) (Pdgfra), and David Wilkinson (National Institute for Medical Research, London, UK) (Epha4). Probes were labeled by random hexamer priming to a specific activity of $\sim 2 \times 10^9$ dpm/μg DNA. Prehybridization and hybridization were carried out at 42°C. The final wash was in 0.5× sodium-saline-phosphate EDTA buffer with 0.1% w/v sodium dodecyl sulfate, at 55°C. After washing, filters were exposed for up to 3 d at −70°C with 2 intensifying screens.

Ligand-responsive calcium fluxes

NC-m4 cells were plated on coverslips and grown for 14 d with FCS, TPA, BDNF, NGF, NT3, and FGF2 to induce neuronal differentiation. The cells were transferred to a perfusion chamber (RC-25;Warner Instruments, Hamden, CT, USA), in a bath solution containing 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES at pH 7.4. (All inorganic reagents were from Sigma-Aldrich.) Cells were loaded with fluo-4 acetoxymethylester (fluo-4 AM; Molecular Probes, Eugene, OR, USA) for 1 h. This and subsequent steps were at room temperature (25±2°C). Residual fluo-4 was washed out, followed by a 15-min pause for deesterification. Capsaicin was added to the bath chamber at 10 μM final concentration from a 10 mM solution in DMSO, or cinnamaldehyde was added at a final 100 μM from a 10 mM solution in ethanol. Ca^{2+} fluctuations were imaged by exciting fluo-4 at 450–480 nm and detecting emitted fluorescence at >520 nm, using an intensified CCD (CoolView IDI camera system; Photonic Science, Sussex, UK) coupled to a Nikon TE-2000 inverted microscope (Nikon, Tokyo, Japan) and controlled by Image-Pro Plus software (Media Cybernetics, Wokingham, UK). Images were acquired at 2 frames/s with 100 ms individual frame exposure time. Data were further analyzed with Origin 5 (Microcal Software, Northampton, MA, USA). Corresponding time traces show normalized fluorescence intensity (ratio of fluorescence to initial fluorescence, F/F_0) in the area of interest.

Whole-cell voltage-clamp recordings

NC-m4 or NC-m6 cells were grown on glass coverslips in the medium specified, for 2 wk. Whole-cell patch-clamp recordings were conducted at room temperature (21°C) using a MultiClamp 700B amplifier and Clampex 9.2 software (both from Molecular Devices Corp., Sunnyvale, CA, USA). Fire-polished electrodes (2.5–2.7 MOM) were fabricated from 1.0-mm borosilicate glass capillaries (Intracel Ltd, Royston, UK) using a P-2000 Puller (Sutter Instrument Co., Novato, CA, USA). In whole-cell voltage-clamp mode, the capacity current was diminished as much as possible with the amplifier circuitry, and series resistance compensation was set at 25–80%. The pipette potential was zeroed before seal formation, and voltages were not corrected for liquid junction potential. Leakage current was digitally subtracted online using hyperpolarizing control pulses, applied before the test pulse (P/N procedure). Recordings were made within 10 min after forming the whole-cell configuration. The pipette solution contained 140 mM CsF, 1 mM EGTA, 10 mM NaCl, and 10 mM HEPES, pH 7.3. Internal fluoride was used to inhibit high voltage-activated Ca^{2+} currents and help to reveal TTX-resistant currents (35, 36). Electrophysiological data were analyzed using Clampfit 9.2 (Molecular Devices) and Origin 5.0 (Microcal Software) software.

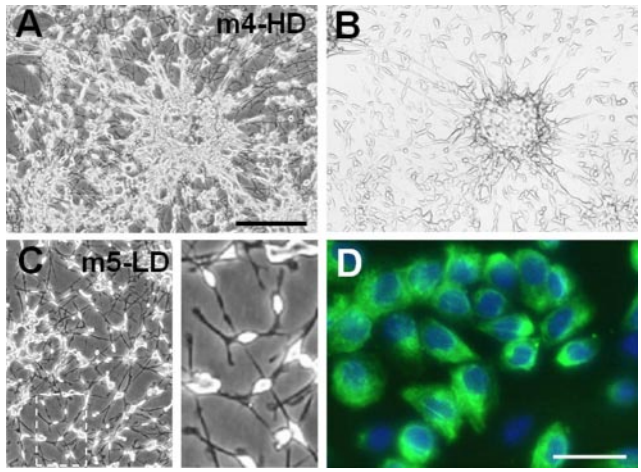

Figure 1. Morphology and immunostaining of NCLSC lines. All 3 lines had a similar appearance and behavior. *A*, *B*) NC-m4 cells are shown at fairly high density (HD) by phase contrast (*A*), showing clumps and strands of cells, and bright-field optics (*B*), showing lack of pigmentation. *C*) NC-m5 cells at lower density (LD) by phase contrast. Outlined area is magnified at right. *D*) Immunostaining of NC-m6 cells for nestin, a stem cell marker. Cells show distinct cytoplasmic immunoreactivity for this marker. Nuclei were counterstained with DAPI. Scale bars = 150 μm (*A–C*); 25 μm (*D*).

RESULTS

Establishment and growth of immortal neural crest-like cell lines from skin

With the initial aim of growing melanoblasts, 3 independent primary epidermal cultures were prepared from separate neonatal mouse skins and plated on keratinocyte feeder cells in a melanoblast medium, which was initially varied slightly to optimize growth (Materials and Methods). From the first passage (after ~4 wk), these cultures were plated onto SC1 fibroblast feeder cells instead, with 10% NBCS and 2 nM 12-*O*-tetradecanoyl phorbol acetate (TPA, a component of the melanoblast medium) and grown thus until immortal cells were obtained (see below). Three parallel *m/m* cultures, grown with keratinocyte feeder cells rather than SC1 cells, all gave rise to melanocyte lines (melan-m1, -m2, and -m3) (31). In all 3 independent cultures grown on SC1 cells, however, an unfamiliar cell type appeared: extremely small, unpigmented, and spindly, with thin processes and a tendency to aggregate into clumps and strands (**Fig. 1**). Growth was initially very slow, and frozen stocks were prepared at passage 6 after 9 mo. Three independent cell lines were established using this method and were later designated NC-m4, -m5, and -m6 (neural crest-like cells-misty, lines 4–6) (Fig. 1 and Supplemental Fig. 1*A*).

It was suspected that these cells were pluripotent, as the cell shape and size resembled those of neural crest cells (37), and colonies of melanoblasts, melanocytes, and other cell types (as detailed below) appeared under some conditions. One of the lines, NC-m6, was cloned by limiting cell dilution, to exclude contaminating cell populations. The same additional cell types continued to appear, however, showing that the small cell type could produce them. After experimentation (described below), growth of the small cell type appeared best in medium with NBCS and 2 nM TPA, in which apparently pure cultures with this morphology could be obtained. These supplements were adopted for routine culture. Since these small cells were found able to produce several neural-crest-derived cell types, they have been designated "neural crest-like stem cells." The NCLSCs showed evidence of bypassing replicative senescence, in that they initially grew extremely slowly, then accelerated somewhat, and now appear immortal. We previously showed that NC-m4 cells express neither p16 nor Arf (effectors of senescence in the mouse) (38). The 2 most extensively characterized lines, NC-m4 and NC-m6, have been grown up to 31 and 30 passages, respectively (≥90 population doublings).

Morphology and substrate adhesion of NCLSCs

NCLSCs in all 3 lines were extremely small, with cell-body diameters of ~6-8 μm (Fig. 1). Confluent cultures contained up to $\sim 3 \times 10^6$ cells /ml (~10 times that of a confluent culture of normal fibroblasts). At high cell density, NCLSCs cohered into clumps and netlike strands (Fig. 1), which tended to detach and float in the medium. Similar cell clumps have been described in embryonic neural crest cultures (*e.g.*, ref. 37), and they are also reminiscent of the spheres produced by SKPs (19) and hair follicle stem cells (20). Substrate adhesion of NCLSCs appeared generally low, potentially explaining the clumping. Tests indicated that adhesion could be increased by several types of protein coating of the plastic substrate, including collagen or gelatin. In medium with NBCS, no difference

in cell number was detectable, but when FCS was used, significantly more cells were obtained in cultures on gelatin ($P<0.01$), and cells remained attached to the plastic rather than forming clusters. Thus, a gelatin substrate was subsequently used for stock cultures grown in FCS, but not for cultures in NBCS.

We immunostained NC-m6 and NC-m4 cells with the well-established stem cell marker nestin (19). The great majority of the cells (384/417; 91.7±1.3%) showed distinct cytoplasmic immunoreactivity for nestin. Control melan-c albino mouse melanocytes demonstrated only background staining (Fig. 1 and Supplemental Fig. 1*B, C*).

Induced differentiation of melanoblasts and melanocytes

Melanocytic determination from cultured embryonic crest can be stimulated by agents, including TPA with a cAMP agonist (6), stem cell factor (SCF) and endothelin 3 (7, 39), and Wnt1 (8). Here we tested for promotion of melanocytic differentiation of NC-m4 cells by FCS, TPA, and the cAMP agonists CT and [4-Nle, 7-D-Phe] α-melanocyte-stimulating hormone (NDP-MSH), a stable analog of αMSH. All cultures with CT initially showed apparent cell death, as indicated by floating, phase-dark single cells and lower cell numbers relative to controls at 2–4 wk ($P<0.001$). In cultures with FCS and 200 nM TPA as well as CT, or sometimes with FCS and 200 nM TPA only, groups and colonies of melanoblast-like cells emerged (larger, bi-, and tripolar). Pigmented cells (melanocytes) were typically seen within the colonies by 10–14 d (**Fig. 2*B*;** compare NCLSCs in Fig. 2*A*). The efficiency of melanoblast/melanocyte production varied in the long term (possibly depending on batch of FCS). These cells were immunostained for melanosomal proteins Si/Silv/gp87/Pmel (silver) and dopachrome tautomerase (Dct), expressed by melanoblasts and melanocytes (26). Most cells in the colonies showed cytoplasmic staining for both Dct and Si (Si immunostain shown as illustration in Fig. 2*C*). After growth of the melanoblast-like cells into lines, these and other lineage markers were corroborated by gene expression studies (see below), confirming that the unpigmented cells were melanoblasts. In comparison, NC-m4 NCLSCs expressed no melanocytic marker. Melanocytes were also obtained when NDP-MSH was used instead of CT. They tended to appear in the centers of colonies, consistent with observations that high local cell density promotes melanocytic differentiation (40, 41).

Likewise, no pigmented cells were visible in the cloned NC-m6 line when grown with NBCS and 2 nM TPA, but these appeared 1-2 wk after changing the supplements to FCS, CT, and 200 nM TPA, with or without additional melanogenic factors, showing that determination rather than selection was occurring. Long-term culture with these factors resulted in the establishment of an apparently pure melanocyte line, melan-m6.

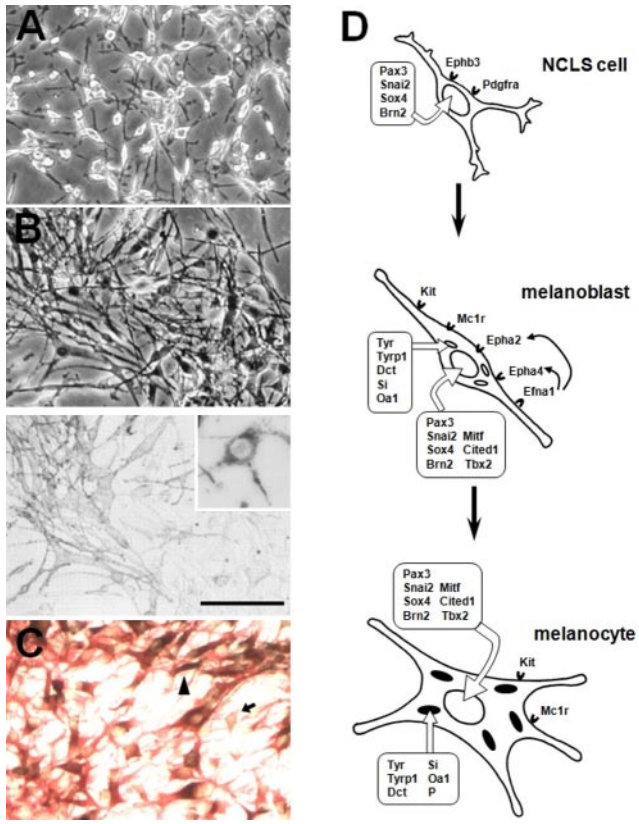

Figure 2. Differentiation to melanoblasts and melanocytes. *A*) Control NC-m4 cells grown with FCS and TPA (2 nM) (phase contrast). *B*) Pigmented melanocytes in an NC-m4 culture plated sparsely (3×10^3 cells/ml) and grown with FCS and TPA (200 nM) for 9 wk, seen by phase-contrast (top) and bright-field optics (bottom) to show melanocytic morphology and melanin pigment. Inset: higher magnification showing clustered melanosomes. *C*) NC-m4 cells after growth with TPA (200 nM) and MSH for 3 wk and immunostaining for melanosomal protein Si; bright-field optics. Apparently all cells showed specific cytoplasmic staining (red); some (melanocytes, arrowhead) had melanin (brown to black) while others (melanoblasts, arrow) did not. Scale bar = 100 μm (except inset). *D*) Diagram summarizing gene expression observed in NCLSC, melanoblast, and melanocyte lines. Findings in Table 1 are summarized. Note that gene products are shown by protein location for clarity only, because mRNA expression does not guarantee the presence of the protein. Filled ovals represent melanosomes; small unfilled ovals represent premelanosomes. Gene products (P, Oa1) associated with melanosome biogenesis are shown in the melanosomal set. Note that expression patterns of 2 genes included here were reported in previous publications before we were certain of the pluripotency of the NCLSCs. At that time the NCLSCs were described as "melanoblast precursors," and the NC-m4 and NC-m6 lines were named only m4 and m6. These genes were Tbx2 (47) and Oa1 (69).

Melanogenic factors similarly induced NC-m5 cultures to produce substantial numbers of melanoblasts; these were grown up giving an apparently pure melanoblast line, melb-m5. In turn, a pigmented melanocyte line, melan-m5, was produced from these melanoblasts by growth in melanocyte medium. A pure line of melanocytes (melan-m4) was also produced from

NC-m4 cells by expansion of one pigmented colony in melanocyte medium. These lines where tested showed typical melanoblast and melanocyte gene expression (next section).

Gene expression in NCLSCs compared to melanoblasts and melanocytes

Since both melanoblasts and melanocytes, including those derived from NCLSCs, can be grown as stable lines, extensive comparisons of gene expression are possible. We used Northern blot analysis and scanning densitometry to give semiquantitative comparisons of expression of a variety of genes, including regulatory transcription factors, other mRNA markers of neural crest or melanocytes, and some genes relevant to melanoma. The results are summarized in **Table 1** and Fig. 2*D*.

Of the genes studied, NCLSCs expressed few mRNAs in common with melanoblasts and melanocytes (Table 1). These were 4 transcription factors: Pax3 (paired-box 3) and Snai2 (Snail2)/Slug, required for neural crest

TABLE 1. *Comparative expression of marker mRNAs in NCLSCs, melanoblasts, and melanocytes*

	Neural crest markers, tyrosine kinases, and others									
Cellline	Pax3	Brn2	Sox4	Snai2	Pdgfra	Ephb3	Epha2	Epha4	Efnal	Cited1
NC-m4	11	+	540	+	+	+	0	0	0	0
NC-m6			+	+	+		0			0
melb-a	45	+	149			0	+	0	+	
melb-s1	54	+	102			0	+	+	+	
melb-p3	298	+	139			0	0	+	0	
melb-m5			+	+	0		0			+
melan-a	100	+	100	+	0	0	0	0	0	+
melan-a2	64	+	25			0	+	0	+	
melan-s1	80	+	22			0	0	0	+	
melan-m1	272	+	+			0	0	0	0	
melan-m5			+	+	0		0			+
melan-p1	298	+	+			0	0			
melan-rs	438	+	+			0	0			
melan-c	177	+	+			0	0			
B16-F1	68	+	+			0	0			
K1735			+		+		+			0
XB2			173				+	+	+	
Liver	0	0	0			0	0			0
Kidney							0			0

	Melanocytic markers							
Cellline	Mitf	Kit	Mclr	Dct	Si	Tyrpl	Tyr	P
NC-m4	0	0	0	0	0	0	0	0
Melanoblasts[a]	82	67	61	63	112	83	57	0
melb-a	31	56	44	44	61	48	28	0
melb-s1	49	101	44	55	55	89	38	0
melb-p3	167	45	95	90	220	111	105	(0)
Melanocytes[a]	146	120	202	178	145	171	243	243
melan-a	100	100	100	100	100	100	100	100
melan-a2	30	50	37	69	25	48	94	25
melan-s1	37	42	32	97	78	79	115	85
melan-m1	195	74	230	303	351	200	313	389
melan-p1	186	281	167	137	203	170	335	(0)
melan-rs	174	174	267	315	160	300	393	360
melan-c	299	+	581	227	98	301	353	501
B16-F1	8	0	40	47	11	30	22	0
Liver	0	0	0	0	0	0	0	0

Quantitation of autoradiographic bands from Northern blots was by laser densitometry. Suitable blots (not overexposed) were selected for each probe shown. Where no blot was suitable for quantitation, data are shown as positive or negative (+ or 0). Readings were normalized to the reading for GAPDH (Gapd) for a given cell line, and then to the level for melan-a as 100%. Blank indicates not done. Lines melan-p1 and melb-p3 have P gene deletions and have no P mRNA, as expected (omitted from means for P). NCLSC, melanoblast, and melanocyte lines are designated NC-, melb-, and melan-, respectively. B16-F1 and K1735 are melanoma lines; XB2 cells are keratinocytes. [a]Arithmetic means of this group of cell lines.

development (42, 43); Brn2/Pou3f2/N-Oct3, an octamer-binding factor found in brain, melanoma cells, melanoblasts, and melanocytes (44, 45); and Sox4 (SRY-box 4), required for cardiac neural crest development (46) although also expressed more widely, including in melanoblasts and melanocytes (Table 1). NCLSCs did not detectably express mRNA for Mitf (microphthalmia-related transcription factor), a "master" gene regulator for the melanocyte lineage (14). Mitf, Pax3, Brn2, Sox4, and Snai2 were expressed in all tested melanocytes and melanoblasts (Table 1). We previously reported 2 other transcription factors found in melanoblasts and melanocytes but not in NCLSCs (called at that time "melanoblast precursors"), namely, Tbx2 (T-box 2) (47) and Cited1 (Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 1) (Table 1), originally called melanocyte-specific gene 1/Msg1, being expressed in melanocytes and well-pigmented melanoma cells (48).

We examined expression of several membrane receptors and one related ligand, with emphasis on those found in melanocytes and melanoma cells. Interestingly, NCLSCs expressed transcripts for 2 receptors found in some melanoma cells but not melanocytes (49) and not detected here in melanocytes or melanoblasts, namely, Ephb3 (Sek4/HEK2), and Pdgfra (platelet-derived growth factor receptor A). It was not surprising that mRNA for receptors Kit and Mc1r were expressed by all tested melanoblast and melanocyte lines, since these cells respond to the respective ligands SCF and MSH (*e.g.,* refs. 24, 41, 50). These mRNAs were not detected in NCLSCs. Two other Eph-family receptors, Epha2 (Eck) and Epha4 (Sek), and a ligand common to both receptors, Efna1 (ephrin A1, LERK1, B61), were all expressed in melanoblasts but not detected in melanocytes or in NCLSCs. In comparison, Epha2 and Efna1 are frequently expressed in melanoma cells but not human melanocytes, while Epha4/Sek appears to be downregulated in some melanomas (34, 49).

We also quantitated transcripts for several proteins specific to melanosomes (matrix protein Si/Pmel and enzymes Tyr/tyrosinase, Tyrp1/tyrosinase-related protein 1, and Dct), or involved in melanosome biogenesis (Oa1/ocular albinism 1 and P protein/pinkeyed dilution), all required for normal pigmentation (reviewed in ref. 13). None of these mRNAs was expressed in NCLSCs. All were detected in melanocyte lines, and in melanoblasts, with the sole exception of P protein. Thus P appears to be one gene that is upregulated on differentiation to melanocytes. Since expression levels appeared to vary between lines of one type, general quantitative comparisons between cell types would require more lines, although mean expression for these melanoblast and melanocyte lines is shown for reference. Levels of expression could be directly compared in the 2 melanoblast lines melb-a and melb-s1 with those in the pigmented melanocyte cultures derived from these melanoblasts by differentiation, melan-a2 and melan-s1. Differences that seemed large enough in both pairs to be likely to reflect real changes on melanocytic differentiation were a fall to around 25% in the level of Sox4 and a rise (~3-fold) in transcription of the rate-limiting melanogenic enzyme Tyr (tyrosinase).

Normal mouse liver or kidney and mouse melanoma cell lines B16-F1 (lightly pigmented) and sometimes K1735 (unpigmented) were included for comparison. None of the transcripts studied except the loading control Gapd was detected in liver or kidney, while gene expression in B16-F1 melanoma cells was very similar to that in melanoblasts (including absence of P protein), except that Kit was not expressed. Kit is commonly down-regulated in advanced melanoma cells (reviewed in ref. 49).

Differentiation of NCLSCs to pre-Schwann cells

TGFβ1 and the related family of bone morphogenetic proteins were reported to promote commitment of primary rat neural crest stem cells to autonomic neuronal lineages and smooth muscle-like cells *in vitro* (16, 51). We therefore tested the effect of TGFβ1 on NCLSCs. Simple substitution of FCS for NBCS had little effect on NC-m4 cell morphology in 1-2 wk (**Fig. 3*A***). In medium with FCS, TPA, and TGFβ1, however, most cells changed within a few days to a larger, flattened cell type somewhat like fibroblasts (Fig. 3*B*). There was little effect on cell proliferation (Fig. 3*F*). Immunostaining of these flat cells for the smooth muscle marker calponin proved negative, suggesting that they were not smooth myocytes. We then hypothesized that they might be related to Schwann (peripheral glial) cells.

Differentiation of glial cells in the peripheral nervous system and from cultured rat neural crest stem cells or glial precursors can be promoted by ß forms of neuregulin 1 (NRG1-β, related to glial growth factor) (3, 11, 52, 53). NRG1 is expressed in developing nerves (3). We examined the effect of NRG1-β1 on the NCLSC-derived flat cells. It did not noticeably change the morphology of these cells, but did increase their proliferation at 10 nM (Fig. 3*C, F*), indicating the presence of receptors for NRG1-β1. A smaller effect of NRG1-β1 at 1 nM was observed ($P<0.01$), and none at lower concentrations ($P>0.1$ for 0.4 nM). NRG1-β1 (10 nM) also significantly increased numbers of stock NC-m4 NCLSCs (grown with TPA and NBCS) over 3 wk ($P<0.001$), so these also possessed receptors.

Since most NCLSCs changed into the flatter cell type in a few days with TGFβ1, we tested whether this was just a reversible effect of TGFβ1 or was stable, like cell determination. NC-m4 cells were grown for 15 d with FCS, TPA (2 nM), and TGFβ1, either with or without NRG1-β1. The cells were then returned for 7 d to medium with FCS and TPA (2 nM) only. As shown in Fig. 3*D, E,* the cells retained the larger, flatter morphology. The increased cell yield with NRG1-β1 was maintained after the week in TPA only (Fig. 3*F; P*<0.001). The flat cells lost the responsiveness to gelatin seen in NCLSCs ($P>0.1$ for cell number) and no longer

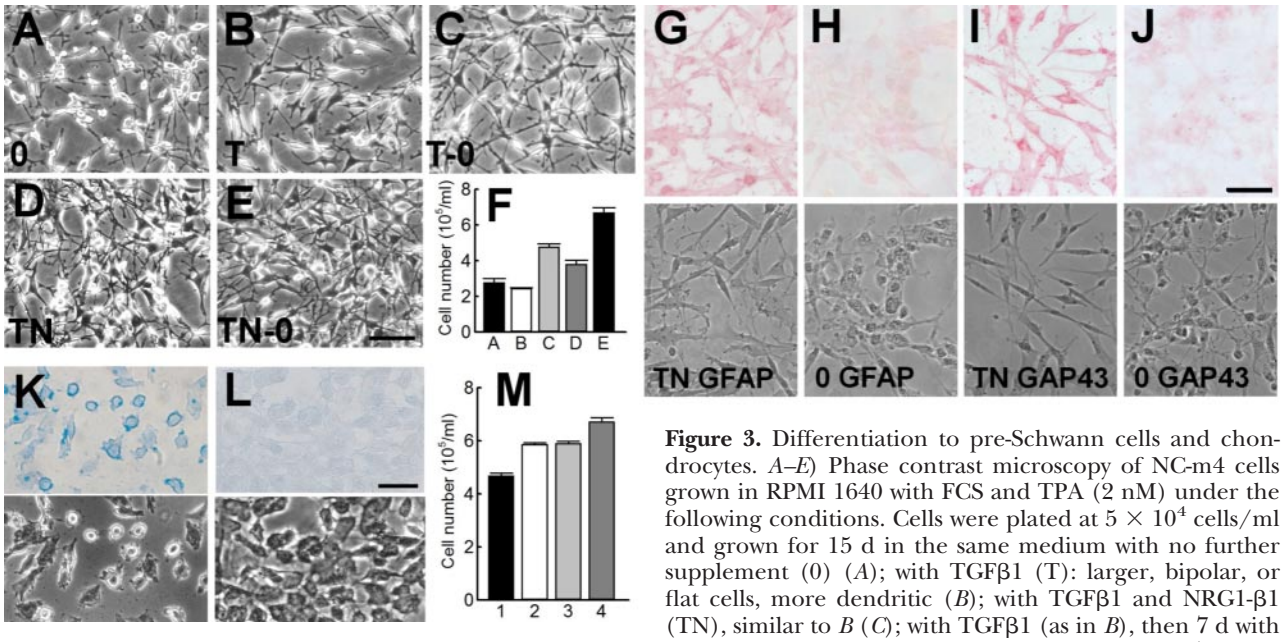

Figure 3. Differentiation to pre-Schwann cells and chondrocytes. *A–E*) Phase contrast microscopy of NC-m4 cells grown in RPMI 1640 with FCS and TPA (2 nM) under the following conditions. Cells were plated at 5×10^4 cells/ml and grown for 15 d in the same medium with no further supplement (0) (*A*); with TGFβ1 (T): larger, bipolar, or flat cells, more dendritic (*B*); with TGFβ1 and NRG1-β1 (TN), similar to *B* (*C*); with TGFβ1 (as in *B*), then 7 d with FCS and TPA only, little or no reversion to NCLSC morphology (*D*); or with TGFβ1 and NRG1-β1 (as in *C*), then 7 d with FCS and TPA only, little or no reversion (*E*). *F*) Cell numbers obtained in cultures after treatments *A–E,* means + SE. Growth was somewhat retarded by TGFβ1 and stimulated by NRG1-β1 in the presence of TGFβ1 ($P<0.001$; Student's *t* test). The pre-Schwann cells survived but doubled less than once in 7 d with FCS and TPA alone. *G–J*) GFAP and GAP43 immunoreactivity in pre-Schwann cells. NC-m4 cells were plated at 5×10^4cells/ml and grown for 10 d as specified, before fixation and immunostaining for GFAP (*G, H*) or GAP43 (*I, J*). Cells grown with FCS, TPA, TGFβ1, and NRG1-β1 (TN) show cytoplasmic reactivity for both markers in most or all cells, indicating pre-Schwann cells (*G, I*). NCLSCs grown with NBCS and TPA only (0) show no more than background staining (like control melan-c melanocytes; Supplemental Fig. 2*A, B*) for both markers (*H, J*). *K, L*) Alcian blue staining for NC-m6 induced to differentiate to chondrocytes (treatment 4 in *M*) (*K*) and untreated NC-m6 showing only background staining (like control melan-c melanocytes; Supplemental Fig. 2*C*) (*L*). *M*) Effect of BMP-2 and ascorbic acid on NCLSC proliferation. NC-m6 cells were plated at 2.5×10^4 cells/ml and grown RPMI with FCS, TPA, and FGF2 (treatment 1), also either with ascorbic acid (treatment 2) or with BMP-2 (treatment 3) or both factors (treatment 4) for 7 d. Data represent mean + SE cell numbers. Growth was stimulated by ascorbic acid and BMP-2 ($P<0.001$; Student's *t* test). Scale bars = 50 μm (*A–J*); 25 μm (*K, L*).

tended to form clumps and detach from the substrate (Fig. 3).

We now immunostained cultures of the NC-m4 flat cells for glial fibrillary acidic protein (GFAP) and GAP43 (growth-associated protein 43), markers expressed by immature and unmyelinated Schwann cells (GAP43 also in axonal growth cones) (11). TGFβ1- and NRG1-β1-treated cultures showed clear cytoplasmic expression of both markers (Fig. 3*G, I*) in ~90% of the cells, indicating that these were pre-Schwann cells. NCLSCs (Fig. 3*H, J*) and control melan-c albino melanocytes (Supplemental Fig. 1*G, H*) were unstained.

Cloned NC-m6 cells grown with TGFβ1 and NRG1-β1 showed a similar morphological response to that by NC-m4, indicating that the response was not due solely to selection or differentiation, but involved cell determination.

Differentiation of NCLSCs to chondrocytes

To determine whether NCLSCs can generate a mesenchymal cell type, we sought to induce differentiation into chondrocytes. BMP-2 was reported to promote commitment of adult neural crest-derived cells to chondrocytes *in vitro* (18, 21). We examined the effect of BMP-2 and ascorbic acid on NCLSCs in the presence of FCS. Both these factors increased cell proliferation (Fig. 3*M*).

We identified chondrocytes (~50% of all cells) in NC-m6 grown in the presence of FCS, TPA, FGF2, ascorbic acid, and BMP-2 for 14 d by Alcian blue staining (1% in 3% acetic acid, pH 2.6) (Fig. 3*K*). Undifferentiated NCLSCs and melan-c melanocytes showed only background staining (Fig. 3*L* and Supplemental Fig. 2*C*).

Spontaneous and induced differentiation of sensory neurons from NCLSCs

In pluripotent mouse mesenchymal stem cells, determination of some cells to several lineages (myoblasts, chondroblasts, preadipocytes) can be induced by growth with the DNA demethylating agent 5-azacytidine (54). We tested whether azacytidine would promote cell determination in NCLSCs. After culture of NC-m4 cells with FCS and 5-azacytidine, there was some cell death. Most surviving cells were unchanged, but we saw occasional single, very large, dendritic cells (**Fig. 4*A***). Similar cells also sometimes appeared in very sparse NC-m4 cultures grown in their standard medium (Fig. 4*B*), so the effect

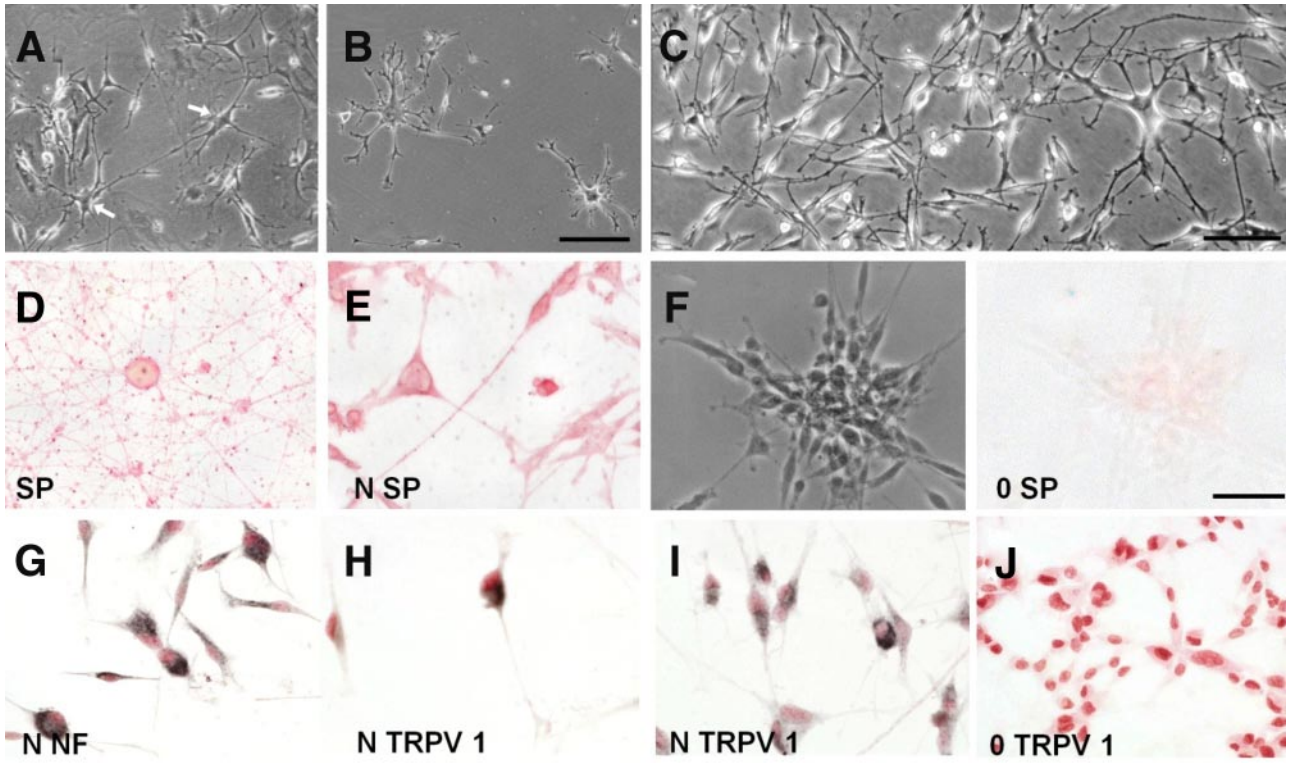

Figure 4. Neuronal differentiation in NC-m4 cultures. *A*) Neuron-like cells in a culture grown with 300 pM 5-azacytidine. *B*) Very large neuron-like cells, diameter 200-300 μm, in a culture plated sparsely and grown only in NBCS and TPA (2 nM). *C*) Numerous bi- to multipolar dendritic cells, diameter up to 400 μm, in NC-m4 culture grown for 14 d with neurotrophic factors (FCS, TPA, BDNF, NGF, NT3, and FGF2). *D–F*) Cultures immunostained for substance P. Control primary rat sensory neurons showed cytoplasmic substance P in cell bodies and dendrites (red) (*D*), and so did the majority of NC-m4 cells grown for 14 d with neurotrophic factors (*E*). Reactivity was most prominent in larger bi- and tripolar cells. *F*) Untreated NCLSCs: negative. *G–I*) NC-m4 cells grown with neurotrophic factors and immunostained for neurofilaments (*G*) and TRPV1, with 2 different antibodies showing distinct cytoplasmic TRPV1 immunoreactivity (*H, I*). Positive staining is gray/black; nuclei counterstained with neutral red. *J*) Untreated NCLSCs were negative for these 3 markers, for example, TRPV1, antibody GSK-C22. Scale bars = 150 μm (*A, B*); 100 μm (*C*); 50 μm (*D–J*). N, neurotrophic factors; 0, untreated; SP, substance P; NF, neurofilaments.

of 5-azacytidine may have been nonspecific. These large, dendritic cells were immunoreactive for neurofilament 200 (a neuronal marker) and substance P (a specific marker of sensory neurons) (Supplemental Fig. 1*D–F*).

We then tested the effects on NC-m4 cells of a combination of 4 neurotrophic factors, each previously reported to promote determination and/or differentiation of sensory neurons: NGF, BDNF, NT3, and FGF2 (55–57). In 2 wk, this cocktail produced a substantial fraction of large cells with morphology suggestive of neurons, either bipolar or stellate, with very long, dendrite-like processes (Fig. 4*C*). The majority of these cells (~90%) showed cytoplasmic immunostaining for substance P, similar to that seen in control primary rat sensory neurons and absent from NC-m4 NCLSCs (Fig. 4*D–F*). They were also immunostained by mixed neurofilament antibodies (Fig. 4*G*), and a proportion of cells by either of 2 antibodies to transient receptor potential channel V1 (TRPV1; see next section) (Fig. 4*H, I*). Untreated NCLSCs were negative for these markers (*e.g.,* Fig. 4*J*).

We then investigated aspects of sensory neuronal function. First, we tested for intracellular calcium responses mediated by TRP channels, ligand-gated ion channels that mediate thermosensation by peripheral sensory neurons (58). TRPV1 is activated by heat and by vanilloids such as capsaicin (59), and TRPA1 by cold and by pungent ligands including cinnamaldehyde (60). NC-m4 cells were treated with the 4 neurotrophic factors for 2 wk as above, loaded with calcium indicator Fluo-4 and fluorescence videos made during perfusion with capsaicin or cinnamaldehyde (separate cultures). Increased fluorescence was observed in some dendritic cells with either agonist, and not with vehicle only (**Fig. 5*A, D*** and Supplemental Videos 1 and 2). The high fluorescence was transient, and could be seen in some areas moving along dendrites (Supplemental Video 2), again with either agonist. Time courses of intracellular calcium concentration in individual cells are shown in Fig. 5*B, E* and resemble those seen in normal sensory neurons. About 5% of all cells showed this functional activity with capsaicin, and ~2% with cinnamaldehyde (Fig. 5*C, F*). No such calcium responses (0) were detected in untreated NCLSCs or in control melan-c melanocytes.

Whole-cell patch-clamp recordings were then used to test for voltage-dependent inward currents, in NCLSCs

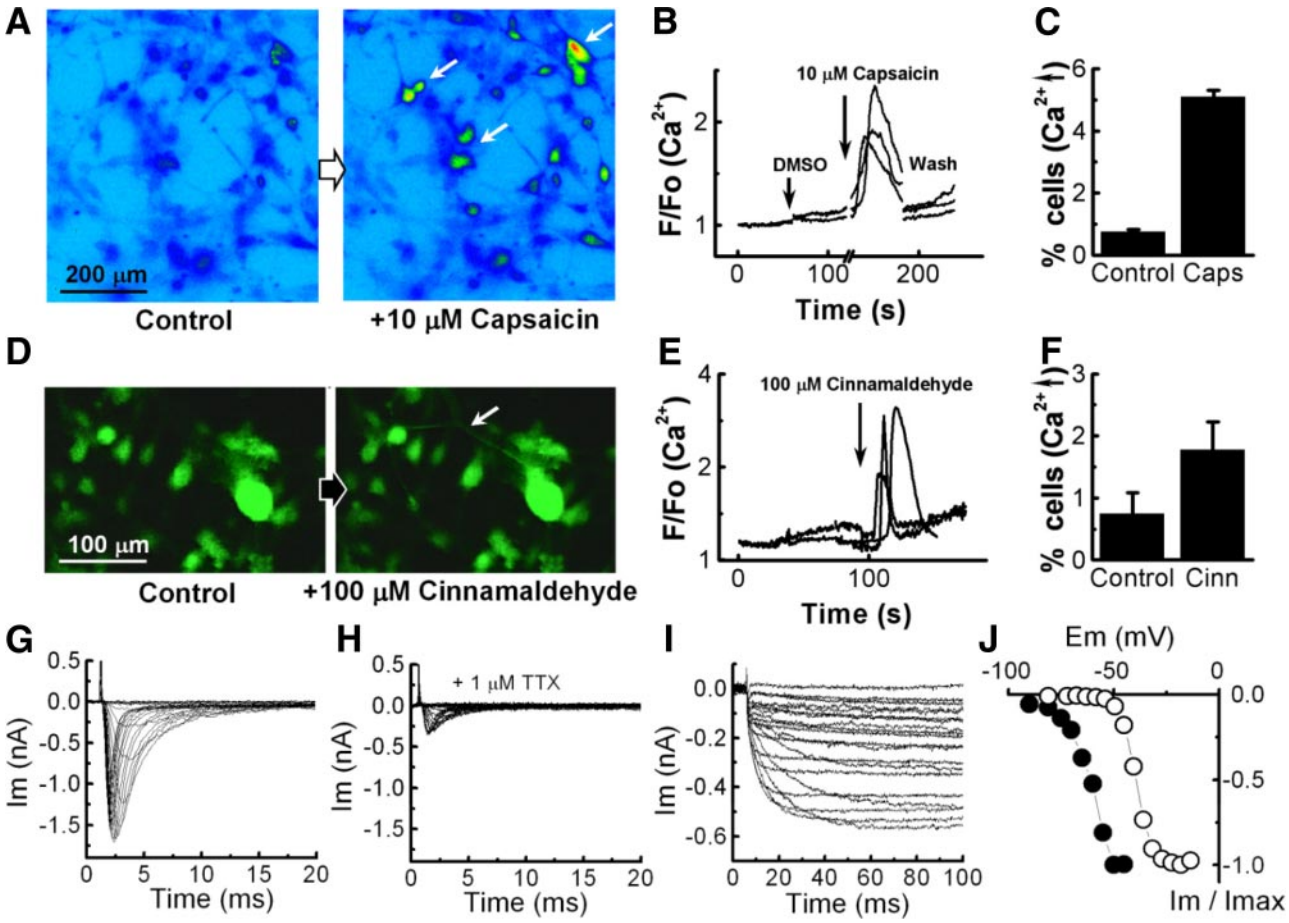

Figure 5. Functional characterization of neurons from NCLCS. *A–F*) Cinnamaldehyde- and capsaicin-induced excitation of NC-m4 cells induced to differentiate by neurotrophic factors (see Materials and Methods). Cultures on glass coverslips were transferred to a perfusion chamber and loaded with the Ca^{2+} indicator Fluo4. Cells were stimulated with capsaicin or cinnamaldehyde preceded by control vehicle solution, and calcium fluorescence was recorded by video fluorescence microscopy and quantitative image analysis. *A*) Stills of fluorescence before and during capsaicin stimulation, showing enhanced calcium fluorescence in a subset of cell bodies and dendrites (arrows). False color: blue (lowest), green, yellow, and red (highest). *B*) Quantitation of fluorescence with time after addition of capsaicin; superimposed recordings from 3 separate neurons. *C*) Percentage of cells showing specific fluorescence in the observed microscope field during recording, with and without capsaicin. *D–F*) As in *A–C*, but using cinnamaldehyde instead of capsaicin. Fluorescence moves along dendrites (arrow), only after stimulation. Note: false color (used in *A*) best discriminates the specific high-fluorescence levels in responding cells but does not show small features like dendrites that are visible in the unprocessed original (used in *D*). *G–I*) Multiple Na^+ currents in individual cells from NC-m4 and NC-m6 cultures. Ca^{2+}-free bathing solutions could not be used to exclude possible Ca^{2+} current components owing to cellular instability, so we used cadmium (Cd^{2+}), a nonselective Ca^{2+} channel blocker, considered to block Ca^{2+} channels preferentially over Na^+ channels. Current recorded with Cd^{2+}in the bath and fluoride in the pipette solution is interpreted as Na^+ current. Total Na^+ currents in vehicle (*G*) or with 1 μM tetrodotoxin (TTX) (*H*), typical only for NC-m6 cells grown with TGFβ1 and NRG1; TTX-resistant, persistent currents characteristic of NC-m4 cells grown with neurotrophic factors (*I*). *J*) Current-voltage relationships for recordings in *G* (-○-) and *I* (-●-).

induced to differentiate either with neurotrophic factors or with TGFβ1 and NRG1-β1 (Fig. 5*G–I*). No such currents were observed in melan-a (0/3) or melan-m5 (0/10) melanocytes or untreated NC-m6 NCLSCs (0/12), and only small currents in a few untreated NC-m4 NCLSCs (5/29; 17.2%). Fast-inactivating inward currents blocked by tetrodotoxin (TTX), indicating TTX-sensitive sodium channels, were recorded only from NC-m6 cells grown with TGFβ1 and NRG1-β1 (27/28; 96.4%) (Fig. 5*G, H*). TTX-resistant inward currents were also detected in these same cultures (Fig. 5*H*). These were Na^+ rather than Ca^{2+} currents, because, although TTX-resistant, they were not sustained. These findings are consistent with cell differentiation under these conditions toward either Schwann cells or neurons (see Discussion). Conversely, the majority of NC-m4 and NC-m6 cells treated with the 4 neurotrophic factors [72/79 (91.1%) and 6/8 (75%), respectively] showed TTX-resistant currents that were not inactivated during the 100-ms depolarization at negative test potentials (Fig. 5*I*). Similar currents were previously recorded in explanted sensory neurons (61) and are referred to as persistent, TTX-resistant currents: the third known class of voltage-gated Na^+ current. They have a negative threshold for activation and a depolarized midpoint of inactivation (61). These

currents were activated between about −80 and −70 mV (Fig. 5*J*), showing slow activation and prominent ultraslow inactivation (Fig. 5*I*). They appeared to be Na^+ currents because of resistance to Cd^{2+} (a Ca^{2+} channel blocker) and fluoride (see Fig. 5 legend).

DISCUSSION

We report 3 lines of NCLSCs, pluripotent stem cells from postnatal mouse skin, sharing biological properties and gene expression with embryonic neural crest cells. These NCLSCs can proliferate indefinitely (self-renewal) and can also differentiate to at least 4 neural crest-derived cell lineages, summarized in **Fig. 6.** We do not yet know whether NCLSCs have all the pluripotency of embryonic neural crest cells; the latter produce further lineages that we have not seen, so NCLSCs may resemble more restricted crest-derived precursors as seen in primary cultures (see ref. 12 and introduction). NCLSCs appear different from SKPs, which can form mesenchymal cells but apparently not melanocytes, and grow in suspension (19). NCLSCs can form at least one mesenchymal cell type, chondrocytes, but conversely, they do produce melanocytes and are substrate attached. Nishimura *et al.* (62) reported postnatal stem cells for melanocytes, found in hair follicles, but those cells expressed Dct and appeared restricted to the melanocyte lineage, unlike NCLSCs. Our NCLSCs apparently have a wider potency than other stem cells explanted from mouse trunk hair follicles, which produced only melanocytes and glia (21). This is plausible: NCLSCs were derived from immature trunk skin epidermis before follicles mature, so they may be developmentally earlier. A likely hypothesis is that these cultures originate from rare crest cells that migrate to the epidermis along with determined melanoblasts, but are not yet themselves determined. Since they come from epidermis, they seem more likely to be precursors of melanocytic stem cells than of SKPs, which are dermal, but this is uncertain because crest cells can cross the basement membrane. Moreover, differentiated avian neural-crest derivatives can sometimes revert to pluripotent precursors *in vitro* (4, 12). Thus, neither we nor others reporting explanted stem cells can exclude the possibility of despecification in culture from more mature cells; but the NCLSC phenotype is at least reproducible (in 3 independent cultures). Another novel feature is that we established immortal lines of the stem cells, which can be frozen, expanded indefinitely, and distributed to others. We report new mRNA markers for NCLSCs, Pdgfra, and Ephb3, and we find that markers Pax3, Snai2, Sox4, and Brn2 are shared with melanoblasts and melanocytes. NCLSC cultures also strongly express the known stem cell marker nestin.

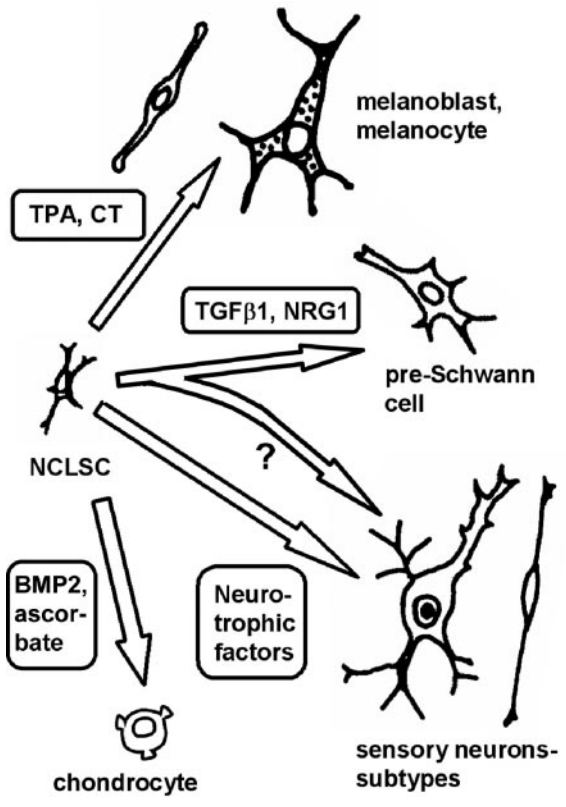

Figure 6. Summary of observed potencies of NCLSCs. Factors known to promote each form of determination are indicated. Longer growth in melanoblast or melanocyte medium produces apparently pure cultures of melanoblasts or melanocytes, respectively. Cells of neuronal morphology are generally observed singly and do not appear to divide; the other cell types proliferate. The branched arrow in the case of TGFβ and NRG1 indicates the possibility that neurons are produced as well as pre-Schwann cells under these conditions.

Melanoblasts and pigmented melanocytes appeared spontaneously under some conditions in NCLSC cultures, including cloned cultures, and apparently pure cultures of melanoblasts or melanocytes could be produced by growth in appropriate media. Gene expression was compared semiquantitatively in NCLSCs, melanoblasts, and melanocytes. No such comparison of gene expression even of melanoblasts with melanocytes has been reported previously, to our knowledge, so this information will be useful for research on melanoblast differentiation. NCLSCs did not express any melanocyte lineage markers. The finding of Pdgfra expression in NCLSCs is consistent with the requirement of the *Pdgfra* gene for normal neural crest development (63).

It was interesting that the mRNA patterns observed in melanoblasts and melanocytes were nearly identical qualitatively. The only clear differences were that P-protein mRNA was found in melanocytes but not melanoblasts, Tyr mRNA levels appeared several-fold higher in melanocytes than melanoblasts, and receptors Epha2 and Epha4 and ligand Efna1 were detected in melanoblasts but not in most melanocyte lines. Low Tyr levels and lack of P could largely explain the lack of pigmentation in melanoblasts. Tyr (tyrosinase) is absolutely required for melanin synthesis: white (albino) mice are null for *Tyr,* and human TYR is also the locus for oculocutaneous albinism type 1 (OCA1) (13). Likewise, *p*-null mice are nearly white, and human *P* is the

locus for OCA2 (13). Eph-ephrin interactions have been implicated in cell migration (64), so their expression in melanoblasts, and Ephb3 expression in NCLSCs, may be connected with the migratory nature of these cells.

NCLSCs and melanoblasts may also be relevant to the phenotypes of melanoma cells. These phenotypes are very variable; for example, cultured melanoma cells vary in cell shape and melanization, and express diverse tyrosine kinase receptors, often unlike those of melanocytes (49). It has long been speculated that some melanoma heterogeneity is due to varying degrees of differentiation (65), and the present findings support this idea. Receptors Epha2 (ECK) and Ephb3 (HEK2) are both expressed in >90% of cultured human melanoma cell lines but not in normal melanocytes, while Pdgfra and the EphA mitogenic and angiogenic ligand Efna1 are also ectopically expressed in some melanomas (49). Here we show expression of both Epha2 and Efna1 in melanoblast lines, with Ephb3 and Pdgfra in the earlier precursors NCLSCs. Lightly pigmented melanoma line B16-F1 showed a pattern of gene expression similar to that of melanoblasts, while unpigmented melanoma line K1735 expressed Pdgfra, like NCLSCs, and also Epha2. There is much scope to expand these comparisons, for example, using microarray analysis, which may yield therapeutic targets or better understanding of melanoma biology.

On addition of TGFβ1, NC-m4 NCLSCs converted efficiently to a larger, flatter cell type, identified as pre-Schwann cells since they expressed GAP43 and GFAP and showed a proliferative response to NRG1-β1. TGFβ was previously reported to promote differentiation of neural crest cells to smooth muscle cells or neurons (16, 51), and under some conditions to reduce Schwann cell differentiation and survival (11). However the effect may differ with conditions, and/or in immortal cells: for example, TGFβ1 promoted proliferation of Schwannoma cells (66). Mature unmyelinated Schwann cells express O4 as well as GFAP and GAP43, while mature myelinated Schwann cells down-regulate these markers and express P_0 (11). These markers will assist future studies on differentiation of the precursors into mature Schwann cell types. Growth of NC-m6 cells with TGFβ1 and NRG1-β1 resulted in cells morphologically similar to those from line NC-m4, and hence believed also to be pre-Schwann cells. These cells displayed TTX-sensitive and some TTX-resistant transient sodium currents when tested. It seems most likely that they were indeed pre-Schwann cells but nonetheless had sodium channels. TTX-sensitive and -resistant sodium channels have both been reported in Schwann cells (67). It is possible that neurons of some type as well as Schwann cells arose in NC-m6 cultures under these conditions; however, it seems unlikely that neurons formed a large fraction of the cultures, since the cells proliferated well in this medium for long periods.

NCLSCs could also generate neurons, especially after growth with a mixture of neurotrophic factors, when most cells expressed neurofilaments and a minority expressed immunoreactive TRPV1 indicating sensory neurons. Likewise, some cells showed ligand-gated cationic channels responsive to either cinnamaldehyde or capsaicin, typical of functional sensory neurons. Most cells also showed typical voltage-dependent inward Na^+ currents of the TTX-resistant, persistent class, a channel type important in sensory transmission and nociception (68). These functional data establish this experimental system as one in which neuronal development and function can be studied without animals, and without the cellular trauma caused by tissue disaggregation. Potential research topics include anesthesia and repair of neuronal injury.

In summary, we report the isolation of established lines of an apparently novel type of neural crest-like stem cell from neonatal mouse epidermis. Functional sensory neurons and growing cultures of melanoblasts, melanocytes, chondrocytes, and pre-Schwann cells were derived from these. These lines provide a new and valuable model for large-scale studies of the biology and gene expression of neural crest stem cells, the regulation of lineage determination, and neuronal biology. FJ

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