Cranial paraxial mesoderm and neural crest cells of the mouse embryo: co-distribution in the craniofacial mesenchyme but distinct segregation in branchial arches

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SUMMARY

The spatial distribution of the cranial paraxial mesoderm and neural crest cells during craniofacial morphogenesis of the mouse embryo was studied by micromanipulative cell grafting and cell labelling. Results of this study show that the paraxial mesoderm and neural crest cells arising at the same segmental position share common destinations. Mesodermal cells from somitomeres I, III, IV and VI were distributed to the same craniofacial tissues as neural crest cells of the forebrain, the caudal midbrain, and the rostral, middle and caudal hindbrains found respectively next to these mesodermal segments. This finding suggests that a basic meristic pattern is established globally in the neural plate ectoderm and paraxial mesoderm during early mouse development. Cells from these two sources mixed extensively in the peri-ocular, facial, perioral and cervical mesenchyme. However, within the branchial arches a distinct segregation of these two cell populations was discovered. Neural crest cells colonised the periphery of the branchial arches and enveloped the somitomere-derived core tissues on the rostral, lateral and caudal sides of the arch. Such segregation of cell populations in the first three branchial arches is apparent at least until the 10.5-day hindlimb bud stage and could be important for the patterning of the skeletal and myogenic derivatives of the arches.

Key words: craniofacial development, neural crest cells, somitomere, paraxial mesoderm, branchial arch, mouse embryo

INTRODUCTION

Craniofacial development is intrinsically related to segmentation along the primary body axis (Hunt and Krumlauf, 1991; Hunt et al., 1991a; Thorogood, 1993). In mammalian embryos, segmentally arranged cranial structures such as the branchial arches and nerve ganglia are built upon this primary metamericism (Keynes and Stern, 1984; Lumsden and Keynes, 1989; Puelles and Rubenstein, 1993). Like most elements that constitute the head and face, the branchial arches are populated by both the paraxial mesoderm and the neural crest cells (Couly et al., 1993). The paraxial mesoderm forms the craniofacial muscles, some skeletal elements and vascular tissues, while cranial neural crest cells form elements of the peripheral nervous system, connective tissues and the cartilage (Le Douarin, 1982; Noden, 1988; Kimmel et al, 1991).

The cranial paraxial mesoderm is organised as a meristic pattern of loosely packed cell clusters called somitomeres (Meier, 1979; Meier and Tam, 1982; Tam and Meier, 1982; Jacobson, 1993). There is an orderly cranial to caudal distribution of the somitomeric mesoderm in the mouse, such that each branchial arch is derived from a set of two consecutive somitomeres (Tam and Trainor, 1994; Trainor et al., 1994). This dual contribution correlates with the ‘two segment periodicity’ that describes the derivation of branchial arch motor neurons from consecutive pairs of rhombomeres. The origin of neural crest cells in the branchial arches and nerve ganglia is related dynamically to the segmental organisation of the hindbrain (Wilkinson et al., 1989; Krumlauf, 1993). Hindbrain segmentation is a direct consequence of lineage restriction and differential activity of a series of regulatory genes (Lumsden, 1990; Fraser et al., 1990; Thorogood, 1993). A number of homeobox genes such as the Hoxb group of genes display precise rostral limits of expression that correspond with the boundaries of rhombomeres (Wilkinson et al., 1989). Neural crest cells which originate from alternate segments of the hindbrain (rhombomeres 2, 4 and 6) have been shown to express a set of Hoxb genes characteristic of their rhombomeric origins (Hunt et al., 1991a; Wilkinson et al., 1989). The crest cells migrate in discrete streams colonising the first three branchial arches in a pattern consistent with their craniocaudal orders in the hindbrain (Hunt et al., 1991b;c; Lumsden et al., 1991; Serbedzija et al., 1992). Unlike the rhombomeres, lineage restriction and molecular heterogeneity of cells in different somitomeres have not been identified. What is clear however, is that the segmental organisation of the cranial paraxial mesoderm and the hindbrain has profound effects on the differentiation and patterning of the craniofacial tissues (Noden, 1983b; Couly et al., 1992; Trainor et al., 1994).

Comparisons of the fate maps of the somitomeres and the
neural crest cells, reveal similarity in the movement and destination of the somitomeric mesoderm and the neural crest cells (Serbedzija et al., 1992; Schilling and Kimmel, 1994; Trainor et al., 1994). However, this has yet to be demonstrated by tracking simultaneously the movement of these two cell populations in the same embryo. If a co-distribution of the somitomeric mesoderm and neural crest cells from the same segmental level occurs, this may imply that firstly, the progenitor cells of individual body segments are laid down in register when the rostral-caudal axis is specified and secondly, this early positional register has a direct impact on the migratory pattern and the acquisition of developmental fate of the mesodermal and neural crest cells.

Interactions between mesoderm cells and neural crest cells are not fully understood, nor is it known how heterologous populations such as mesodermal myogenic primordia, neural crest cells and placodal neuroblasts interact during the formation of the functional nerve-muscle-skeleton relationships (Noden 1988; Wahl et al., 1994). In the present study we have analysed concurrently the spatial distribution of the somitomeric mesoderm and the cranial neural crest cells to craniofacial structures in the mouse embryo. Specifically we aimed to trace the fate of somitomeric mesoderm and neural crest cells to (i) the optic primordium and peri-ocular mesenchyme, (ii) the first three branchial arches and (iii) the otic vesicle and peri-otic mesenchyme. We wanted to determine whether progenitors destined for the same craniofacial elements are initially localised as neighbours in the rostral-caudal axis and whether there is evidence of a distinct segregation of these two cell populations in the branchial arches as previously predicted by Noden (1984).

MATERIALS AND METHODS

Recovery and in vitro culture of host embryos

8.5-day (1-5 somites) embryos were obtained from pregnant female mice of AR/C strain. Following dissection of the conceptuses from the uterus, the parietal yolk sac was removed leaving the embryo proper with an intact visceral yolk sac, amnion and ectoplacental cone. Previous studies on the migration of cranial neural crest cells in the mouse embryo have established that the first population of neural crest cells to commence migration leave the caudal mesencephalic neuroepithelium at the 5-6 somite stage during embryogenesis (Jacobson and Tam, 1982; Chan and Tam, 1988; Nichols, 1981). These neural crest cells are destined for the most distal region of the first arch (Nichols, 1986; Serbedzija et al., 1992). Therefore only donor embryos having less than 5 pairs of somites were used in these experiments to ensure that (i) DiI-labelled mesoderm and grafted paraxial mesoderm were pure mesoderm cell populations that did not contain any migrating neural crest cells and (ii) that labelling the neuroepithelium with WGA-gold or DiI will mark the earliest population of emigrating neural crest cells. After cell grafting or labelling, embryos were cultured for 48 hours under in vitro conditions as described by Sturm and Tam (1993).

Isolating donor transgenic tissue and grafting

8.5-day embryos were obtained from matings of transgenic H253 mice which express a bacterial lacZ transgene under the control of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase promoter (Tam and Tan, 1992). Neuromeric junctions were used as landmarks for isolating wedge-shaped fragments of mesoderm and its associated neuroectoderm and surface ectoderm (Meier and Tam, 1982; Trainor et al., 1994). The embryonic fragments were then incubated in a solution of 0.5% trypsin, 0.25% pancreatic, 0.2% glucose and 0.1% polyvinylpyrrolidone in calcium-magnesium free PBS for 20 minutes at 37°C to loosen the germ layers. The mesoderm was separated from the overlying ectoderm with finely polished alloy and glass needles. The mesoderm was then dissected into fragments, each containing approximately 20 cells, which were grafted orthotopically (back to an equivalent site) in the cranial mesoderm of isochronic host embryos (Trainor et al., 1994).

Preparation and application of the wheat germ agglutinin (WGA)-gold marker

The procedure for the preparation of WGA-gold conjugate followed that described by Tam and Beddington (1987). The lectin (Sigma) was crosslinked to bovine serum albumin (BSA, Miles) in the presence of 0.25% glutaraldehyde. The WGA-BSA complex was then conjugated to the colloidal gold particles (Polysciences, 0.005% of 10-15 nm particles in citrate buffer, pH 5.0) by reacting with polyethylene glycol (Mr 200, Sigma). The conjugate was spun down at 40000 g for 30 minutes at 4°C. The pellet was washed twice with the original citrate buffer. A deep red concentrated WGA-gold preparation was obtained by resuspending the pellet in 100 μl of the original buffer. Approximately 5 μl of the gold label was dispensed by microinjection into the neural crest of the open neural plate.

Preparation and injection of fluorescent dyes for cell labelling

DiI (1,1-dioctadecyl-3,3',3'-tetra-methylindocarbocyanine perchlorate) and DiO (3,3'-dioctadecyloxa-carbocyanine perchlorate, Molecular Probes Inc) were made up as 3 mg/ml solutions in dimethylformamide. Micropipettes were filled with dye solution and the injection was performed in a similar manner to that for WGA-gold labelling. As a rule, DiI was used to label the neuroectoderm and DiO was used for labelling the somitomeric mesoderm (Fig. 3). Previous cell labelling studies using DiI or DiO dissolved in dimethylformamide (Guthrie and Lumsden, 1992; Osumi-Yamashita et al., 1994) reported no indiscriminate transfer of dye between cells nor any adverse affects on the viability of labelled cells.

In order to confirm these observations we tested the viability of cells labelled with DiI and DiO dissolved in dimethylformamide in vivo. The rostral hindbrain neuroepithelium in 8.5-day embryos was labelled with DiI. The adjacent mesoderm in the same embryo was labelled with DiO and these embryos were then cultured for 48 hours in vitro as described by Sturm and Tam (1993). The dye-labelled embryos were either fixed in 4% paraformaldehyde and processed for wax histology or they were washed in sucrose and OCT (Tissue-Tek, Miles), and processed as frozen sections. Wax and frozen sections were examined for the presence of any necrotic, dye-labelled cells. We also tested for the indiscriminate transfer of dye between cells in vitro. The central region of somitomere III was labelled on the left side in 8.5-day embryos with DiI and on the right side in the same embryos with DiO. The labelled mesoderm was then dissected from both sides of the embryo with alloy needles. The DiI and DiO-labelled mesoderm explants were washed with PBI solution and then plated down adjacent to each other on poly-L-lysine coated coverslips in DMEM + 20% foetal calf serum and cultured overnight. The resulting outgrowth was examined confocally for the presence of any double-labelled cells (DiI and DiO) as an indication of label transfer between cells. We also analysed the cultures morphologically for labelled cellular debris as an indication of cell death caused by dimethylformamide.

Mapping the prospective brain segments

The marking of neural crest cells has to be done in early-somite-stage embryos before the final neuromeric pattern becomes apparent in the neural plate. To ensure that the correct neural tube segments were studied for neural crest cell fate, labelling experiments were performed...
to map the boundary of the prospective brain parts in 8.5-day, 5-somite stage embryos. At this stage, conspicuous constrictions in the neural plate have already partitioned the cephalic neural tube into recognisable segments (Jacobson and Tam, 1982). Four sites were studied (Fig. 1C). At each site, a small area of the neural plate close to the midline (the prospective basal plate) was labelled by injections of DiI. Site ‘i’ is at the flexure between the first two segments. Site ‘ii’ is at the constriction between the rostral two-thirds and caudal third of the broad second segment previously identified as the ‘midbrain’ (Jacobson and Tam, 1982). This site also marks the most posterior border of Wnt1-\textit{lacZ} expression (Echelard et al, 1994). Site ‘iii’ is at the pre-otic sulcus and site ‘iv’ is at the otic sulcus. Nearly all the embryos in this study were labelled simultaneously at two sites for a better delineation of the relative displacement of different brain parts during development (e.g. Fig. 1D). After labelling, the embryos were cultured for 48 hours and were then fixed in 4% paraformaldehyde for 30 minutes. The head region of the embryo was then bisected using polished metal needles and was examined by confocal microscopy (see later). The position of the labelled cells was determined with reference to the neuromeric landmarks in the brain (Fig. 1E).

Fig. 1. (A,B) Co-culture of somitomeric mesoderm labelled separately with DiI and DiO. After 24 hours in vitro, labelled mesodermal cells attached and spread on the poly-L-lysine-coated substrate (A, bright-field image). The two labelled populations confronted each other but they remained labelled with only one dye (B, dark-field confocal fluorescent image), suggesting that there was no discriminate transfer of the lipophilic markers among cells. (C-E) Mapping of the prospective segmental borders. (C) A scanning electron micrograph of the medial aspect of the neural plate of a 5-somite-stage embryo. Four different sites (\*) in the ventral part of the neural plate (at axial levels i-iv) were labelled by DiI injection. (D) A confocal image (viewed from the luminal side of the neural tube) of labelled cells in the rhombomeres of an embryo 48 hours after labelling. It shows an example of labelled neuroectodermal cells (arrows) in the ventral region of rhombomeres 2/3 and 5/6 resulted from labelling at sites iii and iv respectively. Details of other results are given in Table 1. Histological examination of similarly labelled specimens revealed no evidence of cell necrosis or dye debris in the labelled tissues. (E) A scanning electron micrograph of the cephalic neural tube of a 19-somite fore-limb bud stage embryo. The neuromeric positions where the labelled neuroectodermal cells were found in the neural tube of the fore-limb bud stage embryo after labelling at sites i to iv (Fig. 1C) are marked (\*). The electron micrographs were taken for a study on mouse neurulation (Tam and Jacobson, unpublished data). Abbreviations for landmarks along the rostral-caudal axis: op, optic vesicle; fb, forebrain; i, forebrain-midbrain junction; mb, midbrain; ii, midbrain-limbbrain junction; r, rhombomeres (numbered); iii, pre-otic sulcus; iv, otic sulcus; md, mandibular process of the first arch; ha, hyoid arch; ht, heart; p1, first pharyngeal pouch; ot, otic vesicle. Bar, 10 \textmu m. (for A,B), and 500 \textmu m. (for C-E).
Strategy for transplantation and labelling experiments

In all the transplantation and labelling experiments described below, the neuroectoderm was marked by injection with DiI or WGA-gold conjugate. In labelling the forebrain, caudal midbrain, rostral, middle and caudal hindbrain neuroectoderm, injections were made into the central regions of the segment away from the sulci or segmental junctions. The labelled neuroectoderm then served as a reference landmark for labelling the mesoderm with DiO or for grafting $\text{lacZ}$-expressing mesodermal cells isolated from specific somitomeres respectively (Fig. 3). In some embryos, a broader crest region was deliberately labelled in order to illustrate the overall distribution of the hindbrain neural crest (e.g. Figs 4, 8 and 10).

1. Optic vesicle and the peri-ocular mesenchyme

The neuroectoderm anterior to site i (forebrain, Fig. 1C) and the mesoderm in the centre of somitomere I which underlies the forebrain (Fig. 2) were simultaneously studied, by labelling or grafting, for their contributions to the optic primordium and the peri-ocular mesenchyme.

Table 1. The localisation of Dil-labelled neuroectodermal cells in the neural tube of the embryo after labelling at the early-somite-stage followed by 48 hours of in vitro development

<table>
<thead>
<tr>
<th>Sites labelled*</th>
<th>No. analysed†</th>
<th>Distribution of labelled cells in:‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fb</td>
<td>Mb</td>
</tr>
<tr>
<td>Site i (n=12)</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Site ii (n=16)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Site iii (n=17)</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Site iv (n=13)</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*See Fig. 1A for the locations of the labelled sites.
†No. of embryos showing segmental distribution of Dil labelled cells.
‡Abbreviations: + = labelled cells present; − = no contribution by labelled cells; Fb, forebrain; Mb, midbrain; r, rhomboomeres numbered in the rostral-caudal order.

Table 2. The distribution of cranial neural crest cells and somitomeric mesoderm to craniofacial tissues during early organogenesis

<table>
<thead>
<tr>
<th>Sites studied*</th>
<th>No. of embryos analysed†</th>
<th>Tissues colonised:‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dil/DiO</td>
<td>WGA/lacZ</td>
</tr>
<tr>
<td>Forebrain</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>Somitomere I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caudal midbrain</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>Somitomere III</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Rostral hindbrain</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>Somitomere III</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>Caudal hindbrain</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*See Fig. 2 for the location of sites for labelling or cell transplantation.
†For dye labelling experiment, the neural crest of specific brain parts was always labelled by DiI and the somitomeres by DiO. For the WGA/lacZ experiments, neural crest was marked by injection of WGA-gold conjugate and lacZ-expressing cells were transplanted to the somitomeres.
‡Abbreviations: + = labelled cells or transgenic cells present; − = no contribution by labelled cells or transplanted cells; Ba2, second branchial arch; Ba3, third branchial arch; Cm, cervical mesenchyme; Fm, facial mesenchyme; Fnm, frontonasal mesenchyme; Md, mandibular process of the first arch; Mx, maxillary process of the first arch; nGV, trigeminal ganglion; nGVIII, facioacoustic ganglion; nGVIX, glossopharyngeal ganglion; Ot, otic vesicle; Op, optic vesicle; Opm, peri-ocular mesenchyme; Ot(m(r)), rostral peri-otic mesenchyme; Ot(m(c)), caudal peri-otic mesenchyme.
2. Mandibular arch and facial mesenchyme
The neuroectoderm anterior to site ii (caudal midbrain) and the neuroectoderm between sites ii and iii (rostral hindbrain, Fig. 1C) were tested, together with ventro-lateral mesoderm of somitomere III (Fig. 2), for contributions to the mandibular arch and facial mesenchyme. The lateral paraxial mesoderm has essentially a myogenic fate (Couly et al., 1992) and our previous study (Trainor et al., 1994) has shown the ventral somitomeric mesoderm contributes to the branchial arches.

3. Hyoid arch and cervical mesenchyme
The neuroectoderm between sites iii and iv (middle hindbrain, Fig. 1C) was examined concurrently with the ventral mesoderm of somitomere IV, which underlies the middle hindbrain (Fig. 2), for their respective contributions to the hyoid arch and cervical mesenchyme. The lateral paraxial mesoderm has essentially a myogenic fate (Couly et al., 1992) and our previous study (Trainor et al., 1994) has shown the ventral somitomeric mesoderm contributes to the branchial arches.

4. Otic vesicle and peri-otic mesenchyme
The neuroectoderm between sites iii and iv (the pre-otic sulcus and the otic sulcus) was examined in conjunction with the dorsal region of somitomere IV for a contribution to the rostral peri-ocular mesenchyme. The neuroectoderm that lies posterior to site iv (otic sulcus; Fig. 1C) gives rise to the caudal hindbrain. Neural crest of this brain region was examined in conjunction with dorsal half of somitomere VI (Fig. 2) for a contribution to the caudal peri-ocular mesenchyme. Somitomeres V, VI and VII are of approximately equal size and underlie the caudal hindbrain (Meier and Tam, 1982; Trainor et al., 1994). In order to ensure that somitomere VI and not somitomeres V and VII were being tracked, the central dorsal region of the paraxial mesoderm equidistant from the rostral and caudal boundaries of the caudal hindbrain was labelled or grafted.

Analysis of embryos in cell transplantation and WGA-gold labelling experiments
Host embryos were harvested after 48 hours of in vitro culture and grossly abnormal embryos were discarded. Embryos were fixed in 4% paraformaldehyde for 15 minutes and stained by X-gal histochemistry to reveal the β-galactosidase activity (Tam and Tan, 1992). Embryos containing blue X-gal stained cells were processed for wax histology. Serial transverse and sagittal sections (8-10 μm) were mounted on gelatin-coated slides. Neuroectoderm-derived cells marked by the WGA-gold conjugate, were then visualised by silver impregnation. De-waxed X-gal stained sections were incubated in a mixture of 0.11% silver lactate and 0.85% hydroquinone in pH 3.9 citrate buffer (Tam and Beddington, 1987). The sections were treated with 5% aqueous sodium thiosulphate for 5 minutes to fix the silver grains and were then counter-stained with nuclear fast red.

Analysis of dye labelling experiments
Embryos cultured in vitro for 48 hours were fixed in 4% paraformaldehyde and viewed as whole mounts with epifluorescence under a Diaplan Confocal Laser Scanning Microscope (Leica). The specimens were examined by taking optical sections of the craniofacial regions at different depths, from the lateral to medial regions of the embryo. The DiI and DiO signals were detected with rhodamine and FITC filters respectively and the general morphology of the specimen was studied under bright-field illumination. Computer images of the fluorescent signals were generated and assembled using CMUE 101 Confocal Imaging Analysis software package (Wild Leitz). Photographs of the computer-enhanced images were taken.

Fig. 3. An 8.5-day, 5-somite embryo showing DiI-labelled neural crest region in the rostral hindbrain (arrowhead) and DiO-labelled cells in the 3rd somitomere (sm III, arrow). Bar, 100 μm.

Fig. 4. Distribution of cells derived from somitomere III to the maxillary prominence and the mandibular process (md) of the first branchial arch of embryos that had been cultured for 48 hours. (A) A composite picture of the bright-field and the confocal image of labelled cells localised in the core region of the mandibular process but spread wider in the maxillary prominence (same specimen as in Fig. 10). (B) The restricted distribution of lacZ-expressing cells in both the maxillary prominence and mandibular process of the first branchial arch. fb, forebrain; ha, hyoid arch; ht, heart; md, mandibular process; mx, maxillary prominence; op, optic vesicle; ot, otic vesicle. Bar, 500 μm.
Dimethylformamide does not appear to affect cell viability nor cause indiscriminate dye transfer between cells in vivo and in vitro

Morphological analysis of both wax and frozen sections of 16 embryos injected with DiI and DiO did not reveal any significant cell death in the neural crest mesoderm or neural tube that may be attributable to dimethylformamide. In 25% (4/16) of cases there appeared to be some localised damage to cells at the injection site. This occurred primarily when an excessive amount of dye was injected into a discrete region. This is perhaps not unexpected given the known toxicity of dimethylformamide to cells. A similar phenomenon has been demonstrated with DiI dissolved in 100% ethanol (Serbedzija et al., 1989) causing localised damage to the neuroepithelium but having no adverse affects on neural crest cell migration or viability. To minimise the potential damage at the injection site the quantity of dye injected has been kept to a maximum of approximately 5 nl.

Analysis of the 12 explant pairs grown overnight demonstrated that there was no indiscriminate transfer of label (DiI or DiO) between cells. The individually labelled DiI and DiO explants plated adjacent to each other, grew extensively and fused, producing regions of intermixed DiI and DiO-labelled cells (Fig. 1A,B). Examination of 103 pairs of cells in which a DiI- and a DiO-labelled cell lay next to each other revealed only one cell (<1%) that appeared to be double labelled. There also appeared to be no effect on the outgrowth of explants as a result of labelling with either carbocyanine dye. Therefore dyes delivered in the dimethylformamide preparation used in our study appear not to affect cell viability nor diffuse between cells.

Mapping the prospective neuromeric junctions in the early neural plate

Fig. 1C shows the initial injection sites in the early somite-stage embryo. Table 1 summarises the localisation of DiI-labelled neural plate cells from four injection sites in the cephalic neural tube after 48 hours in vitro culture. Site ‘i’, the junction of the first two segments, was marked with DiI in 12 embryos (Fig. 1C). Labelled neural plate cells were subsequently found in both the caudal forebrain and rostral midbrain in 58% (7/12) of analysed embryos. 25% (3/12) of embryos exhibited labelled cells in the forebrain only and 17% (2/12) of embryos displayed labelled cells in the midbrain only. Site ‘i’ therefore marks the junction of the forebrain and midbrain. Site ‘ii’ was labelled in 16 embryos (Fig. 1C). In 9 of 16 (56%) cases, labelled cells were localised in the caudal midbrain and the first rhombomere (r1). 25% (4/16) of embryos contained labelled cells in r1 only and 19% (3/16) of embryos contained DiI-labelled cells in r3 only. Hence the pre-otic sulcus (site ‘iii’) marks the junction of the second and third rhombomeres. The neural plate ventral to the pre-otic sulcus (site ‘iv’) was labelled in 13 embryos (Fig. 1C).
After 48 hours culture, labelled cells were found in the ventral region of r4 and 5 in 62% (8/13) of embryos. 23% (3/13) of embryos displayed labelled cells in r4 only and 15% (2/13) of embryos contained labelled cells in r5 only. The otic sulcus in 8.5-day embryos therefore represents the presumptive junction of r4 and r5. Fig. 1E is a summary diagram using an SEM picture of a fore-limb bud stage embryo showing the approximate location of labelled cells relative to the neuromeres. Our results agree with those obtained in similar dye-labelling experiments performed by Osumi-Yamashita et al. (unpublished results, personal communication) and with the regionlisation of the midbrain and rostral hindbrain as revealed by the expression of \( Wnt-1 \), \( RAR\beta \) and \( Hoxb \) genes (Conlon and Rossant, 1993; Echelard et al., 1994). On the basis of these findings, the five neural crest regions tested in the present study are designated as forebrain, caudal midbrain, rostral hindbrain, middle hindbrain and caudal hindbrain. Our results thus point to an inaccuracy in the previous identification of the presumptive midbrain-hindbrain junction in the neural plate of the early-somite-stage embryo (Jacobson and Tam, 1982; Meier and Tam, 1982). Furthermore it implies that in the mouse the third cranial somitomere is associated with both the caudal midbrain and the rostral hindbrain instead of just the caudal midbrain (Tam and Trainor, 1994; Trainor et al., 1994).

**Dual contribution of somitomeric mesoderm and cranial neural crest cells to the craniofacial mesenchyme**

Table 2 summarises the pattern of distribution of transplanted cells, DiI/DiO-labelled cells and WGA-gold-labelled cells to craniofacial structures in the mouse embryo after 48 hours of in vitro culture. Results of transplantation and labelling experiments demonstrate a dual contribution of cranial paraxial mesoderm and neural crest cells to formation of the branchial arch, facial, cervical, peri-ocular and peri-otic mesenchyme. The mesoderm and neural crest progenitors destined for their respective cranial regions were located in register along the rostral-caudal axis.

**Distinct segregation of neural crest and mesoderm cells during branchial arch formation**

To analyse the distribution of neural crest cells and mesoderm destined for the first arch, the rostral hindbrain of 8.5-day host embryos were first labelled with WGA-gold. \( LacZ \)-expressing mesodermal cells from somitomere (sm)-III were then orthotopically grafted to sm-III in the WGA-gold-labelled embryo. Alternatively, the neuroectoderm was labelled with DiI and the somitomeric mesoderm in the same embryo was labelled with DiO (Fig. 3). As expected there was a high degree of similarity in the results obtained by WGA/\( LacZ \) cell marking.

![Fig. 7](image7.jpg)  
**Fig. 7.** A sagittal section through the facial mesenchyme and the first branchial arch showing the somitomere-III derived mesodermal cells (dark blue) in the core and the silver-labelled neural crest cells (arrowheads) localised peripherally in the arch. Bar, 150 \( \mu \)m.

![Fig. 8](image8.jpg)  
**Fig. 8.** A confocal image taken at a deep level of the branchial arch regions showing the localisation of the DiI-labelled neural crest cells from the hindbrain to the branchial arches. A wider region of hindbrain neural crest were labelled in this embryo to demonstrate that the peripheral localisation of neural crest cells is common to all three branchial arches. ha, hyoid arch; md, mandibular process. The arrow points rostrally. Bar, 500 \( \mu \)m.

![Fig. 9](image9.jpg)  
**Fig. 9.** Confocal image of neural crest cells (red) and somitomeric mesoderm (green) in the lateral half of the first branchial arch. This picture represents an end-on view of a stack of confocal images obtained by optical sectioning in the sagittal plane of the arch. The lateral border of the branchial arch is outlined. This image shows the preferential localisation of the neural crest cells (red) to the lateral (l), rostral (r) and caudal (c) regions superficial to the somitomeric mesoderm (green); b, the buccal/medial side of the arch. Yellow signal is generated as a result of image enhancement. It represents the spatial overlap of the two cell populations, but not double-labelled cells.
compared with DiI/DiO labelling. Following 48 hours of in vitro culture, X-gal staining, silver enhancement and confocal microscopy demonstrated that sm-III-derived mesoderm principally colonised the maxillary and mandibular regions of the first branchial arch and the related facial mesenchyme (Table 2, Fig. 4A,B). Similarly the neural crest cells derived from the rostral hindbrain colonised the maxillary and mandibular regions of the first arch but they also contributed to the trigeminal ganglia (Table 2, Fig. 5). In 11% (2/19) of cases the neural crest was labelled in the immediate vicinity of the rostral hindbrain-middle hindbrain junction and this has resulted in the distribution of labelled neural crest cells to the hyoid arch (Fig. 5). The distribution of neural crest cells from the caudal midbrain were also traced in 23 dye-labelled and 21 WGA-gold-labelled embryos in which sm-III mesoderm underneath this brain region was also marked. Similar to the rostral hindbrain, neural crest cells were found in the maxillary and mandibular prominences of the first arch. However, in contrast to the hindbrain neural crest cells, the midbrain neural crest cells also colonised the facial mesenchyme caudal to the optic vesicle but were not found in the trigeminal ganglion (data not shown). Sm-III-derived mesoderm contributed extensively to facial and branchial arch mesenchyme.

Sm-IV-derived mesoderm and middle hindbrain-derived neural crest cells co-colonised the hyoid arch mesenchyme (Figs 6A,B and 16) and the adjacent cervical mesenchyme. The middle hindbrain neural crest cells also contributed to the facioacoustic ganglion and to the rostral and ventral peri-otic mesenchyme (Table 2).

Analyses by histology and confocal microscopy revealed that the somitomeric mesoderm destined for the mandibular and hyoid arches predominantly colonised the core mesenchyme in the central and medial parts of the branchial arches (Figs 7, 9, 10). The neural crest cells derived from the neural segments associated with these somitomeres appeared to migrate along a sub-ectodermal pathway and formed a cohesive sheet of cells as far as the distal regions of the mandibular and hyoid arches. The neural crest cells (marked by WGA-gold or DiI) were localised peripherally in the lateral, rostral and caudal aspects of the arches (Figs 7, 8, 10). Labelled neural crest cells were often localised immediately beneath the branchial arch surface ectoderm, but

Fig. 10. Three series of optical sections taken from the lateral region (B,F,J) to the medial region (E,L,M) of the branchial arches. (A) Bright-field image of the specimen (fm, facial mesenchyme; ha, hyoid arch; md, mandibular process; mx, maxillary prominence). (B-E) Images of the neural crest cells. (F-I) Same sections as B-E but showing the somitomeric mesoderm. (J-M) Computer-generated images combining series B-E with F-I. The combined images illustrate the localisation and overlapping distribution of somitomere III mesoderm and rostral hindbrain neural crest cells in the first arch. A computer-assigned false colour scheme is used for J-M: neural crest cells in red, somitomeric mesoderm in green and overlapping cells in yellow. The yellow colour does not represent double labelled cells but is a product of computer enhancement. The somitomeric mesoderm colonises the core of the branchial arch, which is enveloped by neural crest cells. A few neural crest cells are found in the mesodermal core. Bar, 500 μm.
Somitomere and neural crest cells were never found to contribute to the surface ectoderm of any branchial arch (data not shown). A perpendicular view of sagittal confocal sections of the mandibular process clearly shows that the neural crest cells are found superficially to the mesodermal population (Fig. 9). Neural crest cells were rarely found in the medial region of the branchial arches but small clusters of neural crest cells were present in the core mesoderm (Fig. 10). Our results therefore show unequivocally that the somitomeric mesoderm and neural crest cells are segregated spatially in the developing branchial arches.

The facial mesenchyme surrounding the proximal region of the mandibular arch is also derived from sm-III and from neural crest cells of the caudal midbrain and rostral hindbrain. The cervical mesenchyme surrounding the hyoid arch is derived from sm-IV and from middle hindbrain neural crest cells.

Fig. 11. A sagittal section of the facial region showing the mixing of the somitomeric mesoderm (blue X-gal stained cells) and neural crest cells (silver-labelled; arrowheads). Bar, 50 μm.

Fig. 12. A sagittal section through the cervical mesenchyme dorsal to the hyoid arch showing the interspersion of somitomeric mesoderm (blue X-gal-stained cells) and neural crest cells (silver-labelled; arrowheads). Bar, 50 μm.

Fig. 13. Colonisation of the peri-ocular mesenchyme (opm) by cells derived from somitomere I (blue, X-gal-stained cells in A, and green, DiO-labelled cells in B) and the forebrain neural crest cells (red, Dil-labelled cells in B; op optic vesicle). C is a bright-field picture of the optic vesicle (op) and peri-ocular mesenchyme (opm) and D is a confocal image of the same specimen showing the co-distribution of the two cell populations. Red, Dil-labelled cells are found in the neural crest-derived mesenchyme and in the neural epithelium of the optic evagination. Green, DiO-labelled somitomeric mesoderm is found in the peri-ocular mesenchyme. Yellow signal is generated as a result of image enhancement. It represents the spatial overlap of the two cell populations, but not double-labelled cells. The arrow points rostrally. Bar, 500 μm.
cells. However, in both the facial (Fig. 11) and cervical mesenchyme (Fig. 12) extensive mixing of the somitomeric mesoderm and neural crest cells was found, suggesting that cells may behave very differently outside the branchial arches.

Mixing of neural crest cells and mesoderm in the peri-ocular and peri-otic mesenchyme

The differential contribution of somitomeric mesoderm and neural crest cells to the peri-ocular mesenchyme were examined. Mesoderm derived from sm-I contributed extensively to the peri-ocular mesenchyme with the majority of the cells localised to the rostral and ventral peri-ocular mesenchyme (Table 2; Fig. 13A). The distribution pattern of forebrain-derived neural crest cells was very similar to that displayed by sm-I mesoderm. WGA-gold- and DiI-labelled neural crest cells derived from the forebrain contributed to the frontonasal mass and the peri-ocular mesenchyme. The majority of the forebrain neural crest cells populated the rostral and ventral peri-ocular mesenchyme (Table 2, Figs 5, 13; see also Osumi-Yamashita et al., 1994). In 27% (6/22) of cases the optic evagination was also colonised by labelled neural crest cells (Figs 13B, D and 14). These cells were probably derived from labelled cells that remained in the neural plate, after the departure of the neural crest cells, which is then pinched off to form the optic evagination. The mesoderm cells and forebrain derived neural crest cells were interspersed in the peri-ocular mesenchyme (Fig. 13B, D). The somitomeric mesoderm did not contribute cells to the neuroepithelium of the optic vesicle nor the lens placode but contributed to the endothelium of the blood vessels surrounding the optic evagination (Fig. 14).

The distribution of mesoderm and neural crest cells destined for the peri-otic mesenchyme was also examined. Transplanting and DiO labelling of somitomere IV- and VI-derived mesoderm resulted in labelled mesoderm cells populating pre-
dominantly the rostro-ventral (Fig. 15A) and caudal peri-otic mesenchyme respectively. Somitomere VI also contributes to the third branchial arch (data not shown). Middle hindbrain-derived neural crest cells colonised the rostral and ventral peri-otic mesenchyme and the facioacoustic ganglion (Table 2, Fig. 15A,D). Caudal hindbrain-derived neural crest cells contributed to the caudal peri-otic mesenchyme and the glossoharyngeal ganglion (Table 2, Fig. 15B; Osumi-Yamashita et al., personal communication). Caudal hindbrain neural crest cells populated the third arch and some also colonised the second arch (data not shown). Similar to the facial, cervical and peri-ocular mesenchyme there was widespread mixing between the mesoderm and neural crest cells in the peri-otic mesenchyme (Fig. 15A,D). A small number of ectodermal cells derived from the middle hindbrain and the caudal hindbrain contributed to formation of the otic vesicle as shown by the presence of WGA-gold- and Dil-labelled cells (Fig. 15). Whether this is due to placodal contribution or neural crest colonisation is not known. The otic vesicle however, was devoid of a mesodermal contribution. Therefore in the more dorsal craniofacial regions there is co-distribution and mixing of somitomeric mesoderm and neural crest cells, whereas in the more ventral regions (branchial arches) there is segregation of the two cell populations (Fig. 16).

**DISCUSSION**

The vertebrate head is constructed according to a basic blueprint preserved through evolution (Northcutt and Gans, 1983; Northcutt, 1990). In particular the neural tube, paraxial mesoderm, branchial arches and nerve ganglia are all segmentally organised and the related segments are meristically matched during craniofacial morphogenesis (Thorogood, 1993). To date, we have a limited understanding of the mechanisms controlling how patterns at one level, such as within the hindbrain, are developmentally transposed into patterns at another level, such as the branchial arches. We also do not know the source of the programme responsible for each of the craniofacial structures developing in its proper position within the head and with the appropriate shape, alignment and attachment. Following analysis of the patterns of mesoderm and neural crest cell distribution during craniofacial morphogenesis, it has become apparent that the alignment and co-migration of precursor tissues might be important for generating an anatomically and functionally integrated head.
Co-distribution of somitomeric mesoderm and cranial neural crest cells from similar rostral-caudal positions

Results of cell transplantation and dye labelling experiments revealed that there is a general concordance between the distribution of somitomeric mesoderm and cranial neural crest cells from the same axial level during craniofacial morphogenesis. Mesoderm derived from sm-I and the adjacent forebrain-derived neural crest cells co-distribute to the periorcular region. Mesoderm of sm-III, and neural crest cells of the caudal midbrain and rostral hindbrain both migrate into the facial mesenchyme and the mandibular arch. Mesoderm of sm-IV and middle hindbrain neural crest cells colonise the hyoid arch. For the peri-otic mesenchyme, the rostroventral population is derived from sm-IV mesoderm and middle hindbrain neural crest cells, while the caudoventral population is derived from sm-VI and caudal hindbrain neural crest cells.

The co-localisation of mesoderm and neural crest cells thus provides strong evidence to support the concept that progenitors of individual segments are in register along the rostrocaudal axis (Schilling and Kimmel, 1994). Segmental and lineage restrictions have also been identified during pharyngeal arch development in the zebrafish (Schilling and Kimmel, 1994) and the chick (Lumsden et al. 1991; Bagnall et al., 1989). Noden (1993) has shown that the branchial motor axons and muscles develop at the same axial level with neural crest cells that co-populate each branchial arch. Similarly a conserved registration between limb muscle and motor neuron primordia has also been described (Lance-Jones; 1988). In contrast, Wahl et al. (1994) have demonstrated that there is a lack of registration of the motor neuron and myoblast precursors that constitute the VIth nerve-lateral rectus/pyramidalis complex. Previous studies have suggested that the cranial mesoderm from different anterior-posterior levels is not pre-patterned (Noden, 1988; Trainor et al., 1994) and that neural crest cells from different anterior-posterior levels can respecify the fate of the mesoderm (Noden, 1988). Therefore the real key to anterior-posterior patterning and cell lineage co-ordination is the neural crest cell population, which is in turn established by the neural ectoderm.

Segregation of somitomeric mesoderm and neural crest cells during the initial phases of branchial arch morphogenesis

Somitomeric mesoderm and cranial neural crest cells participate in branchial arch formation. Somitomeric mesoderm colonises the core mesenchyme of the mandibular and hyoid arches. Concurrently neural crest cells migrate along a subectodermal pathway moving between the lateral surface of the somitomeric mesoderm and surface ectoderm (Fig. 17a). The neural crest cells moving into the mandibular and hyoid arches are confined to the periphery of the arch, predominantly on the lateral side and envelop the mesodermally derived branchial arch cores (Fig. 17b). The spatial distribution of mesoderm (core) and neural crest cells (periphery and lateral side) in the branchial arches appears to correlate with the gene expression patterns of Myf-5 (myogenic marker) and Dlx-2 (neural crest marker) respectively (Ott et al., 1991; Bulfone et al. 1993; Robinson and Mahon et al., 1994).

The segregation of somitomeric mesoderm and cranial neural crest cells is a phenomenon confined to the branchial arches only. Outside the branchial arches, there is extensive mixing of somitomeric mesoderm and cranial neural crest cells particularly in the periorcular and peri-otic mesenchyme and in the facial and cervical mesenchyme. Neural tube transplantation experiments performed in the chick suggest that the neural crest cells provide the spatial cues for establishing the three dimensional organisation within the developing head (Noden, 1988). When presumptive first (mandibular) arch neural crest primordia are grafted in place of presumptive second (hyoid) or third (branchial) arch primordia, grafterived neural crest cells migrate normally into the closest arch but therein form a jaw skeleton complete with Meckel’s cartilages (Noden, 1988). If the graft is placed anterior or posterior to the hindbrain, the neural crest differentiates but does not form first arch structures, which implies that there is a particular dependence on the competence of the surface ectoderm of the branchial arches required for neural crest to elaborate its patterning programme. Our results indicate that the neural crest remains closely associated with the surface ectoderm suggesting that signalling between the two tissues may be important. Outside the branchial arches, the neural crest and mesoderm are closely associated with the neural ectoderm. Noden (1988) has proposed that they receive instructive signals from the neural tissue rather than the surface ectoderm. This may explain why it is possible for the mesoderm and crest to intermingle more freely in the anterior regions, because the surface ectoderm has a different role in this region. Studies performed in the chick suggest that the regional specific organisation of muscle depends on the connective tissue pattern set up by the neural crest cells (Chevalier, 1979; Chevalier and Kieny, 1982; Noden, 1983a; 1986). If intimate cell to cell contact is required for specifying a connective tissue fate, then it is likely that the neural crest cells that do not mix with the somitomeric mesoderm, such as those in the periphery of the branchial arches, will ultimately give rise to the craniofacial skeleton.

Despite the migration of the cranial neural crest cells as a continuous sheet (Johnston, 1966; Noden 1975; Lumsden et al., 1991; Sebedzija et al., 1992) a small population of crest cells breaks free from this sheet and invades the branchial arch cores and craniofacial mesoderm. Noden (1988) proposed that this population of cells subsequently forms the medial boundary of the developing branchial arch muscle masses and that the neural crest cells are instrumental in segregating the superficial myogenic population from other tissues types of the somitomere. The connective tissue mesenchyme derived from the neural crest therefore provides the patterning cues necessary to direct the distribution and alignment of myogenic precursors during craniofacial morphogenesis (Jacob et al., 1983).
REFERENCES


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