Migration and proliferation of cultured neural crest cells in W mutant neural crest chimeras

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Summary

Chimeric mice, generated by aggregating preimplantation embryos, have been instrumental in the study of the development of coat color patterns in mammals. This approach, however, does not allow for direct experimental manipulation of the neural crest cells, which are the precursors of melanoblasts. We have devised a system that allows assessment of the developmental potential and migration of neural crest cells in vivo following their experimental manipulation in vitro. Cultured C57BI/6 neural crest cells were microinjected in utero into neurulating Balb/c or W embryos and shown to contribute efficiently to pigmentation in the host animal. The resulting neural crest chimeras showed, however, different coat pigmentation patterns depending on the genotype of the host embryo. Whereas Balb/c neural crest chimeras showed very limited donor cell pigment contribution, restricted largely to the head, W mutant chimeras displayed extensive pigmentation throughout, often exceeding 50% of the coat. In contrast to Balb/c chimeras, where the donor melanoblasts appeared to have migrated primarily in the characteristic dorsoventral direction, in W mutants the injected cells appeared to migrate in the longitudinal as well as the dorsoventral direction, as if the cells were spreading through an empty space. This is consistent with the absence of a functional endogenous melanoblast population in W mutants, in contrast to Balb/c mice, which contain a full complement of melanocytes. Our results suggest that the W mutation disturbs migration and/or proliferation of endogenous melanoblasts. In order to obtain information on clonal size and extent of intermingling of donor cells, two genetically marked neural crest cell populations were mixed and coinjected into W embryos. In half of the tricolored chimeras, no co-localization of donor crest cells was observed, while, in the other half, a fine intermingling of donor-derived colors had occurred. These results are consistent with the hypothesis that pigmented areas in the chimeras can be derived from extensive proliferation of a few donor clones, which were able to colonize large territories in the host embryo. We have also analyzed the development of pigmentation in neural crest cultures in vitro, and found that neural tubes explanted from embryos carrying wt or weak W alleles produced pigmented melanocytes while more severe W genotypes were associated with deficient pigment formation in vitro.

Key words: neural crest cell culture, in utero injection, coat color pigmentation, W mutation.

Introduction

The vertebrate neural crest is a pluripotent embryonic cell population derived from the lateral ridges of the neural plate during closure of the neural tube. Neural crest cells disperse from the dorsal surface of the neural tube and migrate extensively through the embryo, giving rise to a wide variety of differentiated cell types. Neural crest derivatives include all pigment cells of the skin, most neurons of the peripheral nervous system and their supporting cells, adrenomedullary cells, and skeletal and connective tissue of the face and head (see Harrison, 1938; Le Douarin, 1982, and Weston, 1983, for reviews). For the most part, analyses of neural crest cell migration and differentiation have been carried out in avian and amphibian embryos, owing to their accessibility to experimental manipulation. In particular, the ability to transplant neural crest cells into avian embryos in situ (Weston, 1963; Le Douarin, 1973) has proven to be a powerful tool in the analysis of neural crest development. Mammalian embryos are far less tractable to direct experimental intervention, being implanted within the uterus and surrounded by fetal membranes at the time of neural crest migration [embryonic day (E) 8.5–12 in the mouse]. This has largely limited analysis of the mammalian neural crest to descriptive morphology (e.g. Erickson and Weston,
microinjection technique and used it to characterize exogenous melanoblasts in normal embryonic development. It was found that over 30% of the animals showed pigmentation were consistent with participation of the microinjected embryos after birth, it was examined in rodent embryos in vitro to study early developmental fates and migratory pathways of cranial crest cells.

The approach that we have taken is to introduce cultured murine neural crest cells, derived from explanted neural tubes, into midgestation mouse embryos in utero as a means of assessing neural crest development in vivo (Jaenisch, 1985). We have been using this approach to focus on the differentiation of a subset of the neural crest cell population: melanoblasts, the precursors of the pigment-producing melanocytes. Melanoblasts, or their neural crest precursors, are segregated very early in migration from other neural crest progenitors. Marking experiments in both avian (Weston, 1963; Le Douarin, 1973) and mammalian (Serbedzija et al. 1990) embryos have defined two primary pathways of trunk neural crest migration after cells have left the neural tube. One route leads ventrally, between the neural tube and the somites, and is taken by neural crest cells giving rise to sensory and autonomic ganglia, Schwann cells and the adrenal medulla (reviewed by Le Douarin, 1982). The other pathway leads dorsolaterally around the periphery of the embryo, through the dermal mesoderm underlying the epidermis (Mayer, 1973), and is travelled by melanoblasts. These cells eventually invade the epidermis and colonize the skin and hair follicles where they differentiate into melanocytes (Rawles, 1947). The dorsolateral migration of melanoblasts has been visualized in the coat color patterns of murine aggregation chimeras (Mintz, 1967; McLaren and Bowman, 1969). These chimeras, composed of two genotypes derived from mice differing in coat color, are characterized by transverse stripes of pigmentation with a sharp line of demarcation along the dorsal midline between left and right sides. Mintz (1967) interpreted each unit-width stripe as representing the clonal progeny of a single primordial melanoblast.

In a previous report (Jaenisch, 1985), we described the microinjection of C57B1/6J neural crest cells into albino embryos. Neural crest cells were cultured from C57B1/6J embryos and injected in utero into Balb/c embryos between E8.75 and E9.25. When offspring of microinjected embryos were examined after birth, it was found that over 30% of the animals showed evidence of pigmentation, and that the patterns of pigmentation were consistent with participation of the exogenous melanoblasts in normal embryonic development.

In this report, we have refined the in utero microinjection technique and used it to characterize pigment reconstitution in murine W mutant neural crest chimeras. Mutations at the murine W locus (dominant white spotting) have pleiotropic effects on the development of melanoblasts, germ cells and hematopoietic cells (see Russell, 1979; and Silvers, 1979 for reviews), giving rise to mice with varying degrees of deficiency in the affected cell populations, depending on the severity of the mutant allele. Of particular relevance to this study is that the lack of coat pigmentation in W mutants results from a complete absence of melanocytes from the hair follicles of the skin (Silvers, 1956) owing to a cell autonomous defect in the melanoblast population (Mayer and Green, 1968). In contrast, albino mice (such as Balb/c) contain a full complement of endogenous melanoblasts in their skin, but are white because of a deficiency of tyrosinase, the key enzyme in melanin synthesis (Silvers, 1979). In parallel with microinjection of C57B1/6J neural crest cells into W mutant embryos, we have also injected crest cells into Balb/c embryos, in order to allow a direct comparison of the pigmentation of W mutant and albino mice by exogenous melanoblasts. Lastly, we have analyzed the effect of the W mutation on pigment formation in cultures of explanted neural tubes.

**Materials and methods**

**Cells**

y2 cells (Mann et al. 1983) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (CS). Neural crest cultures were prepared from C57B1/6J and DBA/2J embryos isolated on day 8.75 and 9.5 of gestation, respectively. Embryos ranging from 8 to 22 somites were used; cultures derived from embryos younger than approximately six somites proved incapable of producing pigmentation in recipient embryos (data not shown). Embryos were dissected free of fetal membranes in DMEM, incubated on ice in Hepes containing 1% trypsin for 3 min, then washed several times with DMEM/10% CS. Neural tubes were dissected free of surrounding tissue, not all adhering somites were necessarily removed. The tubes were cut into two or three pieces and plated in 35 mm Primaria tissue culture dishes (Falcon) containing DMEM supplemented with 5% fetal calf serum (FCS) and 5% horse serum. Cells could be seen migrating from the neural tube within 6 h. Neural tubes, together with adjacent non-adherent tissues, were excised from the cultures after 2 days.

**Melanocyte cultures**

Melanocyte differentiation was examined in cultures of neural tubes prepared as described above. One neural tube, cut into three or four pieces, was plated per Primaria dish and subsequently excised from the cultures 3 days later. The neural crest medium (see above) was then replaced with TAM medium (Tamura et al. 1987) containing 48 nM phorbol 12-myristate 13-acetate, 0.1 mM dbcAMP and 0.2 mM melanocyte-stimulating hormone (oMSH) in Ham's F10 medium supplemented with 10% CS, 10% FCS and antibiotics. In later experiments, oMSH was omitted from the medium with no effect on melanogenesis. Cells were fed with fresh medium every 3 days and cultures were maintained for 30 days in TAM medium. The presence of pigmented melanocytes was scored by microscopic examination of cultures under bright-field illumination.
Neural crest cells and coat pigmentation

Mice

Balg/c, C57Bl/6J, DBA/2J strains and the W^X/+ and W^Y/W^Y mutants were obtained from Jackson Laboratories and bred in our mouse colony. The W^X and W^Y mutations arose spontaneously in the C3H/HeJ and C57Bl/6J inbred lines, respectively, and have been maintained on the strain of origin. The W^X mutation (Russell et al. 1957) is indistinguishable from the classic W mutant phenotype (Little, 1915): heterozygotes display a large white belly spot and homozygotes are non-viable, dying either in utero or perinatally from severe macrocytic anemia. W^Y/W^Y homozygotes are both viable and fertile, and are characterized by a flecked pattern of coat pigmentation (Gessler et al. 1981). Homozygous sash (W^S) mutant mice were generously provided by Dr Verne Chapman of Roswell Park Memorial Institute and bred in our mouse colony. The W^Sm mutation arose spontaneously in (C3H X 101 F^1) hybrids (Lyon and Glenister, 1982) and has subsequently been introduced into C57Bl/6J (bg') mice. The homozygotes are fully fertile and virtually devoid of coat pigmentation.

Female mice were mated with males in the evening, and successful matings were identified the next morning by vaginal plug formation. The day of plug formation was counted as day 0 (E0) of gestation. Doubly heterozygous W mutant embryos for neural crest cell injections were produced by mating of W^Y/+ females with either W^X/W^X males or W^Y/W^Y males.

Microinjection of medgestation embryos

Neural crest cultures were harvested by trypsinization on the second day of culture, unless otherwise indicated, collected in DMEM/10% CS and concentrated by centrifugation into 40–80 µl of DMEM/10% CS containing 1–2 x 10^5 cells. ψ2 cells were similarly harvested and concentrated to 10^5 cells ml^-1 in DMEM/10% CS. Concentrated cells were deposited in approximately 10 µl drops on 35 mm Petri dishes and overlaid with light mineral oil. The plates were placed on ice and cells were allowed to settle prior to microinjection. For co-injection of C57Bl/6J and DBA/2J neural crest cells, cells were mixed in equal numbers following trypsinization, concentrated by centrifugation and plated as described above.

For microinjection, previously published procedures were used (Jaensch, 1980, 1985, Compere et al. 1989). Pregnant females were anesthetized with metomidate hydrochloride (hypnobil) at 20–25 µg g^-1 of body weight, and ether, prior to injection of the embryos. Neural crest cell injections were carried out between E8.75 and E9.0 (i.e. between 6:00 p.m. and midnight of E8); ψ2 cells were injected at three time: E8.75 (6:00 p.m.), E9.0 (midnight of E8/9) and E9.5 (noon). Laparotomy was performed by a long ventral incision and the uterus held by forceps during injection of each embryo. Neural crest, or ψ2, cells were drawn into a glass micropipette of approximately 55 µm diameter with a closed sharp tip and a side hole of 40–50 µm diameter (the pipet was drawn on a needle puller and modified with a de Fonbrune microforge [Beaudoin, France]). The pipette was manually inserted into the ventral third of the decidua swelling and 0.2–0.5 µl was introduced per injection. Between 1000 and 2400 neural crest cells, or between 2000 and 5000 ψ2 cells, were injected per embryo. The survival rate to weaning of injected embryos was 60–70% (data not shown).

Immunohistochemistry

ψ2 cells were localized in microinjected embryos by immunohistochemical staining techniques to detect retroviral antigens that were used as a cellular marker. Mouse embryos were fixed in Carnoy’s fixative overnight at 4°C at 5–14 h after microinjection (embryos injected at E8.75 and E9.5 were fixed at 5 h postinjection and those injected at E9.0 were fixed at 14 h postinjection). Embryos were dehydrated in graded alcohols and xylenes and embedded in Paraplast plus Senal 4 µm sections were cut (approximately 200–250 per embryo) and heat-baked onto glass slides coated with Eimer's glue (Borden). Immunohistochemistry was performed as described previously (Compere et al. 1989). For recognition of retroviral antigens in ψ2 cells, slides were incubated with a goat anti-Mo-MuLV antibody raised against Tween–ether-disrupted virus (NIH 81S107; 1:160), followed by a swine anti-goat immunoperoxidase conjugate (1:60, TAGO). Antibody was localized by incubating sections with 0.05 M Tris, pH 7.6, containing 3,3'-diaminobenzidine tetrahydrochloride (Aldrich) (1.5 mg ml^-1) and 0.3% hydrogen peroxide. Slides were counterstained with methyl green (Roboc).

Southern blot analysis of genomic DNA

Genomic DNA was prepared from tail biopsies by overnight incubation of minced tissue in 700 µl of 50 mM Tris–HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate and 500 µg ml^-1 of proteinase K, followed by phenol extraction and ethanol precipitation 6 µg of genomic DNA was digested with 40 units of EcoRI, electrophoresed through an 0.9% agarose gel and transferred to a nylon membrane (Zetabind, Cuno). Membranes were hybridized to the radiolabeled (Feinberg and Vogelstein, 1983) 0.8 kb PrvUI–EcoRI fragment of the pK3A c-kit cDNA (Gessler et al. 1988).

Results

Localization of cells injected in utero

Microinjection of neural crest cells into mouse embryos was performed essentially blindly, since the midgestation embryo cannot be visualized within the uterus. We postulated previously (Jaensch, 1985) that neural crest cells were deposited within the amniotic cavity, rather than the extracoelomic space, yolk cavity or embryo proper, by the microinjection procedure. To visualize directly the site of deposition of cells microinjected between 8.75 and 9.5 days of gestation, the fibroblastic retroviral packaging cell line ψ2 (Mann et al. 1983) was used for microinjection. These cells synthesize large amounts of retroviral proteins which can be visualized by immunohistochemical staining techniques that detect retroviral antigens. Embryos were removed from the uterus 6–14 h after microinjection, fixed and serially sectioned. The location of the microinjected cells was determined by staining of tissue sections with an antiviral antisera.

As summarized in Table 1, the time of microinjection markedly affected the site at which ψ2 cells were deposited. Of 13 embryos injected between E8.75 and E9.0, 11 contained cells in the amniotic cavity (e.g. Fig. 1A, B), and in the remaining two embryos the location of the ψ2 cells could not be determined. Of 15 embryos injected on E9.5, none contained ψ2 cells in the amniotic cavity. Cells were present in the extracoelomic space (Fig. 1C), yolk cavity or decidua (Fig. 1D). In a separate series of experiments, it had been determined that the number of chimeric animals
Wx mice can be readily distinguished from their W41/+ occasionally at the base of the tail. W 41 /Wx mice, of the ear pinnae and to a few hairs on the face and completely white, with pigmentation restricted to the skin coat pigmentation. As is characteristic of all W mutants, WSh/WSh and W x/+ parents, are completely devoid of pinnae, and W Sh/W x mice, derived from mating

Frequency of pigment contribution to BALB/c and W mutant mice microinjected with C57B1/6J neural crest (Silvers, 1979). Both W 41 /Wx and W Sh/WSh derived locally from the optic cup, rather than from the pigmented cells of the retinal epithelium are derived from the mating of W 41/W41 and W x/+ parents, as previously reported (Jaenisch, 1985), the fraction of chimeric animals obtained was strongly dependent on the substrata used for crest cell growth. In our previous study, collagen-coated cultures; the frequency of pigmentation dropped sharply with the use of older cultures (Table 2). This differs from our previous experience in which cells could be cultured for up to 5 days without the loss of developmental potential (Jaenisch, 1985) and is likely attributable to differences in the substrata used for crest cell growth. In our previous study, collagen-coated

Table 1. Summary of tp2 microinjection studies

<table>
<thead>
<tr>
<th>Time of injection</th>
<th>No. of embryos containing cells</th>
<th>Location of cells</th>
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<tbody>
<tr>
<td>E8.75</td>
<td>6</td>
<td>Amnion (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amnion+extra-coelomic space (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Indeterminate (2)</td>
</tr>
<tr>
<td>E9.0</td>
<td>7</td>
<td>Amnion (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amnion+extra-coelomic space (2)</td>
</tr>
<tr>
<td>E9.5</td>
<td>15</td>
<td>Extra-amniotic space (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decidua (7)</td>
</tr>
</tbody>
</table>

generated by neural crest cell injection was highest when crest cells were injected between day 8.75 and 9.25, lower at day 9.5 and very low at later times (Jaenisch, 1985; and our unpublished observations). These data indicate that deposition of neural crest cells within the amniotic cavity is necessary for the exogenous cells to contribute to embryonic development. The difficulty in targeting the amniotic cavity on E9.5 likely reflects ongoing changes in the relative sizes of the extraembryonic cavities during this period of development, resulting in a proportional decrease in the volume of the amniotic cavity.

Frequency of chimera formation

Mouse embryos bearing double heterozygous and homozygous combinations of three mutant W alleles (WSh/WSh, W41/WX, WSh/WX), as well as control Balb/c embryos (+/+), were used as recipients for microinjection of C57B1/6J neural crest cells. The W mutants used were chosen for their lack of coat pigmentation. WSh/WSh homozygotes are almost completely white, with pigmentation restricted to the skin of the ear pinnae and to a few hairs on the face and occasionally at the base of the tail. W41/WX mice, derived from the mating of W41/W41 and WX/+ parents, are pigmented only in the skin of the ear pinnae, and WSh/WX mice, derived from mating WSh/WSh and WX/+ parents, are completely devoid of coat pigmentation. As is characteristic of all W mutants, WSh/WSh, W41/WX and WSh/WX mice have black eyes, since the pigmented cells of the retinal epithelium are derived locally from the optic cup, rather than from the neural crest (Silvers, 1979). Both W41/WX and WSh/WX mice can be readily distinguished from their W41/+ and WSh/+ littermates, respectively, since W41/+ mice are fully pigmented with only a very small belly spot (Geissler et al., 1981) and WSh/+ mice display a characteristic broad white sash extending fully around the mid-trunk area (Lyon and Glenister, 1982).

Donor neural crest cells were derived from the explanted neural tubes of C57B1/6J embryos as described in Materials and methods. The cells were microinjected into recipient Balb/c and W mutant embryos between E8.75 and E9.0. Examination of embryos dissected free of fetal membranes revealed that, during this time period, recipient Balb/c and W mutant embryos were of similar developmental age (data not shown). As shown in Table 2, similar frequencies of pigment contribution were observed using both kinds of recipients (expt 1 in Table 2). As previously reported (Jaenisch, 1985), the fraction of chimeric animals obtained was strongly dependent on the age of the neural crest cultures. Optimal results (pigmentation of 41% of Balb/c and 43% of W mutant recipients) were obtained using 2-day-old neural crest cultures; the frequency of pigmentation dropped sharply with the use of older cultures (Table 2). This differs from our previous experience in which cells could be cultured for up to 5 days without the loss of developmental potential (Jaenisch, 1985) and is likely attributable to differences in the substrata used for crest cell growth. In our previous study, collagen-coated

Table 2. Frequency of pigment contribution to BALB/c and W mutant mice microinjected with C57B1/6J neural crest cells

<table>
<thead>
<tr>
<th>Age of neural crest culture</th>
<th>W genotype</th>
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<tbody>
<tr>
<td></td>
<td>+/+ (Balb/c)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>W41/WX</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>WSh/WX</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>W41/WSh</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>WSh/WX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days</td>
<td>14/34 (41%)</td>
<td>9/24 (37%)</td>
<td>11/17 (65%)</td>
<td>8/24 (33%)</td>
</tr>
<tr>
<td>3 days</td>
<td>0/9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 days</td>
<td>5/42 (12%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 days</td>
<td>5/82 (6%)</td>
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* Expt 2 is an independent series of injections using a mixture of C57B1/6J and Dba/2J neural crest cells
Fig. 4. Pigmentation of $W^{sh}/W^{sh}$ neural crest chimeras co-injected with C57Bl/6J and DBA/2J neural crest cells. (A and B) Dorsal and ventral views of a chimera taken at 16 days of age. (C–F) A selection of chimeras photographed at 30, 38, 17 and 9 days of age, respectively. (G and H) Dorsal and ventral views of the same chimera at 9 days and 9 weeks of age, respectively. (I) The same chimera as shown in C is photographed here at 13 weeks of age, next to an age-matched DBA/2J mouse to illustrate the extent of fading of brown pigmentation in older $W$ mutant chimeras.

Fig. 5. Pigmentation of neural crest cultures in vitro. (A) Three dishes are shown containing melanocytes grown from C57Bl/6J neural tubes. The pigmented patches correspond to the sites at which pieces of neural tube had been plated. (B and C) Bright-field photomicrographs of cultures of pigmented C57Bl/6J and $W^{sh}/W^{sh}$ melanocytes, respectively.
Table 3. Extent of pigmentation in neural crest chimeras

<table>
<thead>
<tr>
<th>Type*</th>
<th>Extent of pigmentation</th>
<th>No of chimeras†</th>
<th>Balb/c</th>
<th>W mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>+</td>
<td>14 (100%)</td>
<td>10 (20%)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>++</td>
<td>19 (38%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>+++</td>
<td>12 (24%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>++++</td>
<td>9 (18%)</td>
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*Type A chimeras showed pigmentation of no more than 10% of their coats, for examples see Fig. 2A,B. Type B chimeras showed 10–30% pigment contribution, see Fig. 2D,E,F. Type C showed 30–50% pigment contribution, see Fig. 2G,H,I. Type D chimeras were pigmented over greater than 50% of their coats, as shown in Fig. 2J,K,L.
†Including all chimeras generated by microinjection of 2-day-old neural crest cultures (see Table 2).

W mutants showed extensive pigmentation by exogenous neural crest cells. Black pigmentation was observed in W<sup>sh</sup>/W<sup>sh</sup> chimeras whereas pigment in W<sup>sh</sup>/W<sup>+</sup> and W<sup>+</sup>/W<sup>+</sup> chimeras showed the agouti pattern (brownish appearance) due to the presence of the dominant A (agouti) allele inherited from the C3H/HeJ (W<sup>/<sup>+</sup>) parent. A representative litter of W<sup>sh</sup>/W<sup>sh</sup> mice reconstituted with C57Bl/6J melanocytes is shown in Fig. 2C, and a selection of W mutant chimeras, representing examples of the various patterns and extents of pigmentation, are shown in Fig. 2D–L. Fig. 2L shows the most highly pigmented mouse generated. As summarized in Table 3, the extent of pigmentation in the 50 W mutant chimeras varied over a wide spectrum, with the majority of animals displaying pigment contribution to 10–50% of their coats. Unlike Balb/c recipients, where pigmentation was largely restricted to the head, pigmentation of the W mutants was evenly distributed between the head and the trunk (Table 4; Jaenisch, 1985).

In many of the W mutant chimeras, the deposition of pigment clearly delineated either, or both, the dorsal and ventral midlines (Fig. 2D,G,H,I). In some cases, the demarcation was quite sharp (as in the cranial pigmentation of Fig. 2G) and, in others, a limited amount of drift appeared to occur across the midline (see Fig. 2H,I). Interestingly, whereas virtually all instances of trunk pigmentation extended fully from the dorsal to the ventral midline, in approximately half of the mice with cranial pigmentation the pigmented area extended variable distances laterally from the dorsal midline, never reaching the underside of the head (e.g. Fig. 4G,H).

The pigment patterns in the vast majority of W mutant chimeras are consistent with entry of the C57Bl/6J neural crest cells at the dorsal midline of the embryos, followed by subsequent proliferation and migration dorsoventrally and laterally. In a few mice, however, small patches of pigmentation were observed that did not connect with the dorsal midline. These mice constituted approximately 10% of the W mutant chimeras and fell into three distinct categories: (1) mice with small patches of pigment on the belly along the ventral midline, (2) mice in which only a single limb was pigmented, and (3) mice with pigment on the side of the face. It is possible that these patches are due to melanoblasts that had undergone limited proliferation after migrating away from the neural tube. In all three...
cases, pigment contribution never exceeded 5% of the coat. These animals represented more than half of the W mutant chimeras categorized as type A (1-10% coat pigmentation) in Table 3.

The pigmented phenotype of the W mutant chimeras was stable; no diminution of pigment was observed over an 8 month period. In contrast, pigmentation in Balb/c mice often began to fade within the first 2 months after birth and was either completely absent or very faint by 8 months of age (data not shown). Similar fading of pigmentation has been observed in aggregation chimeras derived from fusion of Balb/c and C57Bl/6J embryos, and has been attributed to preferential selection for amelanotic melanocytes within hair follicles containing both Balb/c and C57Bl/6J melanocytes (Gearhart and Oster-Granite, 1981).

Southern blot analysis of W mutant chimeras

As mentioned above, W41/Wx and WSh/Wx offspring could be readily distinguished from their W41/+ and WSh/+ littermates, respectively, on the basis of their pigmentation. W41/+ mice, in particular, are essentially fully pigmented and thus easily differentiated from their wild-type, or chimera, W41/Wx siblings. For the most part WSh/+ mice, characterized by a broad white sash completely encircling the midtrunk region, could also be distinguished easily from their double heterozygous WSh/Wx littermates. In the case of the extensively pigmented chimera shown in Fig. 2L, though, it was difficult to discern visually whether the pigmentation observed represented extensive pigment contribution to a WSh/Wx mouse, or more limited contribution to a WSh/+ heterozygote.

To ascertain the genotype of this chimera, genomic DNA was probed with the 0.8 kb PvuII-EcoRI fragment of the c-kit cDNA clone K3A, which detects a genomic rearrangement characteristic of the Wx mutation (Geissler et al. 1988). As shown in Fig. 3, the K3A probe detects three fragments in EcoRI-digested WSh/+ DNA (lane 1) and a fourth fragment of approximately 3.1 kb, indicated by an arrow, in WSh/Wx DNA (Lane 2). Lane 3 contains DNA from the extensively pigmented mouse shown in Fig. 2L, and lanes 4-6 contain DNA from the three other highly pigmented putative WSh/Wx chimeras generated, two of which are shown in Fig. 2J and K. The presence of the diagnostic 3.1 kb fragment in all four samples verifies the presence of the Wx allele and the doubly heterozygous WSh/Wx genotype of these mice.

Microinjection of mixed C57Bl/6J and DBA/2J neural crest cells

In an attempt to gain some insight into the size, number and/or distribution of melanoblast clones repopulating the W mutants, a mixing experiment was undertaken using neural crest cells from donor mice with distinguishable pigmentation. Neural crest cells from C57Bl/6J (black) mice were mixed in equal amounts with cells from DBA/2J mice (dilute brown) and co-injected into WSh/WSh embryos. As shown in Table 2 (see Expt 2), 22 chimeras were derived from the crest cell co-injections. The extent of pigmentation and overall distribution of pigment was no different from that observed upon injection of C57Bl/6J cells alone; thus, the data from both injections have been pooled in Tables 3 and 4.

Of the 22 chimeras produced by co-injection of C57Bl/6J and DBA/2J neural crest cells, over half (13/22) were pigmented by only one of the donor cell types (Table 5). The remaining nine chimeras, which included all of the most extensively pigmented animals...
(type D), showed dual color pigmentation. Examples of these latter mice are shown in Fig. 4. In four of these nine mice, no mixing of C57BL/6J and DBA/2J melanocytes was observed, the two colors being physically separated either by the anatomical boundaries of the dorsal and ventral midlines (as in Fig. 4A,B) or by distance (e.g., Fig. 4C). In the remaining five chimeras, however, mixed contribution of brown and black cells to a common anatomical area was observed (four of these mice are shown in Fig. 4D–H).

The mouse shown in dorsal and ventral views in Fig. 4G and H shows what is likely a single clone of C57BL/6J cells as part of the extensive, otherwise DBA/2J-derived, trunk pigmentation of this animal. Fine interspersing of black and brown hairs is evident, attesting to the extensive overlap of this clone with melanocytes from a DBA/2J clone or clones. In the mouse shown in Fig. 4F, the regions colonized by adjacent black and brown melanocytes are more clearly delimited, but considerable intermingling is seen along the boundaries of the two cell types. It should be noted that since this mouse, as well as that shown in Fig. 4G, was less than 2 weeks old at the time of photography, the DBA/2J pigment is gray, rather than the brown color that develops in more mature mice (e.g., Fig. 4C,D,E).

The two chimeras displaying mixed cranial pigmentation (Fig. 4D,E) also showed overlapping of adjacent black and brown clones with extensive intermingling of black and brown hairs in one of the mice (Fig. 4E). Interspersed black and brown hairs can be seen not only in the central patch of predominantly black hairs but also in more equal numbers along the side of the face and as an irregular line of black hairs leading to the small black patch on the right shoulder. It is notable that the patterns of migration in the cranial region, as exposed by the use of distinguishable melanocytes, appear to be more complex than the dorsal-to-ventral banding seen in the trunk. Similar observations have been made regarding cranial neural crest cell migration in in vitro cultures of embryos grafted with exogenous neural crest cells (Tan and Morriss-Kay, 1986).

Fading of brown pigmentation

An unexpected observation arising from these experiments was the lability of DBA/2J pigmentation in WSh/WSh neural crest chimeras. While black coat color was stable, and remained unaltered for at least 4 months, the current age of the oldest mice, all chimeras have shown distinct fading of brown pigment, usually detectable by 6–8 weeks of age. This is manifest either as a patchy appearance of white or light brown hairs or as a dramatic uniform fading of the entire brown area. In both cases, the fading does not appear to progress linearly, but rather as a series of stepwise increments, apparently tied to the cycle of hair growth. An example of fading of brown pigment is shown in Fig. 4I, in which the chimera shown at 4 weeks of age in Fig. 4C is now photographed at 13 weeks of age next to an age-matched DBA/2J control. As is evident from the photographs, no diminution of black pigment is detectable, but a striking loss of brown pigment has occurred. It should also be noted that the chimera shown in ventral view in Fig. 4H, at 9 weeks of age, has also undergone considerable fading of brown pigment (compare with the DBA/2J mouse in Fig. 4I).

Growth of melanocytes in vitro

Mutations at the W locus result in an absence of melanocytes from the skin of affected animals. To examine whether this defect is also manifested in vitro, cultures of neural crest cells were grown from the neural tubes of control and W mutant mice under conditions that promote melanogenesis (TAM medium, see Materials and methods). Under these conditions, cultures of C57BL/6J neural crest cells, derived from individual neural tubes, consistently gave rise to pigmented melanocytes (Table 6). In many cases, outgrowths of melanocytes, identifiable by morphology, were detected several days prior to the appearance of pigmentation (data not shown). Pigmented cells appeared by the second or third week of culture in TAM medium, and increased in number and intensity of pigmentation to the point where, in several cultures, pigment was visible to the unaided eye (Fig. 5A). A photomicrograph of pigmented C57BL/6J melanocytes is shown in Fig. 5B.

To test the ability of W mutant neural crest cells to differentiate into melanocytes in vitro, cultures were established from the neural tubes of W$^{at}$/W$^{at}$ and W$^{Sh}$/W$^{Sh}$ homozygotes. In neither mutant was melanogenesis impaired in vitro and virtually all cultures produced pigmented cells (Table 6), the extent of pigmentation being similar to that observed for C57BL/6J cells. A representative pigmented W$^{Sh}$/W$^{Sh}$ culture is shown in Fig. 5C; the granularity of the pigment, relative to that observed in C57BL/6J cells (Fig. 5B) is due to clumping of pigment granules caused by the bg mutation carried by our W$^{Sh}$/W$^{Sh}$ mice.

Since, as described earlier, both homozygous W mutants display some coat pigmentation, and must therefore retain some melanoblast function, the more severely pigment-depleted F1 animals bearing the W$^{X}$ allele (W$^{at}$/W$^{X}$, W$^{Sh}$/W$^{X}$) were also tested for pigmentation in vitro. Because it was not possible to differentiate between the doubly heterozygous embryos and their heterozygous pigmented siblings (W$^{at}$/+, W$^{Sh}$/+, W$^{at}$/W$^{X}$/+, W$^{Sh}$/W$^{X}$/+)...
mutant or Balb/c embryos that had been injected with later times of injection when chimera production is less successful (Table 1; Jaenisch, 1985).

... localized within the amniotic cavity. This placement allows exogenous neural crest cells direct access to the neural tube would be hindered by the overlying ectoderm formed as a consequence of neural fold fusion. Similar restrictions likely act upon entry of exogenous neural crest cells into W mutant embryos, since they are at the same developmental stage as Balb/c embryos at the time of microinjection. In these chimeras, however, the distinction between entry of cells at the anterior or posterior neuropore is less defined than in Balb/c chimeras, probably because of the greater expansion of donor melanoblasts in W mutants (see below).

The presence of a full complement of endogenous melanoblasts in Balb/c mice can account for the limited pigmentation of these, relative to W mutant, chimeras.
The restriction of C57B1/6J pigment contribution in Balb/c mice can be manifested at two levels. First, experiments carried out by Mayer (Mayer, 1970, 1973) demonstrated that embryonic skin can support only a limited density of melanoblasts; thus, C57B1/6J cells must compete with endogenous melanoblasts for colonization of hair follicles in the epidermis. Second, the clonal expansion of C57B1/6J melanoblasts during migration is likely limited by the population pressure exerted by neighboring Balb/c melanoblasts also spreading through the subepidermal space, accounting for the observed dorsoverentral striping and limited anterior–posterior expansion of pigment (Fig. 2B and Jaenisch, 1985). Similar forces appear to be operative in producing the characteristic dorsoverentral banding patterns seen in allogeneic mice (Mintz, 1967, 1970; McLaren and Bowman, 1969; Cattanach et al. 1972).

In contrast to Balb/c chimeras, pigmentation of the W mutants was not restricted to the head and lower trunk. Assuming that exogenous neural crest cells are unable to penetrate through the epidermal ectoderm into the closed regions of the neural tube in the middle of the embryo, the extensive trunk pigmentation seen in many W mutant chimeras must result from anterior or posterior longitudinal migration of crest cells along, or alongside, the neural tube following entry at one of the neuropores. Longitudinal migration of neural crest cells along the neural tube has been observed in avian embryos, albeit for relatively short distances (Teillet et al. 1987). The apparent rostral and caudal expansion of melanoblasts observed in W mutant chimeras is consistent with the proposal that dispersion of neural crest cells is directed by contact inhibition (Erickson, 1985), which promotes radial movement of cells from areas of high to low cell density (Abercrombie, 1970; Rovasio et al. 1983).

As described previously, the W mutation causes a cell-autonomous defect in the melanoblast population which results in the absence of melanocytes from the skin. This has been attributed to elimination or restricted proliferation of stem melanoblasts (Schaible, 1969), preprogrammed clonal death of melanoblasts prior to migration (Mintz, 1970) or reduced viability of mutant melanoblasts (Gordon, 1977; Mayer, 1979). At issue is whether, or to what extent, melanoblasts in W mutant embryos undergo migration and proliferation. The extensive pigmentation and apparent longitudinal migration of wild-type donor crest cells in our W mutant chimeras suggests that the C57B1/6J cells spread through the dermis as if in an empty space unimpeded by contact with any endogenous melanoblasts. This is most consistent with the interpretation that the W mutation affects migration and/or proliferation of mutant melanoblasts during neural crest dispersal.

Recent studies have identified the c-kit proto-oncogene, a putative tyrosine kinase receptor, as the product of the W locus (Chabot et al. 1988; Geissler et al. 1988), implicating a lesion in a receptor–signal transducing pathway as the molecular defect (Nocka et al. 1989; Reith et al. 1990). We have explanted neural tubes from W embryos in culture in an attempt to assess a possible effect of the mutation on in vitro melanogenesis. While 100% of the cultures derived from WSh/WSh embryos, and virtually all cultures from W11/W11 embryos, developed pigmented melanocyte colonies, as did cultures derived from C57B1/6J control embryos, a significantly lower proportion of cultures established from WSh/Wx and W11/WXx embryos contained pigmented cells. This correlates with the pigmentation seen in mice of the respective genotypes: neither W11/W11 nor WSh/WSh mice are devoid of hair pigmentation; W11/W11 mice are extensively flecked with pigment while WSh/WSh mice show varying degrees of pigmentation of the face and hindmost trunk. In contrast, F1 animals bearing the more severe Wx allele are only lightly pigmented in the skin of the ears (W11/Wx), or are completely white (WSh/Wx). This is consistent with the hypothesis that W mutations can affect proliferation and/or differentiation of pigment cells in vitro. It appears, however, that our culture conditions may partially complement the mutant defect; growth of crest cells on embryonic fibroblasts in minimal medium, as described for W mutant mast cells (Fujita et al. 1988a, b) may serve to uncover more fully the mutant phenotype in vitro.

The mixing and co-injection of C57B1/6J and DBA/2J neural crest cells was carried out in an attempt to obtain information on clonal size and the extent of intermingling of different clones in W mutant chimeras. In the majority of chimeras (13/22), only a single pigment color was seen, while in nine chimeras both donor colors were present in a single animal. In four of the triple-colored animals, the areas of brown and black pigmentation were physically separated by distance or by the anatomical boundaries of the dorsal and ventral midlines. This bias against co-localization of pigment within a common anatomical area can be interpreted as indicating that very few clones contributed to pigmentation. This would imply that individual clones of melanoblasts are capable of extensive expansion, the full capacity of which is not normally realized because of the population pressure exerted by adjacent clones, which restricts expansion to the width of the ‘standard stripe’ (Mintz, 1967) visualized in aggregation chimeras. Alternatively, if the large pigmented patches originated from several clones, our data would reveal that there is a tendency for exogenous cells of like genotype to associate during entry into the embryo, a notion similar to the previous suggestion that neural crest cells of similar genotypes may remain together in aggregation chimeras rather than mix freely (McLaren, 1969). We do not favor this interpretation because in five of the nine triple-colored chimeras produced by co-injection of C57B1/6J and DBA/2J neural crest cells, pigment contribution to a common anatomical area was observed, with extensive intermingling between cells from different clones. We therefore favor the hypothesis that large pigment patches in the chimeras can be composed of descendants of only one or a few neural crest cells with a high proliferative potential. The small patches seen in a number of chimeras may be derived from neural crest cells with a much more limited proliferative
potential at the time of injection, reflecting substantial heterogeneity of neural crest cells in the neural-tube-derived cultures.

We do not know why the brown pigment in the tricolored animals fades with age in contrast to the black pigmentation. It is of interest to note, however, that the black donor cells (C57/B16) were of the same MHC haplotype as the W<sup>Sh</sup>/W<sup>Sh</sup> recipient whereas the brown DBA/2 donor cells were of a different haplotype. While we do not know the mechanism of brown pigment fading, it appears possible that the H-2 incompatible melanocytes were immunologically rejected similar to neuronal graft rejection seen in certain chicken-qual chimeras (Kinutani et al. 1989).

The experimental approach described in this paper combines in vitro culture of neural crest cells with an efficient method for assessment of the developmental potential and migration of the crest cells in the animal. This system provides a means of genetically manipulating crest cell differentiation by introducing genes into cultured cells in vitro and assessing the consequences of gene expression in the animal. For example, this approach could provide a means of directly studying the role of c-kit, the W gene product, in melanoblast development. We have recently established the feasibility of transduction and expression of exogenous genes in neural crest cells in vitro using a retrovirus vector carrying the lacZ marker gene and have further demonstrated expression of the tyrosinase gene in melanocytes in vivo, following retroviral-mediated gene transfer into melanoblasts in situ (manuscript in preparation). These developments, coupled with the reproducibility and efficiency of the neural crest cell microinjection technique, demonstrate the potential of this system for the analysis of the complex biology of neural crest cells at the molecular level.

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Neural crest cells and coat pigmentation

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