Plasticity in mouse neural crest cells reveals a new patterning role for cranial mesoderm

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The anteroposterior identity of cranial neural crest cells is thought to be preprogrammed before these cells emigrate from the neural tube. Here we test this assumption by developing techniques for transposing cells in the hindbrain of mouse embryos, using small numbers of cells in combination with genetic and lineage markers. This technique has uncovered a surprising degree of plasticity with respect to the expression of *Hox* genes, which can be used as markers of different hindbrain segments and cells, in both hindbrain tissue and cranial neural crest cells. Our analysis shows that the patterning of cranial neural crest cells relies on a balance between permissive and instructive signals, and underscores the importance of cell-community effects. These results reveal a new role for the cranial mesoderm in patterning facial tissues. Furthermore, our findings argue against a permanently fixed prepatterning of the cranial neural crest that is maintained by passive transfer of positional information from the hindbrain to the periphery.

The vertebrate hindbrain is a good model system for addressing basic problems of cellular patterning during craniofacial development. Its subdivision into seven rhombomeric (r) segments is an evolutionarily conserved organizational strategy used to initiate the programme that creates the diverse range of nerves and craniofacial structures[1,2]. During rhombomere formation, each segment adopts a distinct set of cellular and molecular properties that are different from those of its immediate neighbours. There is an anatomical and functional registration between rhombomeres and the pathways of neural crest cell (NCC) migration, such that cranial NCCs migrate into the branchial arches as three segmental streams next to the even-numbered rhombomeres[3–5]. Rather than move laterally, NCCs derived from r3 and r5 migrate anteriorly and posteriorly to join the even-numbered streams[6]. NCCs in each branchial arch contribute to specific cranial sensory ganglia, form bone and cartilage derivatives, and influence muscle patterning and branchiomotor targets[7,8]. Hence, hindbrain segmentation plays a key part in head morphogenesis by modulating the formation and properties of neural crest, and it is important to understand the nature and source of signals and interactions that govern the patterning of craniofacial tissues.

Cranial neural crest cells are thought to be prepatterned in a fixed manner before emigration from the neural tube, and control the programmes of cellular differentiation of other tissues such as the mesoderm[9,10]. Gain- and loss-of-function analyses in several vertebrates have underscored the functional importance of transcription factors encoded by *Hox* genes in rhombomeres and cranial NCCs, showing that these transcription factors are essential for regulating head development[11]. Linking these findings, the neural crest prepatterning model predicts that alterations to the spatial organization of rhombomeric tissue would lead to a corresponding reorganization of the neural crest *Hox* code[12] and, ultimately, to craniofacial abnormalities. In support of this model, rhombomere-transposition experiments in the chick have shown that, as NCCs begin to migrate, their anteroposterior identity, as revealed by their *Hox* code, is already fixed and cannot be altered by environmental signals[10]. In contrast, rhombomere rotations have provided evidence for plasticity in NCC *Hox* expression[13,14]. Hence there are conflicting data regarding the degree of autonomy or plasticity in cranial NCCs.

In the hindbrain, there is evidence for both plasticity and autonomy of segmental markers, as shown by transplantation experiments that reveal that the paraxial mesoderm environment and the anteroposterior origin influence the degree of autonomy. In the chick, rhombomeres transposed anterior to the otic vesicle (into the pre-otic region) autonomously maintain the segmental *Hox* expression patterns characteristic of their original anteroposterior origin in the hindbrain[10,15–17]. In contrast, grafts posterior to the otic vesicle (post-otic grafts) result in a reprogramming of *Hox* expression patterns to reflect their new anteroposterior location[18,19], and this plasticity is mediated by somitic mesoderm[19–21]. These differences correlate with differences in the organization and character of paraxial mesoderm in the pre-otic and post-otic environments. Rather than patterning neural tissue, the pre-otic mesodermal populations are thought to have passive roles in head patterning, where they receive signals and information from the neural tube and neural crest[8,9,22,23].

The current models that argue for fixed cell autonomy in the prepatterning of *Hox* expression in pre-otic rhombomeres and NCCs have been derived from analyses of avian embryos that involve manipulations of blocks of tissue generally encompassing multiple segments. Such manipulations could, as a result of signalling between rhombomeres and cell-community effects, mask cellular plasticity. Hence, it is essential to test much smaller populations of cells and whether these models are applicable to other vertebrates. The mouse is a good system for investigating these issues because much is known about hindbrain patterning and *Hox* regulation in the mouse, and because an array of genetic tools, including transgenic and targeted mutant lines, exists.

Here we have developed new techniques for manipulating small groups of genetically labelled mouse rhombomeric tissue and NCCs in cultured embryos. These techniques allow us to study the degree of plasticity or autonomy in small groups of rhombomeric tissue and NCCs, and to assess the influence of the mesodermal environment on these processes in mice. Rhombomere transpositions coupled with lineage analysis of cell fate reveal a plasticity of *Hox* gene expression in NCCs that is dependent upon a combination of the original rhombomeric character and signals emanating from the pre-otic mesoderm. There is also evidence for plasticity in rhombomeric cells that depends upon the size of the cell community.

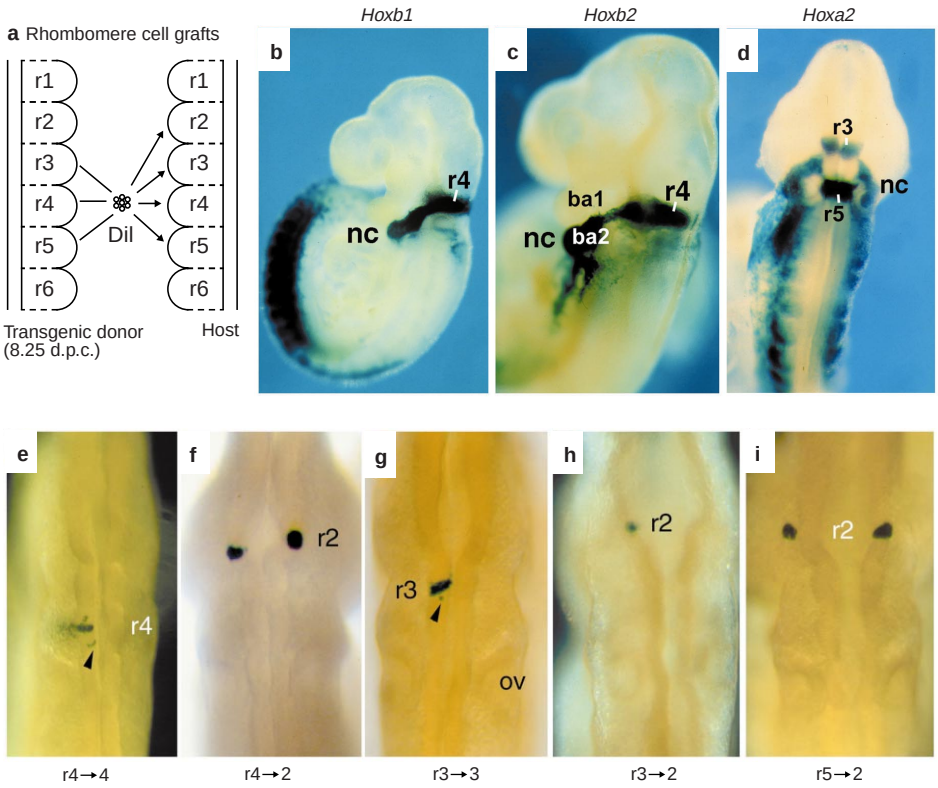

Figure 1 **Assay for autonomy of *Hox* gene expression and mixing in transpositions of murine rhombomeric cells. a**, Diagram of the homotopic and heterotopic transpositions of small groups of DiI-labelled rhombomeric cells. **b–d**, Lateral views of 9.5-d.p.c. embryos from **b**, *Hoxb1* r4, **c**, *Hoxb2* r4, and **d**, *Hoxa2* r3/r5 *lacZ* transgenic lines stained for β-galactosidase. All three lines express the reporter in neural crest cells (nc) populating the second branchial arch (ba2) and in specific rhombomeres. **e–i**, Dorsal hindbrain views of 8.25-d.p.c. (five-somite) host embryos cultured for 24 h after transposition of rhombomere cells and stained for β-galactosidase. Transpositions and lines used were: **e**, *Hoxb1* r4→r4; **f**, *Hoxb1* r4→r2; **g**, *Hoxa2* r3→r3; **h**, *Hoxa2* r3→r2; **i**, *Hoxa2* r5→r2 . In **f**, **i**, bilateral transpositions were performed. On the left in **i**, the graft resides in mesenchyme. Arrowheads in **e**, **g** indicate the higher degree of mixing of cells in homotopic grafts compared with heterotopic grafts. nc, neural crest; ov, otic vesicle; r, rhombomere.

Results

Transposition of rhombomeric tissue in cultured mouse embryos. To test the degree of autonomy or plasticity of rhombomeric tissue and cranial NCCs, we have developed techniques for transposing small numbers of hindbrain cells in cultured mouse embryos. We performed dorsal rhombomere transplantations before emigration of the NCCs, to ensure that grafted cells also contributed to the host NCC populations. We previously generated a fate map of the mouse hindbrain[23], and used transgenic analysis to characterize a number of *cis*-regulatory elements required to direct proper domains of rhombomere-restricted *Hox* expression. These techniques allowed us to take advantage of three transgenic lines carrying a *lacZ* reporter gene under the control of distinct segmental enhancers (those of the *Hoxb1*, *Hoxb2* and *Hoxa1* genes; Fig. 1b–d)[24–26] that genetically mark prerhombomeric cells. We isolated small sub-rhombomeric populations (groups of 10–15 cells) from transgenic donor embryos at 8.25 days post-coitum (d.p.c.; at this stage the embryos consist of five somites), labelled the cell populations with DiI, and grafted them homotopically and heterotopically into stage-matched, wild-type host embryos (Fig. 1a). The analysis of reporter staining, together with lineage tracing, enabled us to monitor the degree of autonomy or plasticity in cell fate, as determined by the *Hox* gene expression patterns in both rhombomeres and NCCs at various stages in culture (24–36 h).

Homotopic grafts show maintenance of *Hox* expression. To verify our approach for transposing small numbers of hindbrain cells, we performed control homotopic grafts of r3, r4 and r5 cells. In all cases, when placed back into the same anteroposterior position in the hindbrain of host embryos, the grafted cells incorporated into the hindbrain, mixed with their immediate neighbours, and exhibited the proper patterns of *Hoxb1*, *Hoxb2* or *Hoxa2* reporter expression (Fig. 1e, g and data not shown). Lineage tracing revealed that dorsally transposed rhombomeric cells also generated NCCs that migrated into the adjacent branchial arch along with the host crest populations and maintained the proper *Hox* gene expression patterns (see below and data not shown).

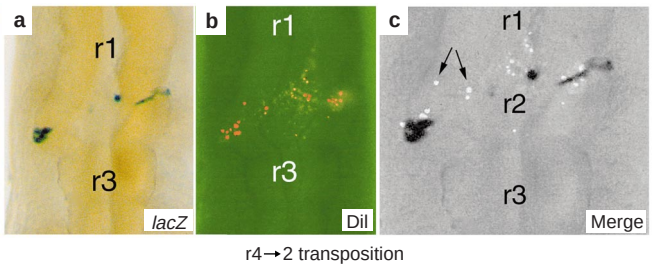

Figure 2 **Cell-community effects and plasticity in transposition of rhombomeric cells.** Dorsal hindbrain views of an 8.25-d.p.c. cultured host embryo after r4→r2 transposition of *Hoxb1*/*lacZ* cells. **a**, Autonomy of *Hoxb1* expression revealed by β-galactosidase staining. **b**, Fluorescent lineage tracing with DiI. **c**, Superimposition of **a** and **b** at high resolution in black and white. Rhombomeric cells that become separated from the primary graft fail to express the reporter transgene (black arrows).

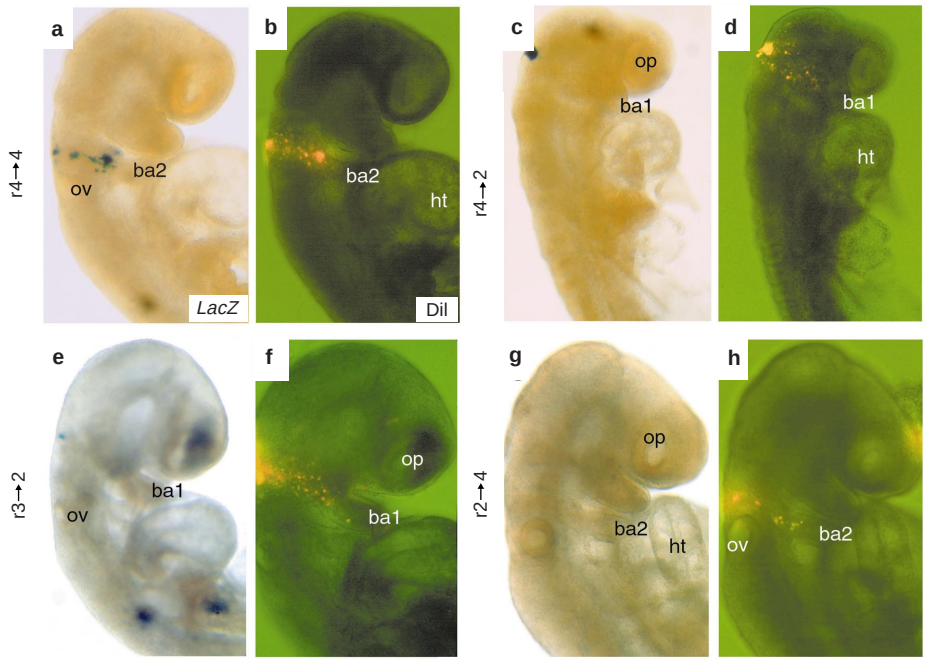

Figure 3 **Plasticity of *Hox* gene expression in cranial neural crest cells.** Paired lateral views of cultured 8.25-d.p.c. host embryos, after transposition of rhombomere cells, assayed for both plasticity of *Hox/lacZ* expression (β-galactosidase staining) and migration (fluorescent lineage tracing with DiI) of graft-derived NCCs. Transpositions and lines were: **a**, **b**, *Hoxb1* r4→r4; **c**, **d**, *Hoxb1* r4→r2; **e**, **f**, *Hoxa2* r3→r2; **g**, **h**, *Hoxb2* r2→r4. ba1, first branchial arch; ba2, second branchial arch; ht, heart; op, optic vesicle.

Heterotopic grafts show plasticity and the importance of cell-community effects. In a series of heterotopic transpositions, in which we grafted r3, r4 or r5 cells into r2, in the pre-otic environment, small groups of donor rhombomeric cells were able cell-autonomously to maintain their anteroposterior character (Fig. 1f, h, i). In heterotopic grafts there was considerably less cell mixing between graft and host populations, and grafted cells were generally distributed as a single coherent group of cells that expressed the reporter gene (Fig. 1e–i). This contrasts with the homotopic grafts, for which we observed dispersed cells, separated from the primary graft, that maintained *Hox* expression (Fig. 1e, g; arrowheads). This raised the question of whether cells that become isolated from the primary graft in heterotopic transplantations alter their identity. In heterotopic transplantations, by simultaneously monitoring gene expression and cell lineage at high resolution, we routinely observed dispersed cells that failed to maintain reporter expression (Fig. 2, arrows). In contrast, cells that remained in a coherent group maintained reporter expression. Despite previous evidence for autonomy of gene expression in rhombomeric tissue, these results show that individual rhombomeric cells can exhibit plasticity with respect to *Hox* expression and change their fate. This indicates that, in ectopic locations, single or dispersed cells lack the signals from their normal neighbours that are needed to reinforce their character, and that cell-community effects play an important part in maintaining an individual cell's identity.

Plasticity of *Hox* expression in cranial neural crest cells. The transgenic lines used above also contain regulatory elements that mediate *Hox* expression in NCCs (Fig. 1b–d)[24–27], and grafted rhombomeric cells will contribute to the host NCC population, allowing us to assay the degree of autonomy in cranial NCCs. In homotopic r4 grafts using the *Hoxb1* and other lines, labelled cells migrate from r4 into the second branchial arch (ba2) and maintain the appropriate patterns of reporter gene expression (Fig. 3a, b). Heterotopic grafts of r3, r4 or r5 cells into r2 show that these cells are capable of generating NCCs that join the host population, migrating from r2 into the first branchial arch (ba1), and there is no evidence of contributions to more posterior arches (Fig. 3c–f and data not shown). There is, however, a complete absence of reporter expression in graft-derived NCCs that migrate into ba1 (Fig. 3c–f). Hence, NCCs are plastic with respect to their patterns of *Hox* gene expression. This shows that their anteroposterior character is neither permanently fixed nor passively transferred from the hindbrain to the migrating neural crest, but instead is influenced by environmental signals.

Are branchial-arch signals permissive or instructive for Hox expression? *Hox* genes are not normally expressed in the first branchial arch, but when r3, r4 or r5 cells from the transgenic lines are placed in ba1 they remain as a coherent group and cell-autonomously maintain their identity regardless of their location within the arch (see Supplementary Information). Hence, the environment of the first branchial arch does not preclude the ability of some cells to express specific *Hox* genes. To investigate the possibility that branchial arches provide instructive signals that mediate neural crest patterns of *Hox* expression, we grafted cells derived from r2 of a *Hoxb1* transgenic embryo posteriorly into r4 of a wild-type host embryo (Fig. 3g, h). r2 cells do not normally express the *lacZ* reporter gene (Fig. 1b). In agreement with the evidence for rhombomere autonomy described above, the reporter was not activated in the r2 cells ectopically located in r4 (Fig. 3g). Use of the lineage tracer indicated that grafted r2 cells can generate NCCs that join the host r4 stream and migrate into ba2 (Fig. 3h); however, they fail to activate *Hoxb1* reporter expression. This shows that the second-arch environment alone is not sufficient to induce *Hoxb1* expression in NCCs, and that the rhombomeric character from which the NCCs are derived is an important factor.

Mesoderm of the second arch is needed to maintain *Hox* expression in NCCs. The absence of reporter expression in NCCs derived from transposed rhombomeric tissue could represent a failure in activation and/or maintenance of *Hox* expression programmes. We never detect reporter expression in the neural crest, even in newly migrating cells adjacent to the neural tube, indicating that the neural crest may fail to activate *Hox* reporter expression properly. To

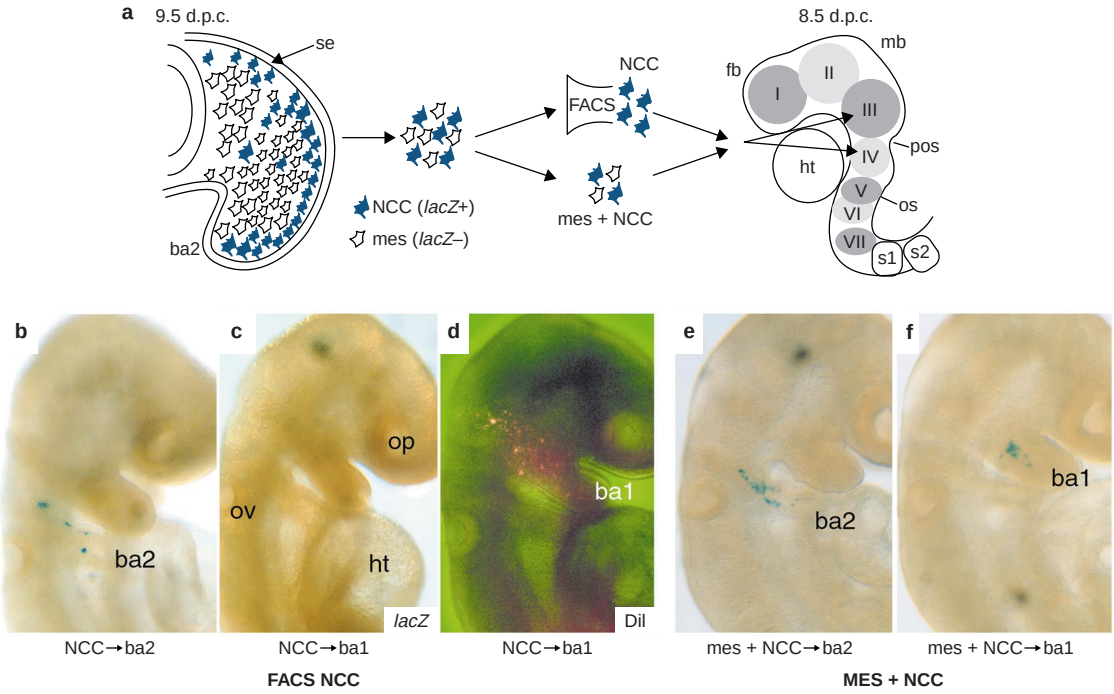

Figure 4 **Cranial mesoderm is required to maintain *Hox* gene expression in the neural crest cells of ba2. a**, Scheme for the isolation and transplantation of a pure population of second-arch neural crest cells (NCC) and second-arch mesenchyme (mes). **b**, **c**, Lateral views of 9.5-d.p.c. embryos assayed for *Hoxb1* gene expression by β-galactosidase staining, after transplantation of NCCs from ba2 into presumptive ba2 (**b**) and ba1 (**c**). **d**, Fluorescent DiI lineage tracing image of graft-derived cells in the first arch; the embryo is the same as that shown in **c**. **e**, **f**, 9.5-d.p.c. embryos exhibiting maintenance of *Hoxb1/lacZ* expression, after transplantation of second-arch mesenchyme into presumptive ba2 (**e**) and ba1 (**f**). fb, forebrain; ht, heart; mb, midbrain; os, otic sulcus; ov, otic vesicle; pos, pre-otic sulcus; s, somite; se, surface ectoderm; I–VII, somitomeres; FACS, fluorescence-activated cell sorting.

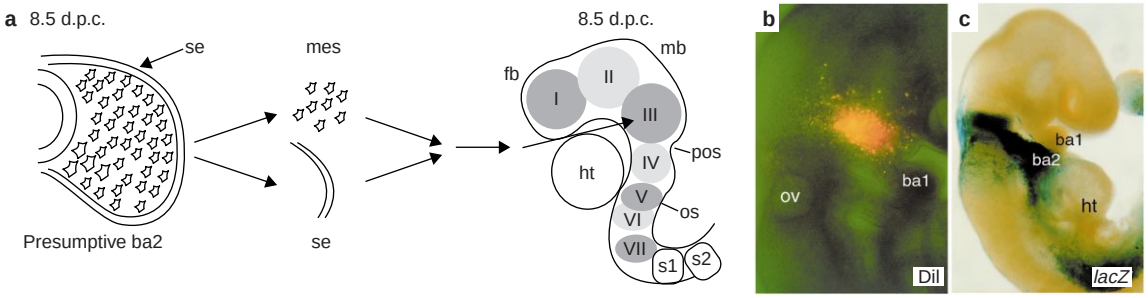

Figure 5 **Cranial mesoderm is insufficient to induce *Hox* gene expression.** **a**, Scheme for assaying for the induction of *Hox* gene expression by the transplantation of wild-type presumptive second-arch mesoderm into presumptive ba1 of isochronic transgenic host embryos. **b**, **c**, Paired lateral views of 8.25-d.p.c. embryos cultured for 24 h after transplantation and assayed for the distribution of DiI-labelled, graft-derived cells by fluorescence (**b**) and the absence of induction of *Hoxb2* gene expression (**c**).

test whether migratory NCCs can maintain *Hox* expression once activated, we transposed pure populations of migratory NCCs into ba1 and ba2 (Fig. 4a). We isolated migratory NCCs from the second arch by fluorescence-activated cell sorting, taking advantage of a vital β-galactosidase stain and the fact that the *Hox/lacZ* reporters are expressed only in the neural crest component of ba2 (ref. 27). In control grafts, when these purified NCCs were placed back into presumptive ba2 of a wild-type host, they colonized the periphery of the arch and retained their identity (Fig. 4b). In contrast, second-arch NCCs grafted into presumptive ba1 dispersed and populated the arch, but reporter expression was completely lost (Fig. 4c, d). This shows that second-arch tissues can maintain the proper *Hox* expression patterns in NCCs following activation, and that the first arch lacks the ability to both activate and maintain these patterns.

To investigate whether cranial mesoderm could account for these environmental differences in the branchial arches, we grafted a combination of second-arch neural crest and mesoderm cells into presumptive ba1 and ba2 (Fig. 4a). In control grafts of these cells into presumptive ba2, expression of the *Hoxb1* transgenic reporter was properly maintained in the NCCs, which populate the arch periphery (Fig. 4e). Surprisingly, heterotopic transplantation of these cells into ba1 also resulted in the proper maintenance of reporter expression (Fig. 4f), in contrast to transplantations of neural crest alone (Fig. 4c, d). Hence, second-arch mesoderm is able to provide the signals necessary to maintain NCC *Hox* expression in an ectopic branchial-arch environment.

This result indicates that cranial mesoderm may normally play an important part in modulating *Hox* expression in cranial NCCs. To test whether second-arch mesoderm is sufficient to induce reporter expression in ba1, we grafted small populations of pre-

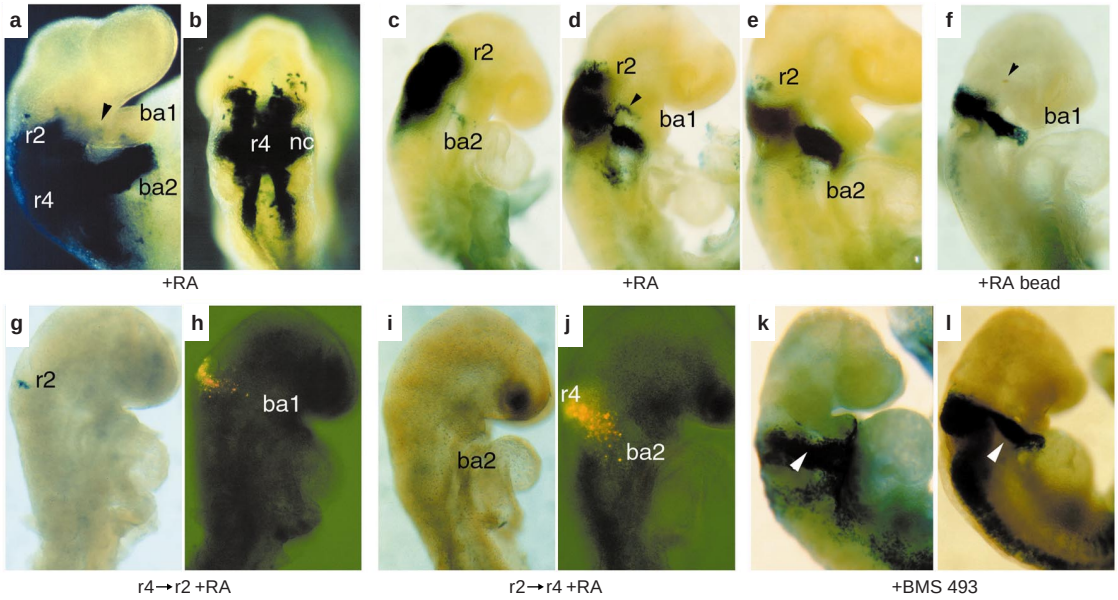

Figure 6 **Retinoic acid is not required for second-arch patterns of *Hox* gene expression. a**, Lateral and **b**, dorsal views of a 9.5-d.p.c. embryo, showing the induction of *Hoxb1/lacZ* expression in the anterior hindbrain but not in ba1 (arrowhead in **a**) following treatment with retinoic acid (RA) at 7.5 d.p.c. *in utero*. **c–e**, Lateral views of 9.5-d.p.c. *Hoxb1/lacZ* embryo littermates, showing varying levels of reporter induction after exposure to exogenous RA at 8.0 d.p.c. and the effect of RA on the pathways of migration of NCCs from ba2 (arrowhead in **d**). **f**, Lateral view of a 9.5-d.p.c. transgenic embryo cultured for 24 h after implantation of an RA-soaked bead fragment into presumptive ba1 (arrowhead). **g–j**, Paired lateral views of 9.5-d.p.c. wild-type host embryos, cultured in the presence of RA after r4→r2 (**g**, **h**) or after r2→r4 (**i**, **j**) transplantation. Note *Hoxb1* expression in grafted r4 (**g**) but not in the graft-derived NCCs migrating into ba1 (**h**). **k**, **l**, *Hoxb2/lacZ* (**k**) and *Hoxb1/lacZ* (**l**) embryos cultured from 7.5 d.p.c. in the presence of the RA inhibitor BMS493 faithfully retain their proper patterns of *Hox* gene expression in ba2 NCCs (white arrows).

sumptive ba2 mesoderm from wild-type donors into ba1 of *Hox/lacZ* transgenic embryos (Fig. 5a). As a control, we also transplanted second-arch surface ectoderm into ba1. In all cases, second-arch mesoderm (Fig. 5b, c) and ectoderm (see Supplementary Information) failed to activate ectopic reporter expression in ba1. This result is consistent with our finding that, in r2-to-r4 transpositions, r2-derived NCCs in ba2 fail to activate appropriate *Hox* gene expression patterns even in the presence of normal neural crest and mesodermal cells (Fig. 3g, h). Therefore, mesoderm appears to play a part in maintaining rather than instructing the cell fates and patterns of *Hox* gene expression in the branchial arches.

Retinoic acid is not the mesoderm signal influencing NCC Hox expression patterns. Having identified the cranial mesoderm as important in mediating cranial neural crest patterns of *Hox* gene expression, we next investigated potential roles for retinoic acid in this process by using a combination of approaches to increase or block retinoic-acid signalling. Treatment of 7.5–8.5-d.p.c. embryos with retinoic acid *in utero* expanded *Hoxb1* expression anteriorly into r2 (Fig. 6a–e) and transformed r2 to an r4-like character[28]. However, widespread *Hoxb1* expression was not induced in the NCCs migrating from the transformed territory, indicating that the neural crest *Hox* code in ba1 is unchanged (Fig. 6a–e). Occasionally, we observed a few cells in the first arch ectopically expressing the *Hoxb1/lacZ* reporter gene (Fig. 6a, arrowhead). However, this situation arose as a result of the misrouting of some NCCs emigrating from r4 into ba1 (Fig. 6d, arrowhead), as also described after treatment of cultured rat embryos with retinoic acid[29]. In addition, we transplanted transgenic r4 tissue into r2 and cultured the embryos in the presence of retinoic acid to determine the effect on the ability of the NCCs to maintain *Hoxb1* expression. However, reporter expression was not induced in the graft-derived NCCs migrating into the first arch (Fig. 6g, h). Similarly, transplantation of r2 cells from a *Hoxb1/lacZ* embryo into r4 of wild-type hosts, followed by treatment with retinoic acid, did not induce reporter expression in the transplanted rhombomeric tissue or in graft-derived NCCs migrating into ba2 (Fig. 6i, j). Retinoic-acid-impregnated beads transplanted into ba1 were also unable to induce *Hoxb1* expression in NCCs (Fig. 6f, arrowhead). These results indicate that the rhombomeric and NCC populations respond to retinoic acid independently. Finally, in embryos cultured in the presence of a compound (BMS493)[30] that blocks retinoid signalling, reporter expression in NCCs and arches was unaffected (Fig. 6k, l), indicating that retinoic acid was not required. Together, these results show that retinoic acid is not the second-arch mesodermal signal required to induce and maintain *Hoxb1* expression in NCCs migrating from r4 into ba2, and that as-yet-unidentified factors residing in the cranial mesoderm mediate these processes.

Discussion

Our development of techniques that allow the transposition of cells between mouse embryos provides new insights into the patterning mechanisms controlling vertebrate craniofacial morphogenesis. These techniques have made it possible to analyse changes in gene expression in hindbrain and neural crest cells in ectopic environments in the mouse. Our results have revealed that a balance between permissive and instructive signals is essential for regulating *Hox* gene expression programmes during murine craniofacial morphogenesis. Transposition analyses using small numbers of cells in combination with genetic and lineage markers have uncovered a surprising degree of plasticity with respect to *Hox* expression in both rhombomeric tissue and cranial NCCs. With respect to rhombomeric cells, *Hox* expression patterns can be autonomously maintained in ectopic locations within the pre-otic region, in agreement with previous analyses of avian embryos[15–17]. However, this autonomy is observed only in cells that remain in a coherent group. Analysis at higher resolution indicates that cells that become separated from the primary graft and intermingle with the surrounding populations are plastic and unable to maintain their appropriate *Hox* expression patterns. Therefore, cell-community effects are impor-

tant for reinforcing regional identity in rhombomeric populations. This result has important implications for the generation of the sharp segmental boundaries of *Hox* expression in normal development. Repulsive cues involving bidirectional signalling between Eph receptors and their ligands, ephrins, act to separate distinct rhombomeric territories as they form[31,32]. The plasticity uncovered here indicates that, once boundaries are formed, dispersed cells in an inappropriate location may be able to change their fate. Together these two processes provide a mechanism for the progressive generation of precise rhombomeric domains of gene expression.

In contrast to the hindbrain, cranial NCCs exhibit a high degree of plasticity in gene expression. They migrate as a dispersed cell population and are unable to maintain their proper *Hox* expression programmes in an ectopic branchial-arch environment. Thus, NCCs rely on distinct environmental cues in the branchial arches to elaborate their regional identity properly. Cranial mesoderm from the same axial level appears to be a source of the signals needed to maintain *Hox* patterns, as it supports *Hox* expression in NCCs when co-transplanted into ectopic branchial-arch environments. The mesodermal signals appear to be permissive rather than instructive for *Hox* expression, as they are unable to induce *Hox* expression in NCCs. These results indicate a model for regulation of the anteroposterior identity of NCCs in normal development, whereby the specific origin of the rhombomeric cells provides them with an initial anteroposterior character that prepares them to respond to a particular set of environmental signals in each arch. Hence, segmental organization functions in the hindbrain to maintain the proper anteroposterior registration between neural tissue, mesoderm and branchial arches. This registration is essential to coordinate the interplay between the signals needed to elaborate the proper *Hox* expression and morphogenetic programmes. When transposed to an ectopic environment, NCCs fail, as a result of the absence of the appropriate regional signals, to activate and/or maintain *Hox* expression characteristic of their origin. This fact raises several important questions with respect to the mechanisms governing autonomy versus plasticity in neural crest patterning.

The central dogma in the prepatterning model formed on the basis of experiments in avian embryos holds that the anteroposterior identity of cranial NCCs is fixed before they emigrate from the neural tube, and that their *Hox* code should not be altered by the environment[9,10]. However, our results show that the mouse cranial neural crest is plastic and is not prepatterned in a fixed manner. Our findings, together with the results of transgenic analyses that indicate that separate regulatory elements mediate *Hox* expression in neural crest and rhombomeres[27], shows that positional information is not simply set in the hindbrain and passively maintained during migration into the branchial arches.

These differences between mouse and chick may reflect experimental differences related to the size of the cell community being challenged. Autonomy in mouse rhombomere transpositions was dependent on the grafted cells remaining in a coherent group, but the few graft-derived mouse NCCs were widely dispersed and constituted a minor component of the total branchial-arch NCC population. From time-lapse studies in the chick there is evidence for cell communication between migrating NCCs through an extensive network of filipodial connections[33]. Thus, transposed mouse NCCs may be altering their gene expression and identity because they rely on signals from both their neighbours and the mesodermal environment to sustain their anteroposterior character. In the chick assays, transpositions involving multiple rhombomeres resulted in branchial arches being populated primarily by graft-derived NCCs[10,17]. This facilitates communication between axially related populations of migrating NCCs, enhancing their ability to maintain the proper anteroposterior patterns of *Hox* expression. But the observed autonomy of *Hox* expression in chick NCCs can be lost when analysed over longer time frames[14]. Therefore, we favour the idea that there is a similar degree of plasticity in cranial neural crest in both mouse and chick, but that this plasticity is masked in the chick by cell-community effects resulting from the transposition of larger blocks of tissue.

Our results show that cranial mesoderm is necessary for maintaining *Hox* gene expression in the second-arch crest. This is surprising given that pre-otic mesodermal populations were thought to play a passive part in head patterning, and to receive signals and information from the neural tube and neural crest[9,22]. We detected regional differences in the patterning properties of distinct cranial mesoderm populations, but it is not known when these are first established. *Hox* genes are regionally expressed in the presumptive cranial mesoderm, before their activation in the neural epithelium, and may have a role in establishing these regional differences[34].

Our analysis indicates that signals from cranial mesoderm have a permissive rather than an instructive role in regulating *Hox* expression in the neural crest. This result highlights the importance of the initial anteroposterior programme established in the rhombomeres in regulating the ability of NCCs to respond to environmental signals in the branchial arches. Furthermore, there is evidence that signals from the rhombomeres can influence the character of the environment through which the NCCs migrate. In ErbB4-deficient mutants, the loss of ErbB4 signalling in r3 and r5 results in the ectopic anterior migration of r4-derived NCCs[35]. This abnormal migration is accompanied by a downregulation of *Hox* expression in the ectopic location, in agreement with our findings on plasticity of transposed NCCs. Hence, there is an interplay between instructive signals in the neural ectoderm and permissive mesodermal signals in the branchial-arch environments that establish and maintain the anteroposterior identity of cranial NCCs and regulate their pathways of migration[35].

This study, together with our previous analysis of somitic mesoderm[19,21], reveals that both head and trunk mesoderm is involved in regulating patterning processes through the *Hox* genes. Cranial mesoderm is distinct from trunk mesoderm, as it is a loosely packed mesenchymal population that does not form epithelial-like condensations or express somitic markers. Unlike the cranial mesoderm, somites can provide the instructive signals required to establish *Hox* expression in the hindbrain, and this ability is dependent upon retinoid signalling pathways[19,21,36]. In contrast, by applying exogenous retinoids or blocking retinoic-acid signalling, we have shown that retinoic acid is not involved in the cranial mesodermal mechanisms that regulate *Hox* expression in NCCs. Thus the signals in these different mesodermal populations are distinct. It will be important that we determine the precise nature of the signal(s) and how the patterning potential is first established in the cranial mesoderm.

Finally, our results argue against a fixed prepatterning of cranial neural crest cells and indicate that there is not a passive transfer of anteroposterior identity or positional information from the hindbrain to the periphery. The approaches that we have taken have proved valuable in understanding the nature of patterning defects in *erbB4*-deficient and *kreisler* mutant embryos[35,37] and will be useful in studying other craniofacial abnormalities in the expanding array of mouse mutants. □

Methods

Donor and host embryo isolation and culture.

Embryos were obtained from timed matings of CBA × C57/Bl6 mice or from homozygous crosses of transgenic males to F_1 females. Lines were as follows: *Hoxb1*, construct number 1 (ref. 24); *Hoxb2*, construct number 1 (ref. 25); *Hoxa2*, construct number 2 (ref. 26). Host embryos (at 8.25–9.5 d.p.c.) used in culture experiments were dissected from the uterus with an intact visceral yolk sac, amnion and ectoplacental cone[23]. 8.25-d.p.c. host embryos (which had five somites) were cultured *in vitro* for 6–24 h in DR50 medium in a 5% O_2, 5% COs, 90% N_2 atmosphere[38]. 9.5-d.p.c. host embryos were cultured *in vitro* for 24 h in DR75 medium in a 20% O_2, 5% CO_2, 75% N_2 atmosphere[38]. To catch the earliest waves of migrating NCCs in rhombomere-grafting experiments, we used 8.25-d.p.c. donor and host embryos having five or fewer pairs of somites. In each of the grafting, cell-labelling and retinoic-acid/BMS493 treatment experiments described below, we used a minimum of ten embryos. In all types of experiment, embryos were analysed for β-galactosidase activity as described[39].

Isolation of rhombomeres.

A fate map and consistent neuromeric landmarks were used to identify the rhombomeric source of tissue

to be grafted and the correct site of transplantation, as described[40]. Finely polished alloy and glass needles were used to separate the neuroectoderm from adjacent tissues. Rhombomeres that could not be cleanly separated from adjacent tissues were then incubated in 0.5% trypsin, 0.25% pancreatin, 0.2% glucose and 0.1% polyvinylpyrolidone in PBS for 3 min at 37 °C or in Dispase for 3 min at 37 °C to ensure a pure population. Isolated rhombomeres were then washed in DMEM medium before being labelled.

Isolation of mesenchyme from the second branchial arch.

The second branchial arch was isolated from 9.5-d.p.c. embryos using finely polished alloy needles. Two transverse cuts were made along the junctions of ba2 with ba1 and ba3. A longitudinal cut was then made to ensure that ba2 was completely separated from any neuroectodermal tissue. ba2 was then incubated in either 0.5% trypsin, 0.25% pancreatin, 0.2% glucose and 0.1% polyvinylpyrolidone in PBS for 5 min at 37 °C, or in Dispase for 5 min at 37 °C to loosen the ectoderm. Ectodermal tissue was then teased away with glass needles, leaving the mesenchymal core, which consists of mesoderm and NCCs. The mesenchymal cores were then washed in DMEM before being labelled.

Isolation of NCCs from the second branchial arch.

ba2 mesenchyme from transgenic 9.5-d.p.c. embryos was isolated as described above and washed in 250 µl MAH medium (DMEM plus 5% fetal calf serum, 10 mM HEPES, pH 7.2). The tissue was then spun at 3,000 r.p.m. (Sigma model 3K10) at 4 °C for 5 min before being resuspended as single cells in 50 µl prewarmed MAH medium for 10 min. After the tissue was equilibrated in 1 mM chloroquine diphosphate (Molecular Probes), prewarmed 0.2 mM CMFDG (green fluorescence *lacZ* gene expression reagent; Molecular Probes) was added to the suspension, which was then incubated at 37 °C for 10 min. 2 ml ice-cold MAH was then added to the cells to stop the labelling reaction. The mesenchymal suspension was spun at 3,000 r.p.m. for 5 min at 4 °C and then resuspended in 2 ml MAH. The suspension was then incubated at 37 °C for 30 min before fluorescence-activated cell sorting (FACS) using a FACStar (Becton-Dickson) with an argon laser at 488nm. The *lacZ* transgene is expressed only in arch NCCs and not in the mesoderm, enabling a pure population of NCCs to be collected.

Labelling of tissue fragments.

Isolated rhombomeres and ba2 mesenchyme were labelled with a 1:1 mix of DiI:DR50 for 2 min, washed in DMEM and then dissected into smaller fragments consisting of ~10–15 cells suitable for transplantation[37]. FACS-sorted NCCs were labelled with DiI (5µl per ml collected cells), washed in MAH, concentrated by centrifugation at 3,000 r.p.m. and then resuspended in 25µl MAH ready for transplantation. DiI labelling was assessed primarily by fluorescence microscopy (Zeiss Axiovert) but also with a Leica TCS NT confocal microscope (567-nm excitation).

Transplantation experiments.

Small groups of ~15 cells from r2, r3, r4 and r5 of 8.25-d.p.c. (five-somite) donor embryos were orthopically transplanted back to the same site or heterotopically transplanted into other sites in isochronic host embryos. These embryos were cultured for 6–30 h *in vitro*. Groups of 15 cells from r3, r4 and r5 of 8.25-d.p.c. transgenic embryos were transplanted into the first branchial arch of 9.5-d.p.c. host embryos and cultured for 24 h. NCCs or mesenchyme, sorted by FACS, from the second branchial arch of 9.5-d.p.c. transgenic embryos were transplanted into the presumptive first and second branchial-arch environments of 8.25-d.p.c. host embryos and cultured for 24–36 h. Small populations of presumptive second-arch mesoderm and ectoderm were isolated from 8.25–9.0-d.p.c. embryos and heterotopically grafted into the presumptive first arch of 8.25-d.p.c. embryos, then cultured for 24–36 h.

Treatment with retinoic acid and the retinoic-acid inhibitor BMS493.

7.5–8.5-d.p.c. transgenic embryos were treated with all *trans*-retinoic acid *in utero* by oral gavage of pregnant females, using 15µl stock retinoic acid (25 mg ml^{-1}) mixed with 200µl sesame seed oil[28]. Ion-exchange beads (BioRad) were soaked in retinoic acid (1 mg ml^{-1}) for 15 min, washed in DMEM, and then crushed with a pair of forceps into small pieces suitable for grafting into the first branchial arch of 8.5-d.p.c. transgenic embryos. For application in embryo-culture experiments, retinoic acid (25 mg ml^{-1}) was diluted 1,000-fold directly in DR50 culture medium, and BMS493 stock solution (10 mM) was diluted to a final concentration of 1µM DR50 (ref. 30). All embryos were collected at 9.5 d.p.c. for analysis.

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