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Neural crest progenitors of the melanocyte lineage: coat colour patterns revisited

Alison L. Wilkie, Siobhán A. Jordan and Ian J. Jackson

MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU, UK

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SUMMARY

Neural crest-derived melanoblasts are the progenitors of melanocytes, the pigment cells of the skin, hair and choroid. Previous studies of adult chimaeric mice carrying different coat colour markers have suggested that the total melanocyte population is derived from a small number of melanoblast progenitors, each of which generates a discrete unilateral transverse band of colour. This work also suggested minimal mixing of cells between clones. We have used two complementary approaches to assess the behaviour of migrating clones of melanoblasts directly in the developing embryo. First, we made aggregation chimaeras between transgenic *Dct-lacZ* and non-transgenic embryos, in which *lacZ* is a marker for melanoblasts. Second, we generated transgenic mice carrying a modified *lacZ* reporter construct containing a 289 base pair duplication (*laacZ*) under the control of the *Dct* promoter. The *laacZ* transgene is normally inactive, but reverts to wild-type *lacZ* at low frequency, labelling a cell and all of its progeny at random. Mosaic embryos containing labelled melanoblast clones were generated. In contrast to previous data, chimaeric and mosaic embryonic melanoblast patterns suggest that: (1) there is a large number of melanoblast progenitors; (2) there is a pool of melanoblasts in the cervical region; (3) different cell dispersion mechanisms may operate in the head and trunk regions; and (4) there is extensive axial mixing between clones.

Key words: Melanoblast, Transgenic, *Dct*, Clonal, Mouse

INTRODUCTION

The lineage of individual cells during embryogenesis is one of the fundamental questions in developmental biology. The number of progenitors for any cell population, and the patterns of proliferation and interaction within the population, play an important role in determining the final structure of the organism. Most tissues in mammalian embryos are multiclonal in origin. This may provide a selective advantage: the accidental loss of one progenitor does not affect the development of the tissue as a whole (Mintz, 1971) and developmental plasticity allows modification of developmental mechanisms through evolution.

The developmental biology of mouse melanocytes offers an ideal model for studying cellular interactions in development, partly because of the abundance of coat colour mutants (Jackson, 1994; Jackson, 1997). Indeed, the first lineage analysis of mammalian tissues was carried out on melanocytes almost 40 years ago. Melanoblasts give rise to melanocytes, and they are a subpopulation of neural crest cells that emerge from the dorsal neural tube at around embryonic day 8.5 (E8.5). Melanoblasts migrate along the dorsolateral pathway between the dermatome and the overlying ectoderm, and from E10.5 migrate ventrally through the developing dermis. At E14.5, they begin to invade the overlying epidermis and then migrate into the developing hair follicles, where they continue to proliferate and differentiate, before beginning to synthesise pigment at around postnatal day 4 (Mayer, 1973; Jordan and Jackson, 2000b). Neural crest-derived melanoblasts delaminate from the trunk neural tube at all axial levels, and are clearly derived from multiple progenitors. However, several key questions remain unanswered. How many melanoblast progenitors are there and how are their progeny distributed? Specifically, how much mixing is there between cells from different progenitors, how large a region can one melanoblast progenitor populate and what is the proliferative potential of a single melanoblast progenitor cell?

Genetic chimaeras have been used to address these types of questions in many tissues of the mammalian embryo (Mintz, 1967; McLaren and Bowman, 1969; Mintz, 1971; Lewis, 1973; McLaren, 1976; Rossant, 1987; Rossant and Spence, 1998; West, 1999). However, it is difficult to use chimaeras to estimate the number of progenitor cells in an organ for several reasons. There may be genotype-dependent tissue-specific selection, resulting in cells of one embryonic origin being favoured to contribute to a particular lineage (Rossant, 1987; Rossant and Spence, 1998; West, 1999). The contribution of one progenitor cell may be over-represented in the mature organ because of differences in proliferative potential (Rossant, 1987; Rossant and Spence, 1998; West, 1999). Cell death during organogenesis may also complicate the interpretation. Indeed, the very term ‘progenitor cell’ can cause confusion

(McLaren, 1972; Lewis et al., 1972). Ideally, a progenitor cell may be defined as a cell whose descendants all contribute to the tissue in question, but whose parent cell contributed to more than this tissue. In practice, this can be a difficult cell to identify, especially when performing a retrospective analysis with a cell-specific marker. Normally, these analyses provide information about a cell, perhaps only some of whose descendants contribute to the tissue. In this paper, we have used the term 'progenitor' to refer to a cell that contributes some of its descendants to the melanoblast lineage. Despite these limitations, mosaics and chimaeras are used successfully to study other patterns of tissue growth, including cell mixing, proliferation, and migration within and between clones.

Mintz performed the first lineage analysis using chimaeras by aggregating genetically distinct morulae differing at various coat colour loci to study melanoblast development (Mintz, 1967). Adult coats showed a spectrum of phenotypes, depending on the degree of chimaerism, but generally appeared to be made up of broad transverse bands of colour, with each side of the animal being patterned independently. These phenotypes were interpreted to reflect the migratory history of melanoblasts in the embryo. By assessing the patterns of many chimaeric coats, Mintz proposed that if the bands were arranged alternately by chance, the maximum number that could be generated on either side of the body was 17: three in the head, six in the body and eight in the tail. She therefore concluded that the mouse embryo had 34 melanoblast progenitors in total (Mintz, 1967; Mintz, 1971). Furthermore, she proposed that cells would proliferate predominantly laterally, with only limited expansion longitudinally as a result of body growth (Mintz, 1967), and that cell mixing would be limited to clonal boundaries (Mintz, 1971).

Similar coat colour patterns in chimaeric mice were observed by others (McLaren and Bowman, 1969), but they were interpreted differently. Although a tendency for transverse bands was recognised, these were frequently interrupted and broken by neighbouring bands, suggesting individual melanoblast progenitors did not generate discrete bands and that extensive mixing occurred at the boundaries (McLaren and Bowman, 1969; Wolpert and Gingell, 1970; Lewis, 1973; Nesbitt, 1974; West, 1975; McLaren, 1976). Several investigators subsequently developed simulations or mathematical approaches to estimate the number of progenitor cells, in order to model the relationship between clones and patches, and to estimate the degree of cell mixing in chimaeric and X-inactivation mosaic tissues (Lyon, 1969; Wolpert and Gingell, 1970; Mintz, 1971; Nesbitt and Gartler, 1971; McLaren, 1972; Lewis et al., 1972; Lewis, 1973; West, 1975; Tachi, 1988). Several approaches indicated that there were likely to be many more clonal progenitors than Mintz had reported (Lyon, 1969; Wolpert and Gingell, 1970; West, 1975; McLaren, 1976; Tachi, 1988). In particular, it was noted that transverse bands seen in chimaeric coats represented patches rather than clones (West, 1975). However, despite conflicting interpretations, Mintz's proposal that there are 17 melanoblast progenitors on either side of the embryo was recognised to have become widely accepted (West and McLaren, 1976).

All of the classical analyses of the melanoblast lineage rely on extrapolation from the adult coat pattern back to the embryonic pattern. We have shown previously that there are substantial changes in melanoblast distribution throughout embryonic development (Mackenzie et al., 1997), which may confound such extrapolation. Furthermore, we have shown that, at least in mutant embryos, the melanoblast population can undergo considerable spatial expansion late in embryonic development (Jordan and Jackson, 2000a). Analysis of complex adult coat patterns may not provide a detailed account of early embryonic events. In particular, it is unlikely that the number of progenitor cells can be determined by extrapolation from adult patterns. Melanoblast clones must be observed in embryos directly. Here, we have used two complementary approaches to study the melanoblast lineage in embryos at different stages of development. The *Dct* promoter is expressed in melanoblasts from about E10 (Steel et al., 1992; Mackenzie et al., 1997) and *Dct-lacZ* transgenic embryos reveal melanoblasts after staining with X-gal (Fig. 1).

We have carried out chimaeric analysis using *Dct-lacZ* transgenic embryos by aggregating transgenic and non-transgenic morulae and staining for melanoblasts at later embryonic stages. In addition, we have used a modified *Dct-lacZ* transgene (Bonnerot and Nicolas, 1993; Sanes, 1994; Nicolas et al., 1996; Mathis et al., 1997; Mathis et al., 1999) that allows us to generate mosaic embryos containing a single marked melanoblast clone. This permits us to assay the developmental potential of the descendants of a single melanoblast progenitor, and to visualise the expansion of the clone within a normal unmanipulated embryo.

Our analysis of patterns of melanoblast distribution in chimaeric and mosaic embryos indicate that: (1) the melanoblast population, in general, is derived from a large number of progenitors; (2) a large pool of melanoblasts resides in the cervical region of the embryo; (3) the head and face region is populated by a larger number of progenitors than the trunk, with different cell dispersion mechanisms operating in these two regions; and (4) melanoblasts within a clone show extensive longitudinal migration, suggesting there is considerably more axial mixing than was previously thought.

MATERIALS AND METHODS

Our animal studies were carried out under the guidance issued by the Medical Research Council in 'Responsibility in the use of animals for Medical Research' (July 1993) and licensed by the Home Office under the Animals (Scientific Procedures) Act 1986.

Production of Tg(*Dct-lacZ*) ↔ non-transgenic chimaeric embryos

We have previously described transgenic mice containing the *lacZ* reporter gene driven by the *Dct* promoter: STOCK-Tg(*Dct-lacZ*)1Jkn, hereafter Tg(*Dct-lacZ*) (Mackenzie et al., 1997). Chimaeras were made between transgenic and non-transgenic embryos by aggregating embryos of the following strains and genotype: [(C57BL6×CBA) × (C57BL6×CBA)] ↔ [(C57BL6×CBA) × Tg(*Dct-lacZ*)]. (C57BL6×CBA) F_1 females were superovulated by intraperitoneal injection of 10 units of PMS (Intervet) at noon on the first day followed by 10 units of hCG (Intervet) at 2 p.m. 2 days later. Half of the females were mated to (C57BL6×CBA) F_1 males, and half to Tg(*Dct-lacZ*) males. The morning of vaginal plug detection was counted as embryonic day (E) 0.5. Embryos were harvested at E2.5 by flushing the uteri and oviducts with H6 culture media. The zonae pellucidae of eight-cell embryos were removed by incubation in warm Acid Tyrode's solution for a minimal period. Embryos were

aggregated in phytohaemagglutinin (Gibco BRL) in H6, and aggregates were cultured to the blastocyst stage in T6 culture media overnight. Ten to 14 blastocysts were transferred to E2.5 pseudopregnant recipient CD1 females. Chimaeric embryos were re-harvested from E11.5 to E16.5 and stained with X-gal.

Generation of *Dct-laacZ* transgenic mouse lines

The *Dct-laacZ* construct was generated from a plasmid vector containing the *Dct* promoter and the *lacZ* transgene. To create a 289 bp duplication in the *lacZ* sequence, the *Cla*I-*Xho*I fragment was ligated to the *Xho*I-*Eco*RV fragment, thus duplicating the *Cla*I-*Eco*RV fragment (Bonnerot and Nicolas, 1993). The construct was microinjected at a concentration of 2 ng/μl into (C57BL/6×CBA) F_1 × (C57BL/6×CBA) F_1 fertilised eggs. Fifteen out of 58 founders were identified as transgenic by PCR amplification of the duplicated sequence from tail biopsy genomic DNA. Founders were bred onto a mixed (C57BL/6×CBA) F_1-CD1 background. Breeding animals were genotyped by PCR amplification of *lacZ* sequences from genomic DNA using either of the following pairs of primers: 5′GTGACTACCTACGGGTAACA3′ and 5′ATTCATTGGCACCATGCCGT3′, or 5′GAATTATTTTTGATGGCGTT3′ and 5′CGCTGATTTGTGTAGTCGGTT3′.

To generate embryos for X-gal staining, timed matings were set up between CD1 and Tg(*Dct-laacZ*) transgenic animals in either direction. The genetic background of all embryos presented is a mixture of C57BL/6, CBA and the CD1 outbred stock. Half of all progeny were assumed to be transgenic after confirmation that the transgene was transmitted by normal Mendelian inheritance.

X-gal staining of transgenic and chimaeric embryos

Chimaeric embryos were harvested from E11.5 to E16.5; embryos from *laacZ* reversion matings were harvested from E10.5 to E14.5. Embryos were dissected in PBS and stained with X-gal as described previously (Mackenzie et al., 1997).

Recording data from embryos

Clones of cells in chimaeric embryos showed intense X-gal staining. Therefore, embryos were photographed using a Photometrics ICX205 digital colour CCD camera and a Leica M2F2III stereoscopic microscope. Clones of cells in Tg(*Dct-laacZ*) mosaic embryos stained more weakly because only one copy of the transgene would have undergone reversion; these embryos could not be photographed easily. Clones from these embryos were recorded in one of two ways: camera lucida apparatus was used to draw clones directly or the patterns of cells in a clone were copied onto pre-drawn embryo diagrams of the appropriate stage.

In situ hybridisation

Whole-mount in situ hybridisation was performed on E11.5 Tg(*Dct-lacZ*), Tg(*Dct-laacZ*)195Jkn and non-transgenic embryos using a method described previously (Hammond et al., 1998). The *lacZ* riboprobe was derived from a plasmid vector (kindly provided as a gift from W. Skarnes). The vector was linearised to generate a probe of approximately 770 bp in length, and the probe was transcribed with T3 polymerase and labelled with digoxigenin (Boehringher Mannheim DIG RNA labelling kit), according to the manufacturer's instructions.

RESULTS

Distribution of melanoblasts in *Dct-lacZ* transgenic embryos

We have previously described the appearance of X-gal-stained cells in Tg(*Dct-lacZ*) embryos (Mackenzie et al., 1997) and this is summarised in Fig. 1. In addition to expression in melanoblasts, the transgene is expressed in the retinal pigmented epithelium (RPE) and the telencephalon, where the endogenous *Dct* gene is also expressed. Furthermore, ectopic expression of the transgene is seen in caudal nerves, a phenomenon observed with all *Dct-lacZ* transgenes assayed to date (Zhao and Overbeek, 1999; Hornyak et al., 2001).

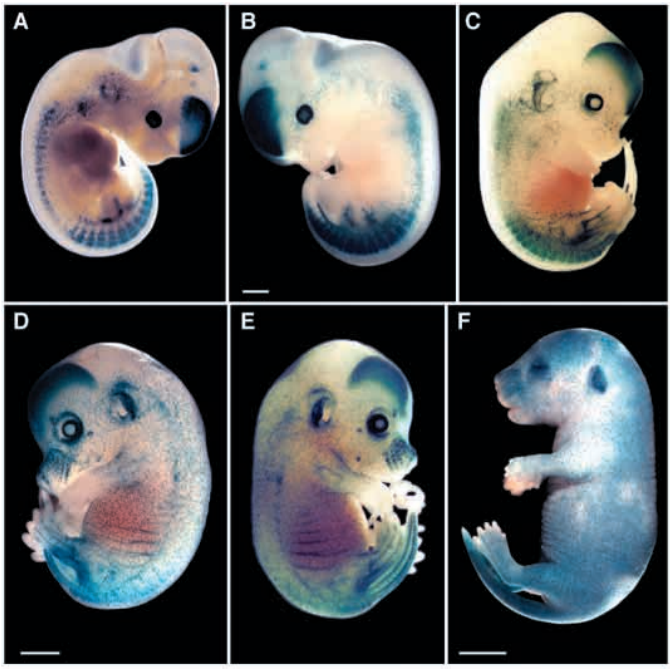

Fig. 1. Labelled melanoblasts in *Dct-lacZ* control embryos of different ages. (A) E11.5. (B) E12.5. (C) E13.5. (D) E14.5. (E) E15.5. (F) E16.5. (A,B) Labelled melanoblasts can be seen at high density flanking the neural tube and in the cervical region. (C) By E13.5, melanoblasts have begun to disperse ventrally, and a significant proportion of the body and face have been populated. (D) By E14.5, melanoblasts are more numerous and almost the entire embryo has been colonised, including the ventrum and crown of the head. (E,F) Melanoblasts continue to increase in number and begin to colonise hair follicles (E15.5-E16.5). Scale bars: 0.8 mm in B; 1.6 mm in D; 3.2 mm in F, respectively.

Expression of the transgene is first seen in the RPE and telencephalon at about E9, and can be observed in melanoblasts at E10.5 (Mackenzie et al., 1997). These early melanoblasts form a cluster in the cervical region very soon after cells begin to be seen leaving the neural crest along the length of the embryo (Fig. 1A). By E12.5, this cervical cluster has apparently dispersed, to give streams of cells leading to the head and face (Fig. 1B). Ventral migration in the trunk is not apparent until E13.5 (Fig. 1C) and at this time there is a lower density of melanoblasts in the trunk region than either rostral or caudal, a phenomenon that has been described previously (Besmer et al., 1993). By E14.5, labelled melanoblasts have populated most of the ventrum of the embryo and are beginning to enter the newly forming hair follicles (Fig. 1D). At E16.5, most of the labelled melanoblasts are found in the follicles (Fig. 1F). The *Dct-lacZ* transgene is a robust marker of melanoblasts, offering a reliable and visual presentation of the spatial arrangement of melanoblasts throughout embryonic development.

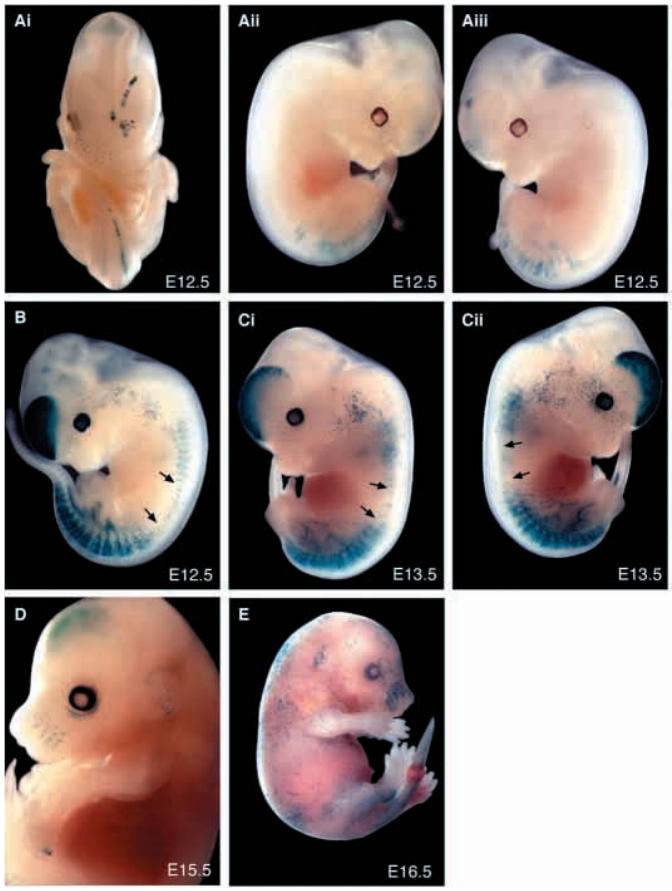

Fig. 2. Examples of *Dct-lacZ* ↔ non-transgenic chimaeric embryos. (Ai) Ventral view of an E12.5 chimaera with labelled melanoblasts in the face and tail. (Aii-Aiii) Right and left side views of the same embryo. (B) E12.5 chimaera showing a dilution of labelled melanoblasts between the limbs (marked by arrows). (Ci-Cii) Left and right sides of an E13.5 chimaera showing distinctive gaps (marked by arrows) lacking labelled melanoblasts between the limbs. (D) Head and face of an E15.5 chimaera showing labelled melanoblasts in the snout, pinna and around the eye. (E) E16.5 chimaera showing discrete patches of labelled melanoblasts.

Distribution of melanoblasts in *Dct-lacZ* ↔ non-transgenic chimaeric embryos

We made aggregation chimaeras between eight-cell Tg(*Dct-lacZ*) embryos and non-transgenic embryos. After transfer to foster mothers, embryos were harvested between E11.5 and E16.5 and stained with X-gal. Chimaeric embryos were analysed by direct comparison with X-gal-stained age-matched Tg(*Dct-lacZ*) embryos. Labelled melanoblasts derive from only one donor embryo, and each chimaera provides lineage information. Those that have a high contribution from the transgenic donor, and have a large proportion of labelled melanoblasts, allow us to identify regions populated by a small number of progenitor cells, as represented by areas that consistently lack labelled melanoblasts. Conversely, we can identify embryonic regions that are populated by a large number of melanoblast progenitors by examining chimaeras with a low contribution from the transgenic donor for areas that consistently contain labelled cells.

A total of 30 chimaeric embryos provided lineage information. Examples of the range of chimaeras we examined are shown in Fig. 2. These chimaeras can be compared with age-matched Tg(*Dct-lacZ*) embryos directly. The left and right sides of chimaeric embryos were patterned independently (e.g. compare Fig. 2Aii with Fig. 2Aiii, and Fig. 2Ci with Fig. 2Cii), consistent with observations made in adult chimaeras. However, in contrast to adult coat-colour chimaeras, embryos that have a high transgenic donor contribution do not show stripes of labelled cells, but rather have an overall dilution of labelled cells when compared with Tg(*Dct-lacZ*) embryos (Fig. 2B,C). The trunk region is almost invariably more diluted, and often lacked detectable transgenic melanoblasts altogether (Fig. 2B,C). Several different mutations of the *Kit* gene produce a similar phenotype. This is evidence that the trunk is populated by fewer melanoblast progenitors than the head and tail region, although we cannot draw conclusions as to the number of such progenitors. At later stages (E15.5-E16.5), some chimaeras begin to have labelled melanoblasts in what appear to be stripes or discrete patches (Fig. 2E), which may be an emergence of the adult pattern. The appearance of stripes corresponds to the second phase of proliferation of melanoblasts that we have previously described (Jordan and Jackson, 2000a) (see below), and may be due to expansion of small clones into stripes or patches.

Almost all chimaeras, even those with a very low transgenic contribution, have a cluster of melanoblasts in the cervical region and the subsequent migratory streams of cells as seen in Tg(*Dct-lacZ*) embryos (Fig. 2Aiii,B,C). This indicates that the head and cervical region is populated by many melanoblast progenitors that mix extensively. Chimaeras almost always had labelled melanoblasts in the head and face, in particular around the eye and pinna, indicating these regions are populated by the daughters of multiple progenitor cells (Fig. 2Aii,D). Studies on adult coat-colour chimaeras have concluded that the head is populated by a small number of melanoblast progenitors. Based on these embryonic analyses, we conclude quite the opposite; the head is populated by a large number of progenitors, relative to the rest of the embryo.

Dct-laacZ mosaic embryos

Information gained by analysing chimaeric embryos is limited because, although the relative contribution of the two donor embryos can be assessed qualitatively, it is not possible to track the descendants of a single progenitor. To overcome this, we have generated transgenic mice in which the progeny of a single cell are marked by expression of β-galactosidase. We modified the *Dct-lacZ* reporter construct by creating a 289 bp duplication in the coding region of the *lacZ* transgene (Bonnerot and Nicolas, 1993). This introduces an in-frame termination codon leading to the translation of a non-functional, truncated β-galactosidase protein. The duplicated sequence is removed at low frequency by homologous recombination, either inter- or intragenically. A reversion event restores the wild-type *lacZ* sequence and a functional protein is produced, marking the founder cell and all of its progeny. Reversion events can occur in any cell and at any time during development (Bonnerot and Nicolas, 1993). It is important to bear this fact in mind in the context of melanoblast development. The melanoblast lineage is considered to be specified relatively late. The neural crest may not be specified until near the time of delamination from the dorsal neural tube

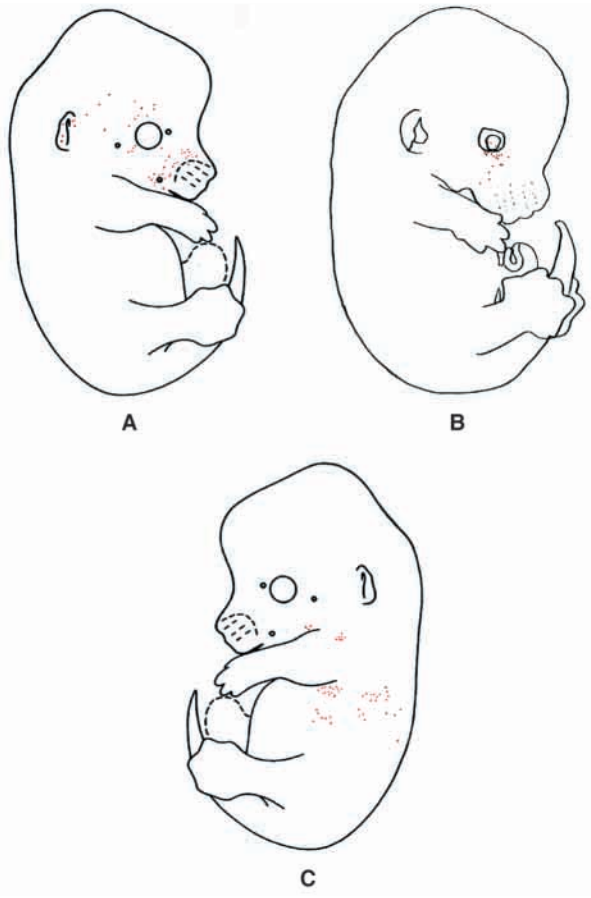

Fig. 3. Examples of melanoblast clones generated in preliminary screening of *Dct-laacZ* transgenic lines. (A) Large E13.5 face clone in line Tg(*Dct-laacZ*)195Jkn. (B) Small E13.5 face clone in line Tg(*Dct-laacZ*)195Jkn. (C) E13.5 clone at the forelimb level in line Tg(*Dct-laacZ*)202Jkn.

at around E9 (Bronner-Fraser and Fraser, 1988; Serbedzija et al., 1994; Ruffins et al., 1998), and neural crest cell sublineages are specified asynchronously thereafter (Henion and Weston, 1997). The *Dct-lacZ* reporter construct used in this study is expressed in melanoblasts from around E10 (Mackenzie et al., 1997).

Fifteen independent transgenic lines were generated. Initially, E13.5 and E14.5 embryos were harvested from each line for analysis by X-gal staining. Lines that failed to produce embryos with labelled clones following analysis of at least 40 embryos were discarded. In order for a cell to be labelled in an embryo, the transgene must integrate into a genomic location at which it can both be expressed and experience recombination. Because reversion events can occur both intra- and intergenically, the copy number as well as the genomic environment is important in determining the frequency of cell labelling; lines with high copy numbers presumably have increased opportunities for reversion. Six transgenic lines generated labelled clones at varying frequencies. Clones were labelled in all lineages that express the transgene: neural crest-derived melanoblasts, melanoblasts of the RPE, and cells in the telencephalon (Mackenzie et al., 1997). Labelled melanoblast clones were only observed in two lines carrying the transgenes, Tg(*Dct-laacZ)*195Jkn and Tg(*Dct-laacZ*)202Jkn, in these initial studies (Fig. 3). Comparison with age-matched Tg(*Dct-lacZ*) embryos (Fig. 1D) suggested unlabelled melanoblasts were intermingled with labelled melanoblasts in mosaic embryos. The line containing Tg(*Dct-laacZ*)195Jkn (hereafter Tg195) produced labelled clones with the highest frequency (albeit less than 3%, see below). This line was therefore selected to generate a large number of melanoblast clones for further analysis.

As different lines carrying the same transgene may show temporally and spatially different expression of the transgene, it was important to establish that Tg195 expressed the *laacZ* reporter in the same pattern as the *lacZ* reporter in control Tg(*Dct-lacZ*) embryos. We carried out whole-mount in situ hybridisation using a *lacZ* riboprobe on embryos from both lines. The *laacZ* reporter in Tg(*Dct-laacZ*)195Jkn embryos is expressed in melanoblasts with the same spatial and temporal pattern as the reporter in control Tg(*Dct-lacZ*) embryos (Fig. 4).

Litters were harvested from line Tg195 between E10.5 and E14.5. Over 3500 embryos were stained with X-gal and screened for the presence of labelled clones. As only half will be transgenic, this corresponds to approximately 1790 transgenic embryos, in which a reversion event can occur. Within these, 50 labelled melanoblast clones were identified (2.79%). This low frequency of labelling indicates that multiple labelling events in a single embryo are unlikely and that labelled melanoblasts represent the clonal descendants of a single revertant cell (the founder cell).

Founder proliferation

The rate of homologous recombination per cell division in cells transfected with the modified *laacZ* construct has been previously reported as approximately 5×10^{-6} (Bonnerot and Nicolas, 1993). The rate of reversion per cell division is not known in Tg195 embryos, but we assume that it remains constant throughout embryogenesis. If cell death is not a significant factor, the frequency of embryos with labelled clones will be expected to increase as later stage embryos are examined. In addition, if proliferation of the melanocyte lineage continues throughout development, then most clones will be small, as later reversions of the *laacZ* gene will occur in cells that will only divide a few times before the embryo is examined.

The occurrence of labelled clones does indeed increase overall during development, but not at a steady rate, which indicates that there are two distinct phases of melanoblast or melanoblast progenitor proliferation. The clone frequency in the melanoblast lineage increased eight fold throughout embryogenesis from 0.008 labelled melanoblast clones per transgenic embryo at E10.5, to 0.06 at E14.5 (see Table 1). Between E10.5 and E11.5, the clone labelling frequency increased fourfold from 0.008 to 0.03, suggesting there is a phase of proliferation in the progenitor population at this time, consistent with the increased number of melanoblasts seen between E10.5 and E11.5 (Mackenzie et al., 1997). During this time, melanoblasts have left the dorsal neural tube and are dispersing onto the dorsolateral pathway between the ectoderm and the dermamyotome. However, over the course of the next few days (E12.5-E14.0), the clone labelling frequency did not significantly increase, suggesting that proliferation has ceased. This is the period during which cells migrate through the

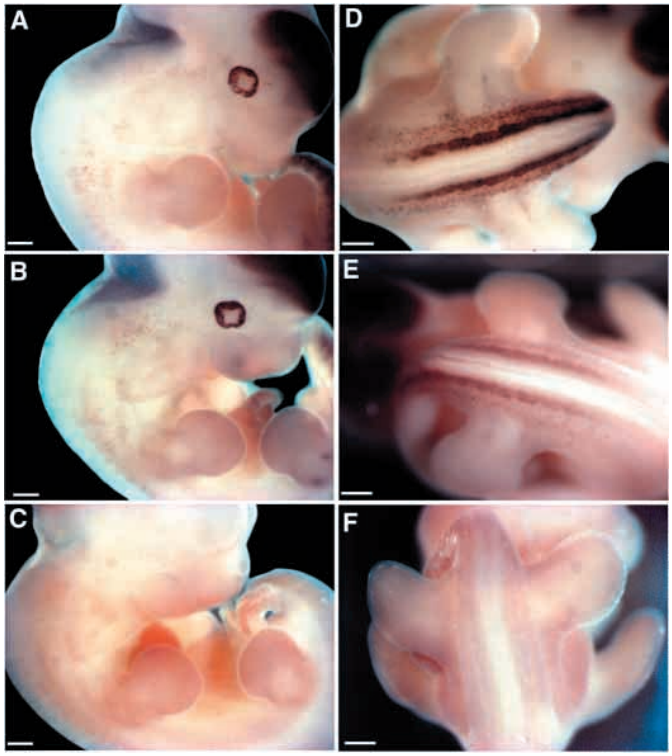

Fig. 4. Transgenes are expressed in equivalent domains in control Tg(*Dct-lacZ*) and Tg(*Dct-laacZ*)195Jkn embryos. Whole-mount in situ hybridisation using a *lacZ* riboprobe. (A,D) E11.5 Tg(*Dct-lacZ*) embryo. (B,E) E11.5 Tg(*Dct-laacZ*)195Jkn embryo. (C,F) E11.5 non-transgenic embryo. Scale bars: 400 μm.

developing dermis towards the ventrum. Subsequently, over just a 12-hour period between E14.0 and E14.5, the clone labelling frequency doubled. At this stage, melanoblasts enter the epidermis prior to colonising the developing hair follicles and they undergo a further phase of proliferation. This latter increase in melanoblast numbers can be visualised directly by comparing X-gal-stained E13.5 and E14.5 Tg(*Dct-lacZ)* embryos (Fig. 1). The impact of this later phase of proliferation can also be seen in the size distribution of clones. The increase in the frequency of embryos with labelled clones between E14.0 and E14.5 is seen principally in an increase of small clones containing fewer than six cells (see Table 1).

Distribution and migratory potential of melanoblast progenitors

In Tg195 *Dct-laacZ/lacZ* mosaic embryos, labelled cells were found in a sufficiently small proportion of transgenic embryos to indicate that each group of labelled cells represented a clone resulting from a single recombination event. The distribution pattern of labelled cells within a single embryo therefore illustrates the proliferative capacity of the founder cell (the cell in which the reversion occurred), and the migratory capacity of its descendants. The distribution of labelled cells among all the embryos reflects the distribution of founder cells within the embryo. Thus, 59% of clones were in the head and face, 27% were in the cervical region and 14% were in the trunk, suggesting a large number of melanoblast progenitors populates the neck, face and head, possibly six times the number that colonises the trunk. Clones were usually, with a few exceptions, confined to either the left or right side of the embryo, supporting the view that melanoblasts migrate independently on the left and right sides of the body and do not normally cross the dorsal midline (Mintz, 1967). A second *Dct-laacZ* transgenic line, Tg(*Dct-laacZ*)202Jkn, shows very similar clone labelling patterns (Fig. 3C).

In the head and face (Fig. 3A,B, Fig. 5A-C) and in the cervical region (Fig. 5D-F) labelled cells were distributed across relatively large areas, covering regions as broad as from the forelimb bud to the eye. Comparison with control Tg(*Dct-lacZ*) embryos (Fig. 1) and with in situ hybridisation in Tg195 embryos (Fig. 4) shows that small numbers of labelled melanoblasts in widespread clones were intermingled with many unlabelled ones, indicating extensive dispersion and mixing of cells. This broad distribution was evident even in young embryos, demonstrating that clonally related cells can become separated early. It is particularly striking in the head that individual clones occupy domains that completely overlap. It is evident that the progenitors in the head give rise to melanoblasts that can populate a wide area of head dermis, and can completely intermingle with melanoblasts deriving from other progenitors. The same seems to be true of melanoblasts in the cervical/forelimb region, although there is little overlap between the two regions.

In the trunk (Fig. 3C, Fig. 5G,H), clones were arranged as broad patches of widely distributed cells, or as transverse bands that appeared to have split into multiple narrower bands. Labelled cells were rarely organised into stripes; in those that were (Fig. 5H), the stripes were broad and cells were widely distributed within the stripe. Again, comparisons with age-matched *Dct-lacZ* embryos suggest there must be extensive longitudinal mixing, with unlabelled melanoblasts being present among labelled clone members. It is formally possible that the clone presented in Fig. 3C resulted from two labelled progenitors, one producing a lower diffuse band, and one producing two small patches at the shoulder. However, this is

Table 1. Summary of clone labelling frequencies in Tg(*Dct-laacZ*)195Jkn and clone sizes at different stages of development

Embryonic day	Number of one- to six-cell clones	Number of seven- to 20-cell clones	Number of >20-cell clones	Total number of clones	Total number of transgenic embryos	Frequency (number of clones/embryo)
10.5	0	1	1	2	240	0.008
11.5	5	1	2	8	264	0.030
12.5	0	4	1	5	273	0.018
13.5	5	2	5	12	411	0.029
14.0	4	0	1	5	297	0.017
14.5	15	0	3	18	302	0.060
Totals				50	1787	0.028

unlikely given this transgenic line [Tg(*Dct-laacZ*)202Jkn] generated labelled melanoblast clones at a frequency of less than 0.01 clones per transgenic embryo. One embryo provides evidence that each axial level in the trunk is populated by multiple melanoblast progenitors. In this embryo (Fig. 5G), three separate patches of melanoblasts were labelled on one side of the embryo, with smaller patches in the same locations on the contralateral side. It appears that in this case, a particularly early reversion event occurred in the ectoderm, giving rise to labelled premigratory neural crest cells at different levels of the neural tube. Mixing must have occurred in the ectoderm, before the left and right sides of the neural crest were separated, so that revertant cells were on both left and right sides of the embryo. The fact that the clones on the left of the embryo are larger than on the right must indicate that, on the right side, the labelled melanoblast progenitors are mixed with unlabelled cells, and hence that at each of these axial levels more than one progenitor contributes to the population of melanoblasts.

DISCUSSION

We have used two different approaches to investigate the distribution of melanoblast clones in the mouse embryo. *Dct-lacZ* ↔ non-transgenic chimaeric embryos and *Dct-laacZ/lacZ* mosaic embryos have shown that the melanoblast population is derived from a large number of neural crest progenitors. Analysis of both chimaeric and mosaic embryos suggests there is a high degree of cell mixing between clones, with cells migrating extensively in an axial direction, in addition to their established lateral migration, such that cells from neighbouring clones normally overlap, and descendants of a single progenitor can be distributed over a large area. A large proportion of progenitors populates the head and face region of the embryo, while melanoblasts in the region between the limbs are derived from fewer progenitors.

Melanoblast progenitors in the head and trunk

In the mouse, unlike in avian embryos (Baker et al., 1997), melanoblasts of the head have not been shown to be derived from the cranial neural crest. Rather, we suggest that the diamond-shaped group of melanoblasts seen in Tg(*Dct-lacZ*) embryos [(Mackenzie et al., 1997) and Fig. 1] and which is present in the cervical region of almost all chimaeras (Fig. 2) is the source of melanocyte progenitors for the head and face. Furthermore, we suggest that the head may be colonised by migratory streams of melanoblasts that are associated with the diamond-shaped region (Mackenzie et al., 1997) (Fig. 2Aiii,D,E). Support for this notion comes from the observation that in the trunk, *lacZ*-expressing cells are first seen near the dorsum and subsequently appear more ventrally, whereas in the head, labelled cells are seen first in the mid-cervical region, followed by the linear streams extending dorsorostrally.

We propose that this source of melanoblasts is derived from a large progenitor pool because chimaeras with low transgenic contributions almost always show labelling in the head and face, and there is a high incidence of labelled clones in the head and face region of mosaic *lacZ/laacZ* embryos. The trunk may be populated by only a quarter of the number of progenitors that populates the head, as evidenced by the severe reduction in the number of labelled melanoblast clones in mosaic embryos in the trunk compared with the head.

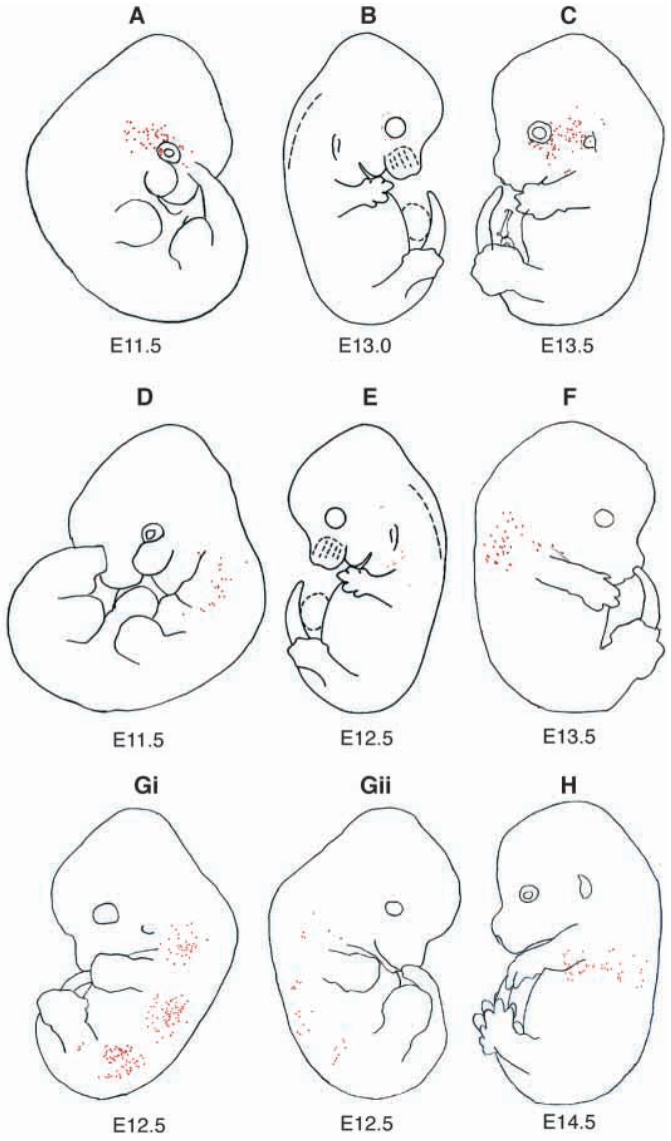

Fig. 5. Examples of melanoblast clones in Tg(*Dct-laacZ*)195Jkn mosaic embryos. (A-C) Face clones. (A) E11.5. (B) E13.0. (C) E13.5. (D-F) Clones in the cervical region. (D) E11.5. (E) E12.5. (F) E13.5. (G,H) Trunk clones. (Gi,Gii) E12.5 (left and right hand sides of the same embryo). (H) E14.5. See text for details and discussion.

It is not clear why different areas should be populated unevenly, although several possibilities have been proposed (Besmer et al., 1993). First, melanoblast progenitors may be produced in different numbers along the body axis, with fewer progenitors leaving the neural crest between the limbs than elsewhere. Second, growth rate variations along the axis may account for differences in melanoblast density. Finally, differences in the migratory environment could affect relative melanoblast numbers in different regions. For example, the trunk dermis, which is derived from the somitic dermamyotome, may offer a different migratory environment

compared with the head dermis, which is derived from the cranial neural crest (Besmer et al., 1993).

It has not been possible to determine exactly the number of progenitor cells there are in the melanoblast lineage, although Mintz's predictions of three progenitors in the head, six in the body and eight in the tail is highly unlikely. Indeed, the extensive mixing of melanoblasts in all regions revealed by chimaeric and mosaic embryos suggests that adult chimaeric coat colour patterns are difficult to interpret. How can we account for the differences between our model and those that propose a much smaller number of progenitors? The difference may lie in the extent of axial migration and mixing that are assumed, and the assessment of the contribution of pigmented cells to a band or patch of coat colour. We demonstrate that melanoblasts from a single progenitor can populate a wide axial domain, although extensively mingled with other melanoblasts. If the contribution of pigmented cells within the mixed population within a band of colour were overestimated, then the number of progenitors will be underestimated. Likewise in the head and neck, if all melanoblasts entering these regions do so via the same pathways, then only a limited number of coat colour chimaeric patterns will be observed, even though a large number of progenitors contribute.

Melanoblast migration and proliferation

Observation of labelled cells in Tg(*Dct-lacZ)* embryos and assessment of the frequency of reversion in Tg195 indicate that melanoblasts undergo an initial period of proliferation followed by a non-proliferative, migratory phase and a second proliferation phase around E14.5. Young *lacZ/laacZ* mosaic embryos often have widely dispersed single cells (Fig. 5Bi,ii), which have undergone migration but no proliferation. Some older embryos have widely dispersed discrete clusters of labelled cells (Fig. 3C), which are a result of subsequent proliferation of these widely dispersed cells.

Although previous investigators re-interpreted Mintz's data and suggested there was extensive cell mixing at clonal boundaries (McLaren and Bowman, 1969; Wolpert and Gingell, 1970; Nesbitt and Gartler, 1971; Lewis, 1973; Nesbitt, 1974; West, 1975; McLaren, 1976), we have found that this mixing is far more extensive than can be visualised from chimaeric coat colour patterns, such that domains populated by single progenitor cells can completely overlap. In the head it appears that all melanoblasts derive from a spatially restricted region and spread over the surface, completely mixing with descendants of different progenitors. In the trunk there is clearly mixing over substantial distances in an axial direction. Is there a mechanism that enables cell mixing until a certain melanoblast density is reached? Studies in which cultured melanoblasts are injected into embryos in utero have suggested migrating melanoblasts respond to melanoblast density in the local environment. Donor melanoblasts can contribute to host pigmentation (Huszar et al., 1991) but show limited contribution in wild-type hosts and migrate predominantly dorsolaterally. However, if the host lacks viable melanoblasts, as in *Kit* mutant embryos, donor cells undergo extensive clonal expansion and longitudinal migration occurs in addition to dorsolateral migration, such that donor cells can pigment more than half of the adult coat. If two populations of differently pigmented melanoblasts are introduced into *Kit* mutant host embryos, the resulting dual-coloured mice exhibit bands with either extensive mixing at the boundaries, or regions of fine intermingling of the two colours within a pigmented patch (Huszar et al., 1991). This fine intermingling suggests that neighbouring bands overlap almost completely. These data also suggest melanoblasts mix extensively between clones and they are consistent with the chimaera and mosaic data presented here.

We have also previously shown a similar phenomenon in mice with chromosomal rearrangements that affect *Kit* expression. Mice carrying the *rump white* or the *patch* mutations have abnormal expression of Kit in the dermamyotome, which is thought to sequester Kit-ligand. *rump-white* adults have unpigmented areas that are devoid of melanoblasts in the rump, while *patch* coats show unpigmented areas of variable shape and size in the abdomen, sometimes with discrete pigmented spots in the middle of an unpigmented area. However, when embryos with either mutation are examined using the Tg(*Dct-lacZ*) reporter, the region lacking melanoblasts is much larger, indicating that the regions devoid of melanoblasts are partially repopulated later in development (Jordan and Jackson, 2000a). This occurs either by migration and proliferation of melanoblasts from regions of high to low cell density, or by rapid clonal expansion of surviving melanoblasts in the middle of regions largely devoid of melanoblasts. In either case there seems to be a mechanism whereby melanoblasts detect unpopulated areas and migrate and/or proliferate to fill them (Wehrle-Haller et al., 1996; Jordan and Jackson, 2000a). Indeed, the availability of Kit ligand, both on the dorsolateral migration pathway and later in the dermal mesenchyme, plays a crucial role in permitting melanoblast survival and promoting proliferation, directing melanoblast migration in the early stages of melanoblast dispersal and promoting melanoblast motility as they colonise hair follicles later (Steel et al., 1992; Mackenzie et al., 1997; Jordan and Jackson, 2000b; Wehrle-Haller et al., 2001).

Our conclusions, based on analysis of mosaic and chimaeric mouse embryos, are fundamentally different from the generally accepted view of melanoblast lineage development. We find that the head is populated by a substantial number of melanocyte progenitors, which mix extensively in a distinctive area of the cervical region. Fewer progenitors give rise to the melanocytes of the trunk, but these cells can show considerable axial migration, such that descendants of a single neural crest progenitor can populate a region extending from the forelimbs to the mid-trunk. Furthermore, we have presented evidence that more than one neural crest cell can give rise to melanoblast progenitors at any one axial level of the trunk. Finally, we have described three distinct phases in trunk melanoblast development: a primary proliferative phase, followed by a migratory phase with little proliferation, leading to a second phase of proliferation of the dispersed cells. Earlier conclusions based on observation of adult chimaera coat colour patterns appear to have greatly underestimated both the number of melanoblast progenitors and the degree of cell mixing that occurs.

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