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ARTICLE

Hoxb1 Neural Crest Preferentially Form Glia of the PNS

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The vertebrate cranial neural crest cells give rise to many complex derivatives of the head, neck, and face, including neuronal and glial cells that act in concert for proper development of the anterior–peripheral nervous system. Several genes have been implicated in the processes of neural crest specification, migration, and differentiation; among these are the hox gene clusters. To determine the fates of hox-expressing cranial neural crest, we describe the results of a genetic lineage analysis by using the Cre/loxP system to drive the activation of different ROSA26 reporter alleles under the regulation of the hoxb1 locus. By targeting the 3′ untranslated region of the hoxb1 gene, we have preserved endogenous gene activity and have been able to accurately follow the fates of the cells derived from the hoxb1 expression domain. Emphasis was placed on identifying the cell and tissue types that arise from the rhombomere 4-derived neural crest. Our results demonstrate that, in addition to forming much of the cartilage, bones, and muscle of the ears and neck, a significant population of rhombomere 4-derived neural crest is fated to generate the glial component of the seventh cranial nerve. *Developmental Dynamics 227:379–386, 2003.* © 2003 Wiley-Liss, Inc.

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INTRODUCTION

The vertebrate hindbrain is organized and patterned as a segmented structure comprised of seven metameric swellings called rhombomeres. Generated early in neural development, these rhombomeres each act as unique signaling compartments and together generate a diverse range of cranial nerves and craniofacial structures (Krumlauf et al., 1993; Kulesa and Fraser, 1998). Along the anterior–posterior (A–P) axis, even numbered rhombomeres harbor progenitor cells of the various brainstem nuclei and serve as the main source of cranial neural crest cells that are destined to populate their corresponding branchial arches (Kandel, 1991; Niederlander and Lumsden, 1996; Kulesa and Fraser, 1998). During rhombomere formation, each segment adopts a unique identity and can be characterized by distinct patterns of gene expression. Among the differentially expressed genes found in the hindbrain, the hox clusters display distinct patterns of rhombomere-restricted expression. Individual hox genes are expressed with segmental periodicity and in a colinear manner along the A–P axis (Duboule, 1994). Thus, each rhombomere expresses a specific combination of hox genes during hindbrain development, generating a "hox code" thought to be in part responsible for determining the identities of individual rhombomeric segments and the tissues originating from them (Wilkinson, 1993).

Mice harboring both individual and compound mutations of the anteriorly expressed hox genes show overt developmental defects in the anterior central nervous system (CNS) and/or craniofacial structures. Hoxb1 null mutants exhibit very distinct effects on the developing facial nerve originating from the rhombomere 4 (r4) domain of the developing hindbrain (Goddard et al., 1996; Studer et al., 1996). Characterized by significant muscular atrophy and facial paralysis, the hoxb1 null phenotype has been attributed to the selective loss of the facial brachiomotor neurons that normally innervate the muscles of facial expression. Of interest, compound mutations in hoxa1 and hoxb1 have directly implicated a functional role

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for hoxb1 in the normal development of r4-derived neural crest and its ability to form second branchial arch tissues (Gavalas et al., 1998; Rossel and Capecchi, 1999). In these mutants, the r4-derived neural crest cells fail to develop and migrate correctly; consequently, all second arch derivatives are missing. Taken together, these models provide genetic evidence that hoxb1 plays a direct role in the development of both central and peripheral components of the developing nervous system at the level of r4. Specifically interested in understanding the roles for hoxb1 in the cranial neural crest cells and how they impinge on the development of the seventh cranial nerve, we set out to conduct a genetic lineage analysis to identify the specific cell types that originate from r4.

Several studies have focused on the specific cell and tissue lineages that are derived from neural crest cells. The most common approach has been to use interspecies transplant studies between chick and quail, generating embryonic chimeras that enable the identification of clonal populations that are derived from either the host or the donor tissues (Le Douarin, 1982; Noden, 1983; Le Douarin et al., 1993). This type of experiment has been extremely valuable in adding to our understanding of patterning and fate specification of the neural crest as a homogeneous population. However, subpopulations of neural crest cells within a given region, unique in specific patterns of gene expression, are difficult to analyze in this manner due to technical limitations. By using a genetic approach, we can overcome these limitations and expand our knowledge regarding the development of neural crest in defined regions of the mouse embryo.

Specification of neural crest-derived structures and associated nervous tissue is known to be a developmentally coordinated program, in which the proper formation of one tissue is directly dependent upon the other (Begbie and Graham, 2001). In efforts to better understand the relationship between the developing seventh nerve and the r4-derived neural crest, we describe the fates of hoxb1-expressing neural crest cells during embryogenesis through Cre-loxP lineage analysis.

RESULTS

Hoxb1 IRES-Cre Recapitulates Endogenous Gene Activity

To follow the fate of hoxb1-expressing neural crest originating from r4, we targeted the hoxb1 locus for gene replacement with an engineered vector containing an internal ribosomal entry site (IRES) driving the expression of bacteriophage P1 Cre recombinase (Cre; Sauer and Henderson, 1988). The IRES-Cre cassette, containing a removable neomycin selection gene, was introduced into the 3′ untranslated region of Hoxb1 (Fig. 1A). Placing the IRES-Cre cassette downstream of the hoxb1 translational stop codon and upstream of the endogenous polyadenylation signal affords us the ability to concomitantly express Cre from the hoxb1 locus without interfering with endogenous hoxb1 activity. In the presence of the appropriate reporter allele, such as ROSA26 (Soriano, 1999) or ROSA-EGFP (Mao et al., 2001), Cre recombinase drives recombination between two loxP recognition sequences, thereby activating the reporter gene and permanently marking the clonal populations of cells derived from the hoxb1-expression domain.

By using standard gene targeting protocols, positive ES cell clones were identified by Southern blotting and hybridization with an external probe 3′ to the region of vector homology, and subsequently verified by probing internally against Cre (Fig. 1B). After blastocyst injection, mice were generated that carry the hoxb1-IRES-Cre driver cassette and were identified by multiplex polymerase chain reaction (PCR) with primers against both wild-type and targeted alleles (Fig. 1C). The neomycin resistance gene was subsequently removed by crossing the hoxb1-IRES-Cre mice to a flp deleter line (Rodriguez et al., 2000), facilitating recombination and excision of sequence between the flanking frt sites. Mice harboring the "clean" allele were tested for appropriate Cre activity by crossing to the ROSA26 reporter strain (Soriano, 1999).

Whole-mount embryos harvested at embryonic day (E) 9.5 were used to characterize the efficacy of the hoxb1-IRES-Cre reporter by comparing X-gal staining patterns, identifying cells derived from the hoxb1 lineage, to the transient expression pattern of hoxb1 by using a polyclonal antibody visualized through horseradish peroxidase activity. The cells and tissues identified through β-galactosidase (β-gal) activity (Fig. 1E) corresponded precisely to the domains expected to be derived from the transient expression domains of hoxb1 (Fig. 1D), verifying that the hoxb1-IRES-Cre driver line faithfully recapitulates endogenous hoxb1 activity and would be useful for conducting an in-depth lineage analysis.

Hoxb1 Is Expressed in Both Mesodermal and Ectodermal Lineages

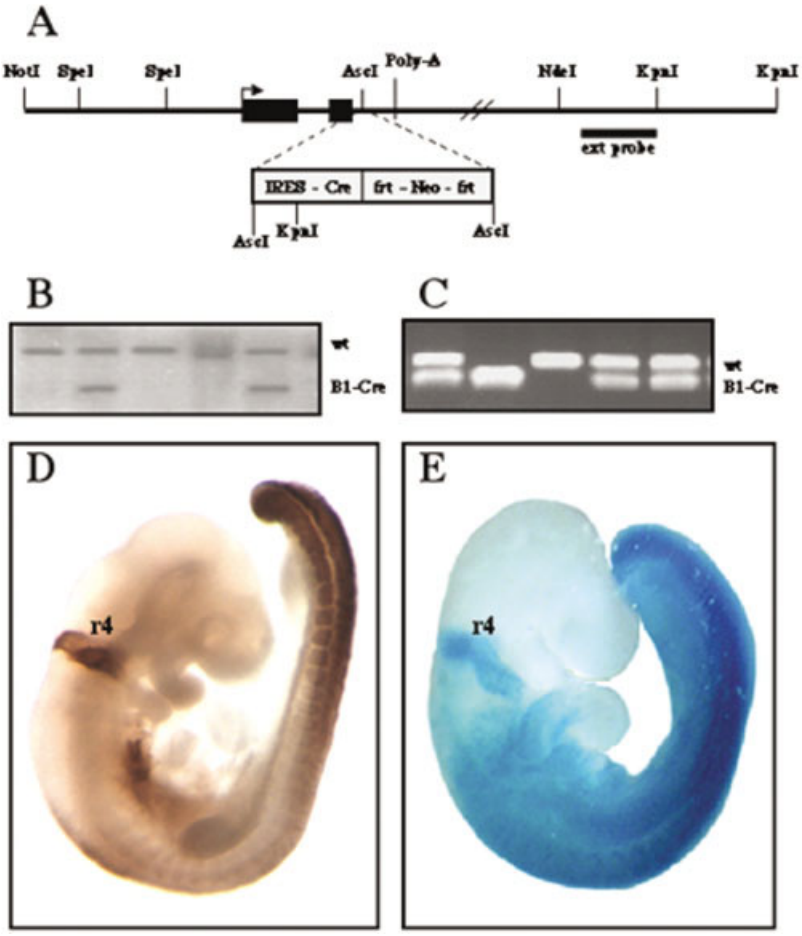
By using the ROSA26 reporter, whole-mount lineage analysis was conducted throughout embryogenesis. X-gal staining was first apparent at E7 in the region of the caudal primitive streak (data not shown), indicating the first activity of the hoxb1 gene. β-gal–expressing cells were easily detectable in mesodermal derivatives that form the somites and caudal tail bud (Fig. 2A). As neurulation proceeds, around the 10- to 11-somite stage, hoxb1-Cre activity is detected in the ectodermal cells of r4. At this point, it appears that the expression in r4 is first detected in the neural crest cells (Fig. 2B), followed by a more widespread expression throughout the entire r4 segment by the time neurulation has completed in this region. By E10.5, the caudal mesodermal derivatives are seen throughout most of embryonic trunk and limbs, with patchy levels of X-gal staining in regions extending rostrally to the caudal hindbrain (data not shown). High levels of β-gal activity persist in the region of r4 through E11.5. This can be seen by the intense X-gal staining within the r4 neuroepithelium and corresponding second branchial arch, which is populated by r4-derived neural crest cells (Fig. 2C,D). By E14.5, hoxb1-deriv-

atives can be seen extending throughout most of the developing embryo, extending caudally from the axial level that was previously demarcated at the r4 boundary (Fig. 2E). In addition to the mesenchymal expression in the body, hoxb1 lineages also give rise to specific ectodermal derivatives associated with the head, neck, and face. In addition to the cartilage and bones of the neck and external ear, cells of the tongue, salivary gland, and pterygopalatine ganglia also exhibited high levels of X-gal staining (data not shown).

As would be expected, due to the high levels of hoxb1 expression in the hindbrain and previously described function in the development of the seventh cranial nerve (Goddard et al., 1996; Gaufo et al., 2000), X-gal staining is strongly localized within cell bodies and axonal tracts of the facial nucleus (Fig. 2F,G, and data not shown). Moreover, high levels of β-gal activity were localized in cells associated with the ventrolateral exit points of the seventh cranial nerve. Adult brain slices stained for β-gal activity do not show any staining rostral to the pons, suggesting that, within the CNS, hoxb1 gene expression is active only during embryonic development and restricted to regions of the developing brainstem (Fig. 2H).

Hoxb1-Expressing Neural Crest Gives Rise to Peripheral Glia

To determine the identity of the hoxb1-expressing r4-derived neural crest that associate with the developing facial nerve, lineage analysis was combined with immunohistochemistry for molecular marker colocalization studies. For this analysis,



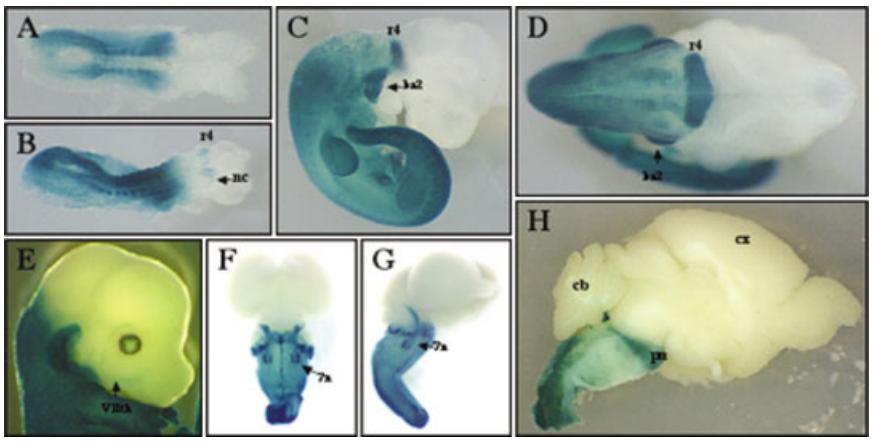


Fig. 1. Hoxb1-IRES-Cre targeting vector design and lineage analysis. **A:** The hoxb1-IRES-Cre targeting vector, harboring the IRES-Cre cassette inserted into the 3′ untranslated region. Bold black boxes represent exons. **B:** Southern blot using external probe fragment shown in A. Wild-type allele (wt) is 12 kb, whereas the targeted allele (B1-Cre) is 9 kb. **C:** Polymerase chain reaction genotyping of hoxb1-IRES-Cre mice. Wild-type (wt) allele is identified as a 301-bp fragment and targeted allele is (B1-Cre) 264 bp. **D:** Wild-type embryonic day 9.5 mouse embryo immunoreacted with an anti-hoxb1 antibody and developed with diaminobenzidine. **E:** X-gal staining of an embryonic day 9.5 hoxb1-IRES-Cre embryo harboring the ROSA26 allele. r4, rhombomere 4.

Fig. 2. Whole-mount lineage analysis in hoxb1-IRES-Cre mice using the ROSA26 reporter. **A:** Dorsal view of a five-somite embryo showing expression in caudal primitive streak and mesodermal components. **B:** Dorsolateral view of a nine-somite embryo showing hoxb1 lineage in the migrating neural crest emanating from r4. **C:** A lateral view of an embryonic day (E) 10.5 embryo. Neural crest derivatives populate the ba2. **D:** Dorsal view of the embryo shown in C. **E:** View of an E14.0 embryo. X-gal staining is observed in mesenchymal derivatives and outgrowing facial nerve. **F:** Ventral view of E14.0 central nervous system. X-gal staining in facial nucleus highlights previous hoxb1 activity. **G:** Lateral view of tissue in E. **H:** Adult brain slice showing restricted lineage contributing to the pons. r4, rhombomere 4; nc, neural crest; 2ba, second branchial arch; VIIth, seventh cranial nerve; 7n, seventh motor nucleus; pn, pons; cb, cerebellum; cx, cortex.

ROSA-EGFP reporter lines (Mao et al., 2001) were crossed to the hoxb1-IRES-Cre mice. Resulting offspring maintain a strong green fluorescent protein (GFP) expression in all cells derived from the hoxb1 expression domain, facilitating the identification of specific cell types by colocalization with various molecular markers. Frozen transverse sections at the level of r4 show that cells of the hoxb1 lineage are detected in both the CNS and peripheral nervous system (PNS), localized primarily to the seventh/eighth sensory ganglion complex and the seventh cranial nerve (Fig. 3A). By using an antibody directed against P75, the low-affinity neurotrophin receptor, neural crest cells can be identified (Mujtaba et al., 1998; Morrison et al., 1999). By colocalization with the GFP reporter, we found that only a subset of neural crest associated with the outgrowing seventh nerve are derived from the hoxb1 lineage (Fig. 3B), suggesting that hoxb1 neural crest cells give rise to specific cell types in the PNS.

Surprisingly, when targeted with antibodies against neuronal markers, very few cells associated with the seventh nerve were neuronal in nature. By using the β-III-tubulin–specific antibody Tuj1, the only regions of colocalization with the hoxb1 lineage are in the cells within the neural tube and outgrowing motor projections of the facial nerve (Fig. 3C). This phenomenon was verified by using the anti-NeuN marker, which localizes to the nucleus of terminally differentiated neurons. We did not observe significant GFP expression in the neuronal cell bodies associated with the sensory ganglia complex (Fig. 3D).

Having determined that very few, if any, hoxb1-expressing neural crest

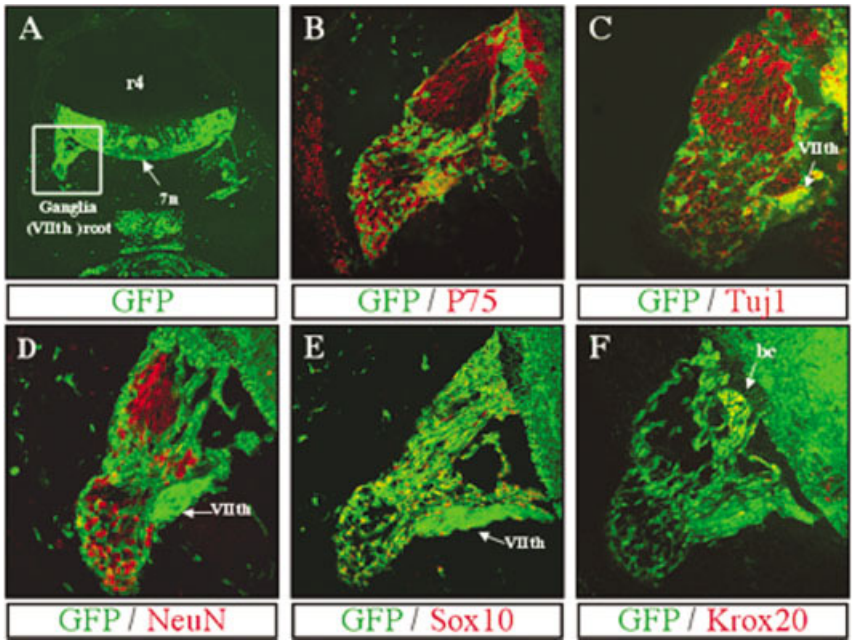


Fig. 3. Marker analysis to identify cell types of the hoxb1 neural crest lineage. Sections were taken from embryonic day 11.5 hoxb1-IRES-Cre embryos that harbor an activated allele of ROSA-EGFP. Various molecular markers (shown in red) are colocalized with green fluorescent protein (GFP) expression for cell fate identification (yellow). **A:** Transverse section through r4, showing domains of hoxb1 lineage through the activated expression of ROSA-GFP (5× mag). Inset represents region of interest in other panels (all shown at 20× mag). **B:** P75 expression in peripheral nervous system (PNS) marking general neural crest-derived cells. **C:** β-III-tubulin (Tuj1) expression in PNS localizing to nerve fibers. **D:** NeuN expression in PNS localizing to neuronal nuclei. **E:** Sox10 expression in glial progenitor cells of the PNS. **F:** Krox20 expression colocalizing to boundary cap cells. VIIth, seventh nerve; r4, rhombomere 4; 7n, seventh motor nucleus; bc, boundary cap cells.

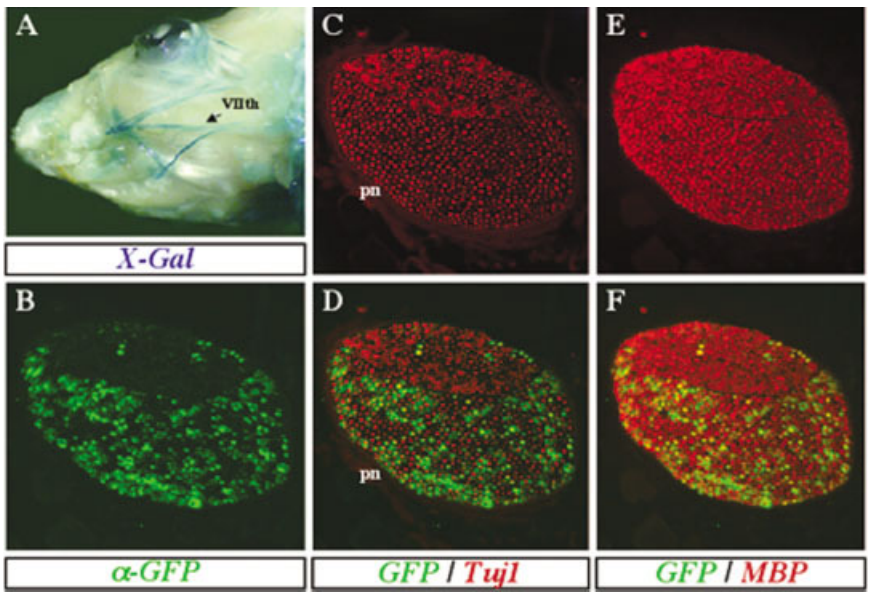


Fig. 4.

Fig. 4. Lineage analysis in the adult peripheral nerve. **A:** X-gal staining in a hoxb1-IRES-Cre; ROSA26 background of an adult head showing specific β-galactosidase activity in the facial nerve. Arrow identifies region of peripheral nerve section in other panels. **B–F:** Marker analysis in hoxb1-IRES-Cre; ROSA-EGFP background of seventh peripheral nerve cross-sections. **B:** Localization of activated EGFP expression to a subset of cells within the seventh nerve. **C:** β-III-tubulin (Tuj1) expression in peripheral nerve cross-section highlighting axonal projections. **D:** Colocalization of EGFP expression to a subset of motoneurons within the peripheral nerve (yellow). **E:** Myelin basic protein (MBP) expression in peripheral nerve cross-section highlighting myelinating glial cells. **F:** Colocalization of EGFP expression with many cells of the peripheral glia. VIIth, seventh nerve; pn, perineurium; GFP, green fluorescent protein.

give rise to neurons of the PNS, we next examined the ability of the hoxb1 lineage to give rise to peripheral glia. By using an antibody directed against the HMG domain containing transcription factor sox10 (Britsch et al., 2001; Paratore et al., 2001), we found perfect colocalization of this marker with the GFP-expressing hoxb1 lineage (Fig. 3E). Within this glial progenitor population, we also observed nested colocalization of hoxb1 neural crest within the presumptive boundary cap cells (Niederlander and Lumsden, 1996), demarcated by the expression of the homeodomain containing transcription factor Krox20 (Niederlander and Lumsden, 1996; Garratt et al., 2000; Fig. 3F). Taken together, these data reveal that the r4-derived, hoxb1-expressing neural crest preferentially give rise to the glial progenitor cells associated with the boundary cap and developing motor component of the seventh nerve complex.

To follow the fate of hoxb1-derived, cranial neural crest and peripheral glial precursors through development, we turned to tissue and cellular lineage analysis in the adult. Adult mice harboring both the LacZ reporter and hoxb1 driver alleles were first studied at the gross anatomic level by means of the readout of β-gal activity. Mice were fixed, washed, and reacted by perfusion of X-gal staining reagents through the circulatory system. Upon removing skin and fascia, tissues were allowed to react further in X-gal staining solution. β-gal was strongly expressed in all branches of the facial nerve, localizing to a subset of unidentified cell types within the peripheral nerve bundle (Fig. 4A). This finding led us to undertake a more detailed analysis of the cells that comprise the peripheral nerve, turning to immunohistochemistry for specific cellular identification.

Again we used mice harboring the GFP-ROSA reporter activated by the hoxb1-IRES-Cre driver. Upon dissection of underlying muscle and associated seventh nerve bundles, tissues were fixed, embedded, and cross-sectioned for immunohistochemistry. By using an antibody directed against GFP to enhance the cellular resolution, we were able to discern at least two different cell types within the main fascicle (Fig. 4B). As expected, strong, punctate regions of GFP expression marked individual motor axons extending from the facial nucleus throughout the area of the nerve bundle. The neuronal nature of these processes was confirmed by colocalization with Tuj1-immunoreactivity (Fig. 4C,D). Next, we addressed the identity of the other cell types within the bundle that also expressed the GFP reporter. By using an antibody directed against the Schwann cell marker, myelin basic protein (MBP), we were able to identify many of the remaining GFP-positive cells as myelinating peripheral glia (Fig. 4E,F). Of interest, GFP-positive glia specifically localized to the motor axons of the facial nerve. Moreover, hoxb1-derived Schwann cells were not found in association with the sensory component of the facial nerve, as indicated by the absence of GFP expression. Taken together, these data reveal that hoxb1 neural crest gives rise to a majority of the cells that make up the seventh cranial nerve and that, amongst these cells, a significant percentage are destined to become peripheral glia.

DISCUSSION

The cranial neural crest is an extremely dynamic population of cells. Giving rise to complex derivatives such as cartilage, bone, muscle, glands, neurons, and glia, the neural crest exemplify a bona fide population of restricted stem cells. Several studies have focused on determining the patterns of gene expression and molecular profiles of neural crest cell pools that make them unique within the developing vertebrate embryo. However, this has proven to be an ongoing challenge, due to the dynamic processes of cell adhesion, migration, and differentiation that the cranial neural crest cells exhibit throughout early development.

Among the genes that have been identified to be important for neural crest development, specific members of the hox gene clusters have been shown to impinge upon these processes. Through gene expression studies, in addition to loss of function in the mouse and gain of function in the chick, it has been demonstrated that the most 3′ hox genes tend to have the most profound effects on development of the cranial neural crest (Manley and Capecchi, 1997; Gavalas et al., 1998; Bell et al., 1999; Rossel and Capecchi, 1999). In many cases, it has been difficult to completely understand the correlation between the results obtained through genetic manipulations and embryonic gene expression studies due to the transient expression patterns of individual hox genes within individual populations of neural crest cells. Hox genes that are turned on in any given pool of neural crest cells are rapidly down-regulated upon completion of migration into their respective branchial arch tissues. One way in which this issue has been addressed has been through transplantation studies in both the chick and mouse model systems using genetically marked cells that can be monitored over time (LeDouarin, 1982; Le Douarin et al., 1993; Trainor and Krumlauf, 2000; Trainor et al., 2002). Although very enlightening, these experiments still present technical limitations associated with transplantation protocols in identifying the ultimate fates of individual cells within homogeneous pools.

Our lab has long been interested in the roles the hox genes play throughout development and how they influence the fates of the neural crest. In this study, we have taken a genetic approach using the Cre/Loxp recombination system (Sauer and Henderson, 1988) to address the issue of cell fate choices in the cranial neural crest, focusing on the population of hoxb1-expressing neural crest cells that are destined to form components of the PNS.

By targeting the hoxb1 locus for gene replacement with an IRES-Cre, bicistronic expression cassette, we have been able to describe the specific cell fates that arise from the r4-derived, hoxb1-expressing cranial neural crest cells. This approach has been extremely valuable for cell lineage studies in populations of cells that express a particular molecular

marker of interest, for a transient period of time.

Hoxb1 lineage analysis in the early embryonic stages faithfully reproduced previously reported expression patterns in the caudal primitive streak and mesodermal derivatives (Wilkinson et al., 1989; Frohman et al., 1990). Lineage tracing confirms that hoxb1 is one of the earliest and most widely spread hox gene to be expressed along the A–P axis of the mouse embryo, a phenomenon substantiated by the X-gal staining pattern through E14.0 (Fig. 2).

Ectodermally, lineage analysis revealed novel information regarding hoxb1 expression in the hindbrain neuroepithelium. It has been previously accepted that hoxb1, after first being expressed in the caudal primitive streak (E7.5), extends in expression domains rostrally from the caudal neuropore to the anterior boundary of r4 within the neural tube (E8.5). Upon initiating an autoregulatory loop within the r4 segment, expression domains regress caudally to a more cervical level, while maintaining high expression in r4 through mid-embryogenesis (E11.0; Wilkinson et al., 1989; Frohman et al., 1990; Gaufo et al., 2000). Our temporal study using the hoxb1-IRES-Cre allele has revealed that, in fact, hoxb1 is initiated within r4, first detectable in the migrating neural crest by the eight-somite stage, followed by maintenance in all layers of the r4 neuroepithelium through E11.0. This is a novel finding, demonstrating that hoxb1 expression is initiated and maintained in r4, before and independent of regulation in more caudal domains. As the hoxb1-expressing neural crest lineage was of primary interest, we turned our attention toward these cells with respect to the outgrowing seventh cranial nerve.

In addition to giving rise to many of the second arch derivatives of the ear, neck, and lower mandible, a specific population of hoxb1-derived cells was identified in close association with the outgrowing facial nerve. It was surprising that very few of these neural crest cells gave rise to peripheral neurons, but instead appeared to selectively generate peripheral glial precursors. Observed to localize at the boundary cap along the region that corresponds to the facial nerve exit point, hoxb1-derived glial precursors are the first cells that the pioneering motor axons of the seventh nerve encounter. As nerve outgrowth continues, individual axons are eventually ensheathed by peripheral Schwann cells that express the activated ROSA reporter. Of interest, in peripheral facial nerve cross-sections, the majority of the facial branchiomotor neurons observed extending to their final targets are insulated by Schwann cells derived from the hoxb1-expressing r4 neural crest.

Previous work has shown that many of the neurons associated with the seventh/eighth complex are of placodal origin, whereas the remainder originates from the neural crest component (Begbie and Graham, 2001). Our results are largely in agreement with previous studies describing the fates of cells originating from the cranial neural crest region. Unexpectedly, we found that very few hoxb1-expressing neural crest gave rise to sensory neurons associated with the seventh ganglia but instead preferentially formed glial precursors. This discrepancy may be reconciled by a couple of possibilities: (1) the timing, efficacy, or expression of Cre recombinase may not be in perfect register with hoxb1 regulation, resulting in a Cre dosage effect and failing to label all cells descendant from the hoxb1-expression domain. (2) Hoxb1 is expressed in restricted subsets of neural crest cells originating from r4, whereas other hox genes, such as hoxa1, may be responsible for the differentiation of sensory neurons of the anterior PNS. Based on existing data describing the hoxa1 and hoxa1/b1 double mutant mice (Gavalas et al., 1998; Rossel and Capecchi, 1999), we favor the latter possibility.

The correlation drawn between the close interactions of r4-derived glial cells and the outgrowing facial nerve presents an intriguing possibility that the molecular program governing the development of the seventh cranial nerve is dependent upon hoxb1, not only in the developing facial branchiomotor neurons but also in the presumptive glial cells that contribute to the seventh nerve fascicle. This prediction would suggest that hoxb1 has nonautonomous roles in facial nerve development and could contribute to the phenotype observed in the hoxb1 null mutant. In this scenario, r4 neural crest are programmed, through the activity of hoxb1, to specifically interact with outgrowing motor axons of the facial nerve to provide support, guidance, or target coordinates for proper neuronal development. A nonautonomous role for hoxb1 may also rationalize the incomplete penetrance associated with the hoxb1 null mutant, a conceivable notion if glial cells derived from other rhombomeres are able to partially compensate for r4 neural crest function (Couly et al., 1996; Voiculescu et al., 2000, 2001). This is a testable hypothesis and is currently being pursued using genetic models.

EXPERIMENTAL PROCEDURES

Cloning and Mouse Breeding

The hoxb1 genomic locus was isolated from an SV 129 lambda library by using oligonucleotide probes to the 3′ UTR. An 11-kb *Not*I-*Nde*I genomic fragment, containing the hoxb1 coding sequence was subsequently shuttled into a pUC backbone harboring the thymidine kinase gene. Introducing an AscI site directly downstream of the translational stop codon, provided a unique cloning site for the insertion of the IRES-Cre cassette. The IRES-Cre cassette was assembled by cloning Cre-recombinase in phase with the IRES element and positioned upstream from a neomycin selection gene flanked with frt sites for subsequent removal by flp recombinase. The entire cassette was inserted into the engineered *Asc*I site and the targeting vector was electroporated into ES cells, followed by culturing under positive and negative selection. Digesting genomic DNA with *Kpn*I, Southern blotting, and hybridizing with a downstream external probe, in addition to a Cre-specific, internal probe identified positive clones. Upon injecting positive clones into host blastulas, resulting chimeric offspring

gave rise to agouti founders that were genotyped for the presence of the IRES-Cre cassette. PCR primers ACGCAGGTGAAGATCTGGTT (for), CTGGGCAGCTCTAAACTGGT (rev), and CTCGACATTGGGTGGAAACA (ICN) were multiplexed to identify wild-type (301 bp) and engineered (264 bp) alleles, respectively. Mice homozygous for the targeted "knock-in" allele resembled wild-type littermates. Lineage analysis was conducted by crossing the hoxb1-IRES-Cre driver line to the previously described LacZ and GFP ROSA reporter strains (Soriano, 1999; Mao et al., 2001).

X-gal Staining

Embryos harboring both the LacZ-ROSA reporter and hoxb1-IRES-Cre driver alleles were removed from pregnant females in phosphate buffered saline (PBS) with 2 mM $MgCl_2$. Embryos were fixed (2% paraformaldehyde, 1.25 mM ethyleneglycoltetraacetic acid, 2 mM $MgCl_2$, 0.1 M PIPES pH 6.9), washed in PBS, 2 mM $MgCl_2$, and moved into X-gal staining solution (15 mM $K_3Fe(CN)_6$, 15 mM $K_4Fe(CN)_6$-^3H_2O, 2 mM $MgCl_2$, 0.01% Na deoxycholate, 0.02% NP40 in PBS, pH 7.2). Adult tissues were fixed by perfusion, followed by rinsing, perfusion, and immersion into X-gal staining solution. All staining was carried out overnight at room temperature while rocking.

Immunohistochemistry

Embryonic and adult tissues were dissected in PBS and fixed for 3 hr at 4°C with 4% paraformaldehyde in PBS, pH 7.2. Samples were washed in PBS, equilibrated to 30% sucrose, and embedded in O.C.T. Serial sections were cut at 10 microns and mounted on Superfrost plus slides. Sections were washed in PBS and immersed in blocking solution (2% bovine serum albumin, 5% normal goat serum, 0.1% Triton in PBS, pH 7.2). Primary antibodies were diluted in blocking solution and incubated on tissue sections overnight at 4°C. anti-GFP polyclonal (Molecular Probes 1:1,000), anti-P75 monoclonal (Chemicon 1:250), anti-Tuj1 monoclonal (Covance 1:1,000), anti-NeuN monoclonal (Chemicon 1:400), anti-Sox10 monoclonal (Dr. Michael Wegner, 1:50), anti-Krox20 polyclonal (Covance 1:300), anti-MBP monoclonal (Chemicon 1:75). Alexa Fluor–conjugated secondary antibodies (anti-Rb or anti-Ms, Molecular probes) were used at a dilution of 1:500 in blocking solution, and incubated at 4°C for 4 hr.

Immunodetection and fluorescence confocal microscopy was carried out by using Bio-Rad MRC1024 instrumentation and software. Whole-mount immunohistochemistry using the anti-Hoxb1 polyclonal (Covance 1:300) was visualized by using the secondary anti–Rb-horseradish peroxidase chromogenic reaction and diaminobenzidine substrate.

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