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Neural crest origin of mammalian Merkel cells

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Abstract

Here, we provide evidence for the neural crest origin of mammalian Merkel cells. Together with nerve terminals, Merkel cells form slowly adapting cutaneous mechanoreceptors that transduce steady indentation in hairy and glabrous skin. We have determined the ontogenetic origin of Merkel cells in Wnt1-cre/R26R compound transgenic mice, in which neural crest cells are marked indelibly. Merkel cells in whiskers and interfollicular locations express the transgene, β-galactosidase, identifying them as neural crest descendants. We thus conclude that murine Merkel cells originate from the neural crest.

Keywords: Neural crest; Merkel cell; Mechanoreceptor; Wnt1; R26R; Cre; β-Galactosidase; Mouse; Mammalian; Whisker

Introduction

Mammalian Merkel cells are localized in the basal layer of the epidermis. Together with the terminals of sensory neurons they form the Merkel nerve endings, slowly adapting cutaneous mechanoreceptors that are activated by steady skin indentation (Iggo and Muir, 1969). The ontogenetic origin of mammalian Merkel cells is controversial. According to one view, they originate from the neural crest and migrate into the mammalian epidermis during embryogenesis (Breathnach, 1978; Breathnach and Robins, 1970; Halata, 1981; Hashimoto, 1972; Winkelmann, 1977). In contrast, the predominant current opinion presumes that mammalian Merkel cells are of epidermal origin (English et al., 1980; Lyne and Hollis, 1971; Moll et al., 1984, 1986, 1990, 1996a). In chick-quail transplantation experiments, the epidermal origin of avian Merkel cells has been excluded (Halata et al., 1990; Grim and Halata, 2000a,b). In chimeric limbs, Merkel cells, together with the crest-derived pigment cells and Schwann cells, originated from the host embryo.

The neural crest forms a transient primordium of the vertebrate embryo that gives rise to a wide array of progeny in the adult organism, including the autonomic and enteric nervous systems, most primary sensory neurons, endocrine cells, and the cephalic mesenchyme (reviewed by Le Douarin and Kalcheim, 1999). Migratory neural crest cells consist of a mixed population of cells that includes pluripotent stem cells, fate-restricted progenitor cells, and cells that are committed to a particular cell lineage (Baroffio et al., 1988; Bronner-Fraser and Fraser, 1988; Henion and Weston, 1997; Ito et al., 1993; Sieber-Blum, 1989; Sieber-Blum and Cohen, 1980; Sieber-Blum and Sieber, 1984; Stemple et al., 1988). Neural crest stem cells, or closely related pluripotent progenitor cells, are present also in target locations in the embryo (Duff et al., 1991; Gershon et al., 1993; Ito and Sieber-Blum, 1993; Richardson and Sieber-Blum, 1993; Sherman et al., 1993; Sieber-Blum et al., 1993) and in adults (Kruger et al., 2002), explaining the extraordinary diversity and plasticity of this embryonic tissue.

We have analyzed Merkel cells in whisker pads of Wnt1-cre/R26R double transgenic mice, in which neural crest cells and their derivatives are marked. In the mouse and in all other vertebrate embryos studied to date, Wnt1 expression during embryogenesis is transient and limited to neural crest cells and some central nervous system cells (Davis et

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al., 1988; Echelard et al., 1994; McMahon et al., 1992; Molven et al., 1991; Wilkinson et al., 1987; Wolda et al., 1993). The Wnt1-cre mouse transiently expresses cre recombinase under the control of the Wnt1 promoter (Danielian et al., 1998). The R26R mouse expresses β-galactosidase conditionally and ubiquitously in a cre-inducible manner (Soriano, 1999). The transient expression of cre recombinase under the control of the Wnt1 promoter in double transgenic mice therefore specifically and permanently activates expression of R26R-derived β-galactosidase in premigratory and early migratory neural crest cells (Chai et al., 2000; Danielian et al., 1998; Echelard et al, 1994; Friedrich and Soriano, 1991; Jiang et al., 2002; Soriano, 1999).

Materials and methods

Genotyping

Heterozygous Wnt1-cre mice were mated with R26R heterozygotes. For genotyping, the DNA was isolated from tail biopsies or extraembryonic membranes. The tissue was treated with 5 μl proteinase K (10 mg/ml; Promega, Madison, WI) in lysis buffer (1 ml Tris, pH 8.5, 10% SDS, 5 M NaCl in H_2O) overnight at 55°C. After centrifugation, the DNA in the supernatant was precipitated with 2-propanol, transferred, and dissolved in TE buffer (10 mM Tris–Cl, 0.5 mM EDTA). One microliter of DNA was placed in PCR buffer [1 M Tris, 2 μl; 2 M $(NH_4)_2SO_4$, 0.25 μl, 14.4 M β-mercaptoethanol, 0.02 μl; 100% DMSO, 3 μl; 0.5 M $MgCl_2$, 0.18 μl; 0.1 M dATP, 0.15 μl; 0.1 M dCTP, 0.15 μl; 0.1 M dGTP, 0.15 μl, 0.1 M dTTP, 0.15 μl; nuclease-free H_2O (Promega), 21.78 μl; Taq polymerase (5 units/μl), 0.16 μl, together with 1 μl of each primer]. The following primers were used. For detection of Wnt1 cre: primer Cre (100 ng/μl): CGTTTTCTGAGCATACCTGGA; primer Cre antisense (100 ng/μl): ATTCTCCCACCGTCAGTACG; primer RARB common (100 ng/μl): GTAGCCATCGAGACACAGAGT; primer RARB WT (100 ng/μl): TGGTAGCCCGATGACTTGTCC (Chai et al., 2000). The amplification conditions were as follows: 1 cycle of 94°C for 30 min, followed by 35 cycles of 60 denaturation at 94°C, 2 min annealing at 55°C, 90 s extension at 72°C, and 5 min final extension at 72°C. For detection of R26R, the following primers were used: primer oIMRO 883 (200 ng/μl): AAAGTCGCTCTGAGTTGTTAT; primer oIMRO 315 (200 ng/μl): GCGAAGAGTTTGTCCTCAACC; primer oIMRO 316 (400 ng/μl): GGAGCGGGAGAAATGGATATG (Soriano, 1999). The PCR buffer was the same as for Wnt1-cre, and the amplification conditions were as follows. One cycle of 19 min at 94°C, followed by 35 cycles of 45 s denaturation at 94°C; 45 s annealing at 53°C, 90 s extension at 72°C, followed by 5 min final extension at 72°C.

Indirect immunocytochemistry and Xgal histochemistry

The tissue was dissected in phosphate-buffered saline (PBS) at RT, fixed for 20 min with 4% paraformaldehyde at RT, rinsed with PBS, incubated in 15 and 30% sucrose in dH_2O for 4–5 h, mounted with OCT compound (Sakura Finetek USA Inc., CA) in vinyl Biopsy Cryomolds (Miles Inc. Elkhart, IN), frozen on dry ice, and stored at −80°C. Twelve-micron cryosections were prepared and placed on "Probe On Plus" slides (FisherBiotech, Fisher Scientific, Pittsburgh, PA). Sections were incubated for 10 min. in PBS, the tissue circled with rubber cement and let dry for 2 min at RT. For blocking unspecific antibody binding, slides were incubated for 30 min at RT with calcium–magnesium-free PBS (CMF-PBS) containing 2% normal goat serum (NGS), 0.2% BSA, and 0.2% Triton X-100. Subsequently, the pooled primary antibodies were added; Troma-1 (1:20, recognizes cytokeratin-8; Developmental Studies Hybridoma Bank, Iowa City, IA) and anti-β-galactosidase rabbit IgG (gift of Joshua Sanes) in CMF-PBS containing 2% NGS, 0.2% bovine serum albumin (BSA), and 0.2% Triton X-100 overnight in the cold. The tissue was then rinsed three times for 10 min with CMF-PBS, and the pooled secondary antibodies were added; fluorescein-conjugated donkey anti-rat IgG (1:200; Jackson ImmunoResearch, West Grove, PA) and ALEXA Fluor 594-conjugated goat anti-rabbit IgG (1:600; Molecular Probes, Eugene, OR), and incubated for 1 h at RT in the dark. Finally, nuclei were stained with DAPI (1:1000; Molecular Probes, Eugene, OR) for 12 min at RT, the tissue rinsed three times for 2 min with CMF-PBS, mounted with ProLong antifade (Molecular Probes, Eugene, OR), and coverslipped.

For combined Xgal histochemistry/TROMA-1 immunocytochemistry, the slides were rinsed three times with 0.005% NP40 and 0.01% sodium deoxycholate in PBS and finally incubated in Xgal reaction solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, and 0.4% Xgal) overnight at RT. The next day, the slides were rinsed twice with PBS, and postfixed for 10 min in 4% PFA at RT. For blocking unspecific antibody binding, slides were incubated for 30 min at RT in CMF-PBS containing 2% NGS, 0.2% BSA, and 0.2% Triton X-100. Slides were then incubated with the primary antibody (TROMA-1 at 1:20; Hybridoma Bank) in 2% NGS, 0.2% BSA, and 0.2% Triton X-100 overnight in the cold. The next day, the slides were rinsed three times for 10 min in CMF-PBS and then incubated with secondary antibody (fluorescein-conjugated donkey anti-rat IgG at 1:200; Jackson ImmunoResearch, West Grove, PA) for 1 h at RT in the dark. The slides were subsequently rinsed three times for 10 min at RT with 0.05% Triton X-100 in CMF-PBS.

In control experiments, either one of the primary antibodies or one of the secondary antibodies was substituted by buffer. In none of the control experiments was significant staining observed (data not shown).

In some experiments, TROMA-1 was detected by immu-

noperoxidase reaction. To this end, Xgal histochemical reaction was performed as described above. Subsequently, TROMA-1 immunoperoxidase reaction was performed as follows. The slides were rinsed once with PBS for 10 min, endogenous peroxidase blocked with 0.6% H_2O_2 in methanol for 20 min at RT. The slides were then rinsed three times for 5 min with PBS and preincubated in 2% BSA in PBS containing NGS (1:20) for 20 min at RT. Subsequently, the tissue was incubated with TROMA-1 (1:10) in PBS overnight at 4°C. The next day, the tissue was rinsed three times for 5 min with PBS. Peroxidase-conjugated goat anti-rat IgG (1:500) in 1% BSA was added for 90 min at RT (Jackson ImmunoResearch, West Grove, PA). This was followed by three rinses for 5 min each in PBS. The staining was developed in the dark at RT for 10 min in the following freshly prepared and filtered solution: 22 ml 0.005 M Tris, pH 7.6–7.8, 17 mg imidazol, 10 mg 3,3′ diaminebenzidine tetrahydrochloride tablets (Sigma), and 5 μl H_2O_2. This was followed by a rinse with H_2O for 5 min. The nuclei were counterstained with nuclear red, the tissue dehydrated, and the slides mounted with DePex (SERVA). Some sections, in which β-galactosidase was visualized by peroxidase reaction product, were processed further for electron microscopy as described (Halata et al., 1999).

Results

The whisker pads of day 16.5 mouse embryos were dissected, frozen, genotyped, and double transgenic tissue processed further for β-galactosidase immunohistochemistry, Xgal histochemical stain, TROMA-1 immunohistochemistry, immunoelectron microscopy, or combinations thereof. The TROMA-1 antibody is a Merkel cell marker that recognizes cytokeratin 8 (Moll et al., 1996a; Vielkind et al., 1995).

Merkel cells are located in the epithelium of the outer root sheet of whiskers, in the rete ridge and in touch domes in the epidermis between hairs (Halata, 1993; Halata and Munger, 1980). Double transgenic Merkel cells, identified by TROMA-1 antibody binding, express β-galactosidase in all three locations, follicular (Fig. 1A–D, L–N), rete ridge (Fig. 1A, E–G), and touch dome (Fig. 1H–K). Cells in which TROMA-1 and β-galactosidase immunoreactivities colocalize appear yellow (Fig. 1A, D, G, K, and N). The majority of the facial mesenchyme is derived from the neural crest (reviewed in Le Douarin and Kalcheim, 1999). Large numbers of β-galactosidase immunoreactive cells form the connective tissue of the facial dermis and the blood sinuses (bls; Fig. 1A), that surround the whisker follicle. We have confirmed colocalization of β-galactosidase and TROMA-1 immunoreactivities in the same optical section by confocal microscopy (Fig. 1L–N). Furthermore, we have combined Xgal histochemistry with TROMA-1 immunocytochemistry (Fig. 2A–E). Cytokeratin 8 immunoreactive cells contain Xgal reaction product (Fig. 2A and B; e.g.,

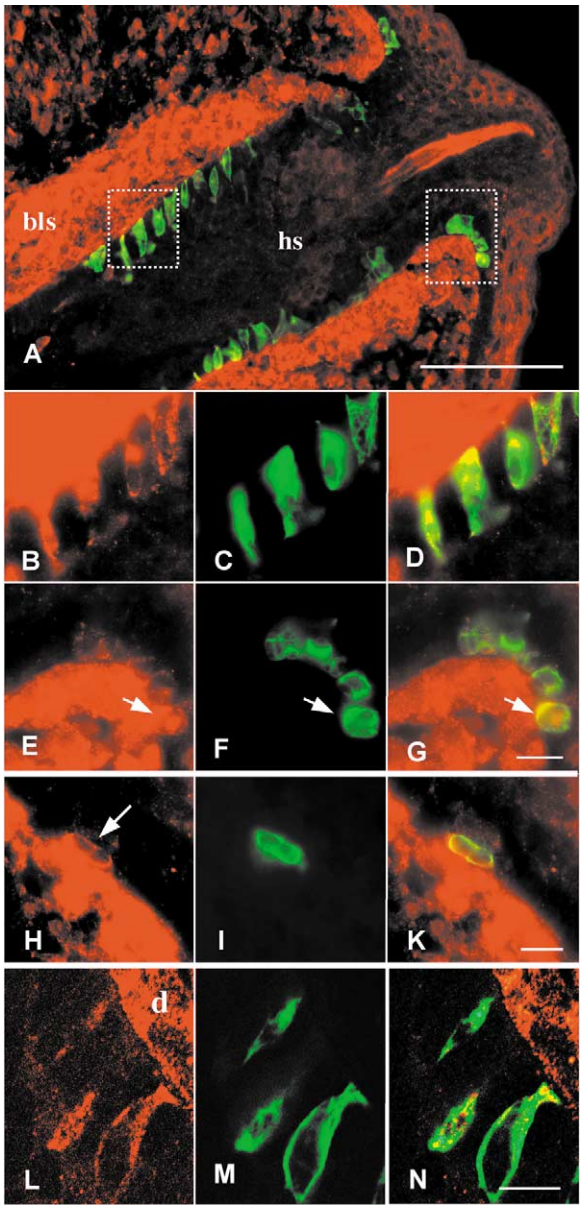

Fig. 1. β-Galactosidase immunoreactivity in Merkel cells in the outer root sheet of the follicle, the rete ridge, and in touch domes. (A) Longitudinal section, double transgenic E16.5 mouse whisker; merged images of double stain with β-galactosidase (red ALEXA fluor 594 fluorescence) and TROMA-1 (cytokeratin 8; green fluorescein fluorescence) antibodies. Merkel cells are located in the outer root sheet (A, B–D) that surrounds the hair shaft (hs) and in the rete ridge (A, E–G). The hair and the stratum corneum of the epidermis show unspecific staining (A). (B–D) Higher magnification of area outlined on the left in (A). All Merkel cells express β-galactosidase (B) and cytokeratin 8 (C). (D) Merged images; cells with colocalizing antibody binding appear yellow. (E–G) Higher magnification of Merkel cells in rete ridge outlined in (A) on the right. (E) β-Galactosidase (ALEXA fluor 594) and (F) TROMA-1 (fluorescein) in the same focal plane; (G) merged images. (H–K) β-Galactosidase immunoreactivity of two cells in an interfollicular touch dome (H, arrow). (H) β-Galactosidase immunoreactivity (ALEXA fluor 594); (I) corresponding TROMA-1 immunoreactivity; (K) merged images; β-galactosidase and TROMA-1 immunoreactivities colocalize (yellow). (L–N) Confocal microscopy of follicular Merkel cells. Three Merkel cells express β-galactosidase (L; red ALEXA fluor 594), as do dermal cells (d) of the sinuses; (M) corresponding TROMA-1 immunoreactivity (green fluorescein fluorescence) in the same focal plane; (N) merged images of same optical section, showing colocalization of cytokeratin 8 and β-galactosidase. Bars, (A) 50 μm; (B–G, H–K, L–N) 10 μm.

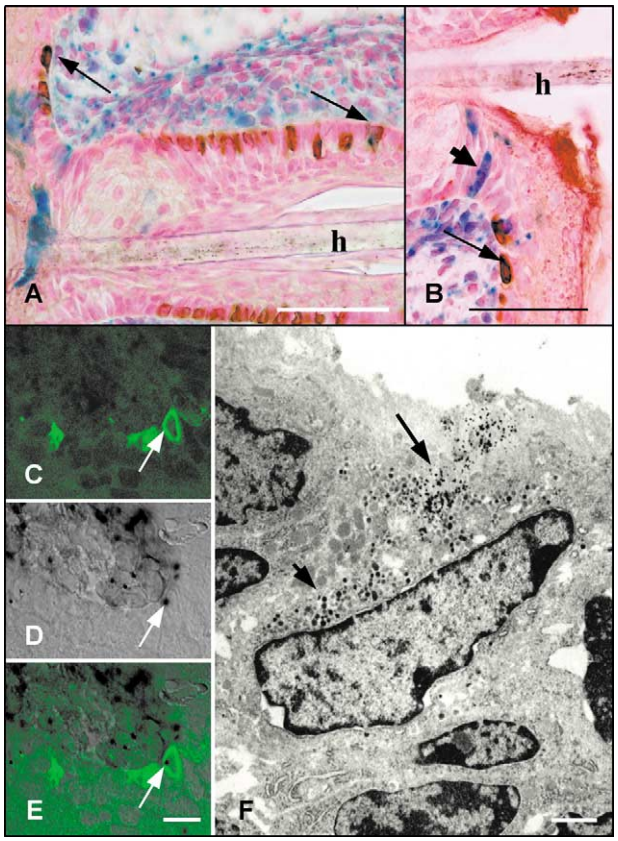

Fig. 2. Colocalization of Xgal histochemistry and cytokeratin 8 in Merkel cells; immunoelectron microscopy of Merkel cells. (A, B) Merkel cells in the follicle and rete ridge express cytokeratin 8 (TROMA-1; brown peroxidase reaction product) and are positive for Xgal reaction product (blue; e.g., long arrows in A and B). (B) Occasional Xgal-positive, TROMA-1-negative cells, putative undifferentiated progenitor cells, or melanogenic precursors, are present also (B; short arrow). (C–E) Confocal images of Xgal/TROMA-1 double stains. In contrast to β-galactosidase immunoreactivity, Xgal stain often does not fill the entire cell, but is located in inclusion bodies that are not seen in all optical planes of a cell, as has been noted also in other studies (see, e.g., Rico et al., 2002). Confocal microscopy thus confirmed the presence of Xgal reaction product (D, black) within TROMA-1-positive cells (C; green fluorescein fluorescence). (E) Merged images showing colocalization of Xgal and TROMA-1 stains in the same cell and focal plane. (F) After peroxidase immunostaining for β-galactosidase, some sections were further processed for electron microscopy. A typical Merkel cell that contains dense core granules (short arrow) also contains fine granular peroxidase reaction product (long arrow), confirming at the electron microscopic level the presence of β-galactosidase in Merkel cells. Bars, (A, B) 50 μm; (C–E) 10 μm; (F) 1 μm.

long arrows). Interestingly, occasional β-galactosidase-positive cells (not shown) or Xgal-positive cells (Fig. 2B, short arrow) that are TROMA-1-negative are present as well. They constitute a small subset of neural crest-derived cells in the outer root sheet. Since whisker development is completed postnatally only, it is conceivable that they are multipotent progenitors or committed Merkel cell precursors. Furthermore, it is possible that they are melanogenic precursors, as has been shown by Peters et al. (2002). We have corroborated colocalization of Xgal stain and cytokeratin 8 immunoreactivity by confocal microscopy (Fig. 2C–E). In a third approach, immunoelectron microscopy showed peroxidase reaction product in β-galactosidase-immunostained Merkel cells (Fig. 2F).

Discussion

We have provided evidence for the neural crest origin of murine Merkel cells by three independent methods. Our findings thus resolve a long-standing controversy.

All Merkel cells expressed β-galactosidase when probed with anti-β-galactosidase antibodies, and immunoreactivity was distributed throughout the cytoplasm. In contrast, in histochemical stains, some Merkel cells appeared Xgal-negative, and the Xgal stain was limited to one or a few inclusion bodies within the cell, as has been observed in other studies as well (e.g., Rico et al., 2002). Thus, there are several reasons for the discrepancy between immunocytochemistry and histochemistry. β-Galactosidase protein expression may differ from β-galactosidase enzyme activity. In fact, it has been shown that a higher number of transgenic cells are β-galactosidase-immunoreactive than Xgal-positive (Couffinhal et al., 1997). The Xgal-positive inclusion body can be in a different focal plane, rendering TROMA-1-positive Merkel cells falsely Xgal-negative. Furthermore, it is conceivable that cre-induced recombination does not take place in every neural crest cell. This appears unlikely as we have observed high intensity of cre-immunoreactivity in migrating neural crest cells of E9.5 double transgenic embryos (data not shown). Several observations are in agreement with our present data. By transplanting Hamburger–Hamilton stage 17–18 (Hamburger and Hamilton, 1951) chick limb buds to replace same-age limb buds of host quail embryos, and vice versa, we have excluded the epidermal origin of avian Merkel cells and suggested their neural crest origin (Halata et al., 1990; Grim and Halata, 2000a,b). Moreover, occasional fetal human Merkel cells have been localized in the dermis, where neural crest cells migrate before they invade the epidermis (Halata, 1981; Hashimoto, 1972).

In contrast, other studies have favored the epidermal origin of Merkel cells. One reason for associating mammalian Merkel cells with the epidermis is their location, as they reside in the basal layer of the epidermis (English et al., 1980; Lyne and Hollis, 1971). Moreover, Merkel cells express low molecular weight cytokeratins, which are characteristic for epithelial cells (Moll et al., 1984, 1986). Third, xenograft experiments in which human fetal skin was grafted onto host nude mice have suggested an epidermal origin of Merkel cells (Moll et al., 1990). There are several problems with the interpretation of these studies. Low molecular weight cytokeratins are characteristic of simple epithelia, not of keratinocytes (Kemler et al., 1981). Neither the location of a cell, nor the expression of a particular gene, or a set of genes, necessarily defines cell type identity. Moll and collaborators have grafted gestational week 8–11 hu-

man fetal skin onto nude mice and observed the development of human Merkel cells in the grafts (Moll et al., 1990). However, Merkel cell progenitors and neural crest-derived glia are present in human fetal skin already at gestational weeks 6–11 (Moll et al., 1996b; Moore and Munger, 1989; Terenghi et al., 1993). Therefore, the human fetal skin xenograft experiments do not provide conclusive evidence in support of the epidermal origin of Merkel cells.

Wnt genes, specifically Wnt3 and Wnt5a, are expressed in the skin (Millar et al., 1999; Reddy et al., 2001; St-Jacques et al., 1998), including in the ectodermal placodes of E16.5 mouse embryos (Fuchs et al., 2001). In contrast, Wnt1 is expressed exclusively in the central nervous system and the neural crest during embryonic development (Davis et al., 1988; Echelard et al., 1994; McMahon et al., 1992; Molven et al., 1991; Wilkinson et al., 1987; Wolda et al., 1993). We have nevertheless confirmed the absence of cre recombinase in E16.5 double transgenic whisker pads by immunocytochemistry with anti-cre antibodies (data not shown).

Taken together, we have shown that Merkel cells in double transgenic Wnt1-cre/R26R mouse embryos express β-galactosidase, identifying them as neural crest progeny. We thus conclude that avian and mammalian Merkel cells share the same ontogenetic origin, the neural crest. According to our findings, Merkel cells can be added to the list of cell types that originate from the neural crest.

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