ALTERED NEURONAL LINEAGES IN THE FACIAL
GANGLIA OF *Hoxa2* MUTANT MICE

by

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Altered Neuronal Lineages in the Facial Ganglia of *Hoxa2* Mutant Mice

Abstract

By

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Neurons of cranial sensory ganglia are derived from the neural crest cells (NCCs) and ectodermal placodes, while the glial cells are exclusively derived from the crest. Neural crest and placodal cells generate different portions of neurons in different ganglia. For example, crest cells of the second branchial arch (BA2) generate very few neurons in facial ganglion and none in the vestibuloacoustic ganglion, but in other branchial arches crest cells generate many sensory neurons. Until now, the mechanisms that control the relative contributions of these sources in different ganglia have not been well understood.

HOX genes play critical roles in controlling the behavior of neural crest cells at different axial levels in craniofacial tissues. A striking example is the requirement of *Hoxa2* function in neural crest that arise from the fourth rhombomere (r4) of the hindbrain to form the mesenchyme of the second branchial arch. Unexpectedly, we found that the facial ganglia of *Hoxa2* mutant mice contain a large population of crest-derived neurons that are not present in wild-type embryos, suggesting that *Hoxa2* normally represses the neurogenic
potential of second arch crest cells.

One paradigm that was used to test this hypothesis was the overexpression of Hoxa2 in cultures of P19 embryonal carcinoma cells. In high density cultures, P19 cells can spontaneously differentiate into neurons as detected by the expression of PGP9.5, a pan-neuronal marker. After over-expressing Hoxa2 in P19 cells, we detected a reduced frequency of spontaneous neuronal differentiation, but only when P19 cells were co-transfected with two Hox cofactors, Pbx1 and Meis1. Interestingly, the inhibitory function of Hoxa2 in neural differentiation could be antagonized by co-expressing one of Hox3 paralogs, Hoxd3.

Finally, ectopic expression of Hoxa2 and its cofactors in chick neural crest cells populating the trigeminal ganglion also reduced the frequency of neurogenesis by neural crest cells in the intact embryo. These data suggest an unanticipated role for HOX genes in controlling the neurogenic potential of at least some cranial neural crest cells, and suggest their possible roles in modulating neuronal differentiation by other populations of crest cells warrants further scrutiny.
Chapter 1. Introduction
General introduction:

Neurons in cranial ganglia are derived from both neural crest cells and ectodermal placodes (D'Amico-Martel and Noden, 1983; Ziller and Smith, 1982). However, the contribution of these two sources varies significantly in different ganglia. The mechanisms that control such variations in different ganglia are not known. HOX genes modulate the mesenchymal fate of the neural crest in many branchial arches (Creuzet et al., 2002; Rijli et al., 1993). Whether HOX genes modulate the neuronal fate of neural crest cells is not known. We observed that Hoxa2 deficient mice have an increased number of crest-derived neurons in the facial ganglion which led us to hypothesize that Hoxa2 function represses neuronal differentiation of neural crest cells. To test our hypothesis, we ectopically expressed Hoxa2 in both P19 cells and chick embryos, and determined the effects of the Hoxa2 over-expression on neuronal differentiation in vitro and in vivo.

General introduction of cranial ganglia

The generation of the proper number of sensory neurons is critical for accurately relaying various stimuli from the environment and non-neural tissues to the central nervous system. Dorsal root ganglion neurons, whose cell bodies are located in dorsal root ganglia (DRG) lateral to the spinal cord, are groups of sensory neurons, and all of them are derived from the neural crest (Schoenwolf, et al., 1985). They transmit information from the neck, limbs, and trunk to the
central nervous system. The organization and embryonic origins of DRGs at all levels of the spinal cord are highly stereotyped. In contrast to the DRGs, there are significant variations among the cranial sensory ganglia in terms of location, embryonic origin and the sensory modalities they attend to.

The most anterior cranial ganglion is the trigeminal ganglion (V) that develops within the first branchial arch, and acts as a relay station for somatosensory stimuli such as temperature, pain and touch from the facial skin, jaws and teeth. In the second branchial arch, the facial ganglion (associated with facial nerve, VII) and the vestibuloacoustic ganglion (associated with vestibule-acoustic nerve, VIII) are adjacent to each other. The facial ganglion (also called the geniculate ganglion) primarily conveys the sensation of taste from the anterior two thirds of the tongue, but a minor component of the ganglion mediates somatosensory sensation from a small region of skin at the back of the ear. The vestibuloacoustic ganglion has two major components, as its name implies. The vestibular component innervates the semi-circular canals, the saccule and the utricle, and relays proprioceptive information. The acoustic component innervates the organ of Corti and mediates hearing.

At more posterior cranial levels, the ganglia associated with glossopharyngeal nerve (IX) and vagus nerve (X) are each divided into two subdivisions: the proximal subdivisions that include the superior (IX), and jugular ganglia (X), and the distal subdivisions that include the petrosal (IX) and nodose (X) ganglia. Both the glossopharyngeal and vagus nerve innervate small patches
of surface skin, the mucous membranes and body organs. Sensory axons in the
glossopharyngeal (IX) nerve innervate taste buds in the posterior one third of the
tongue, and the mucous membranes of the pharynx, a portion of the nasal
cavities and the sinuses. Those in the vagus (X) nerve innervate the mucosa of
the hypopharynx, the larynx, the esophagus, and the thoracic and abdominal
viscera.

Unlike the sensory neurons in dorsal root ganglia which are solely derived
from neural crest cells, sensory neurons in cranial ganglia are mosaic
populations derived from two origins: one being neural crest cells, the other
being ectodermal placodes (Ayer-LeLievre and LeDouarin, 1982; d'Amico-Martel
and Noden, 1983). Gangliogenesis starts with condensation of neural crest cells,
following their delamination from the neural tube and migration towards the
cranial anlagen. The crest cells are subsequently joined by neuroblasts that
delaminate from epidermal placodes (Johnston, 1966; Noden, 1975; Tosney,
1982). Ablation studies in chick embryos have demonstrated that neural crest
cells are required for establishing central connections of sensory neurons
generated by ectodermal placodes (Begbie and Graham, 2001). Conversely,
neural crest-derived neurons fail to mature and establish normal peripheral
projections when their contact with placodal cells is prevented (Hamburger,
1961).

**Placodes:**

1. *Localization of placodes and their derivatives*
The ectodermal placodes were described in 1883 by van Wijhe in shark embryos (Webb and Noden, 1993), although the term first came into use in 1891 (Von Kupffer, 1891). Identified as transient focal thickenings of the embryonic ectoderm, placodes are histologically pseudostratified epithelia that form at the border of the neural plate and epidermis. Neurogenic ectodermal placodes are found exclusively in vertebrates, and underlie the special senses, such as olfaction, hearing, balance and taste (Gans and Northcutt, 1983). In addition to generating neurons of the cranial ganglia, placodes also generate sensory receptor cells and sensory epithelia.

In antero-posterior order, the cranial placodes include the olfactory, hypophyseal, lens, trigeminal, otic, lateral line and epibranchial placodes (Reviewed by Baker and Bronner-Fraser, 2001). The olfactory placode emerges immediately in front of the neural plate and gives rise to the olfactory and vomeronasal epithelia, a population of migratory GnRH neurons, and the glia that will ensheath the olfactory nerve (Brunjes and Frazier, 1986; Dellovade et al., 1998; Chuah and Au, 1991; Couly and Le Douarin, 1985). The hypophyseal placode gives rise to the endocrine adenohypophysis (Eagleson et al., 1986). The lens placode is entirely non-neurogenic and gives rise to the lenses of the paired eyes.

The ectodermal placodes that generate neurons of the cranial ganglia can be divided into dorsal (trigeminal and otic) and ventral (epibranchial) groups that,
in addition to their physical segregation, also require different neurogenic factors to produce neurons (Ma et al., 1998; Fode et al., 1998). Most notably, neurogenesis by the dorsal placodes requires Neurogenin1 function, while neurogenesis by the ventral placodes requires Neurogenin2. The most anterior of the dorsal placodes are the trigeminal placodes that form at the level of midbrain-hindbrain junction. There are actually two trigeminal placodes, the ophthalmic and the maxillomandibular (Verwoerd and van Oostrom, 1979; D'Amico-Martel and Noden, 1983). In anamniotes, these two placodes give rise to separate ganglia while in higher vertebrates the ganglia fuse together (Hamburger, 1961; Northcutt and Brandle, 1995). The trigeminal placodes are solely neurogenic, forming cutaneous sensory neurons in the distal portion of the trigeminal ganglion (Stark, 1997). The other dorsal placodes are the otic placodes in all vertebrates, and the closely associated lateral line placodes in anamniotes (Nothcutt et al., 1994). The otic placodes give rise to the primary sensory cells of the inner ear and vestibular organs for auditory and proprioceptive sensation (Riley and Phillips, 2003), and to all of the neurons of the vestibuloacoustic ganglia. The lateral line placode generates the sensory cells of the lateral line system which include both mechano-and electoreceptors.

The ventral neuron-generating ectodermal placodes are generally referred to as epibranchial placodes, reflecting their position at the dorsal margin of the branchial arches, immediately adjacent to where the pharyngeal pouches contact the ectoderm (Webb and Noden, 1993; Verwoerd and van Oostrom 1979; D'Amico-Martel and Noden, 1983). There are three epibranchial placodes:
geniculate, petrosal and nodose placodes (Northcutt and Brandle, 1995). Like the trigeminal placodes, they are entirely neurogenic. In vertebrates, the most anterior epibranchial placodes (the facial or geniculate placodes) give rise to the sensory neurons in the facial ganglion of cranial nerve VII. The second epibranchial placode (glossopharyngeal or petrosal) gives rise to the distal (petrosal) ganglion of glossopharyngeal nerve (cranial nerve IX) which innervates taste buds on the rest of the tongue, and other visceral organs. The third epibranchial placode (vagal or nodose) gives rise to neurons in distal (nodose) ganglion of vagus nerve (cranial nerve X), which primarily innervates heart and visceral organs. The supporting cells of all of these ganglia are derived from neural crest cells. Neural crest cells also generate some neurons of the trigeminal ganglion and all neurons in the proximal ganglia of the glossopharyngeal and vagus nerves, which is an important point for the studies presented here.

2. Initial formation of the pre-placodal ectoderm (PPE)

It is thought that all placode precursors arise from a common ectodermal domain, the pre-placodal ectoderm (PPE), which is located in the anterior region of the embryo, medial to the epidermis and lateral to both the neural crest and neural plate (Knouff, 1935). The region first develops as undifferentiated non-neural ectoderm that is competent to form many different placodes (Knouff, 1935). Various signals and interactions are involved in the initial formation of the PPE.
Several general hypotheses have been proposed to explain the mechanisms by which PPE is initially induced. First, many signals that are involved in the induction of neural plate, such as bone morphogenetic protein (BMP) antagonists (Noggin, Chordin, etc) and fibroblast growth factors (FGFs), are also responsible for PPE induction (Glavic et al., 2004; Brugmann et al., 2004; ) BMP family members from the nonneural ectoderm are believed to promote epidermal fate by inducing genes expressed by presumptive epidermis (Wilson and Hemmati-Brivanlou, 1995; Suzuki et al., 1997; Feledy et al., 1999; Beanan and Sargent 2000; Lou et al., 2001). On the other hand, several BMP antagonists, such as Chordin, Follistatin and Noggin, are secreted from the dorsolateral mesoderm to promote induction of neurogenic tissue from dorsal ectoderm (Marchant et al., 1998; LaBonne and Bronner-Fraser, 1998; Nguyen et al., 1998). Since BMP antagonists presumably diffuse from the dorsal midline tissue, and PPE originates from an area lateral to neural plate and medial to the ectoderm, it has been suggested that PPE forms in response for intermediate levels of neural inductive signals (Baker and Bronner-Fraser, 2001). Interestingly, neural crest cells are also generated lateral to the neural plate, but medial to PPE region. Therefore, the same signals are also responsible to the induction of neural crest cells, but likely at different concentrations.

A particularly striking difference between the domains of these two neurogenic systems (PPE and neural crest cells) is that they are distributed in different but overlapping areas along the anterior-posterior axis. Neural crest
cells are excluded from the most anterior area of the neural tube while placodes
are restricted to the cranial region. Various studies indicate that posteriorizing
factors (Wnt, FGF, retinoic acid) initially promote neural crest fate over placodal
fate (Gould and Grainger, 1997; Brugmann et al., 2004; Popperl et al., 1997;
LaBonne and Bronner-Fraser, 1998). Importantly, two dorsal neural tube markers,
Pax3 and Slug, are thought to distinguish placodes and neural crest cells at later
developmental stages (Buxton et al., 1997). Slug, a member of the Snail family of
zinc-finger transcription factors, is expressed by pre-migratory and migrating
neural crest cells, while Pax3 is expressed in the ectodermally derived cells from
some placodes (Nieto et al., 1994; Liem et al., 1995; Buxton et al., 1997; Stark et
al., 1997; Sefton et al., 1998).

In addition to the BMP gradient, interactions between the neural plate and
epidermis are also required for the generation of PPE (Selleck and Bronner-
Fraser, 1995&2000; Woda et al., 2003; Mancilla and Mayor, 1996). First, placode
and neural crest induction seems to require more than just an intermediate
concentration of a BMP antagonist. This is because the BMP4 transcript is most
highly expressed at the epidermal/neural plate border (Streit and Stern, 1999).
This is in conflict with a simple gradient model for neural crest and placode
induction in which the level of BMP4 should be the highest at the lateral
epidermis, and only moderate at the epidermal/neural plate border. However, it
should also be noted that the transcription levels are not always a reflection of
the protein concentration or functional level. Additionally, transplantation studies
have shown that grafting neural plate tissue into non-neural ectoderm in several
vertebrate embryos causes the ectopic induction of neural crest and placodal markers (Streit and Stern, 1999; McLaren et al., 2003), implying that the interaction between neural plate and epidermal tissue is sufficient to induce the neural crest and PPE border. Presumably, this interaction modulates the relative sizes of the different ectodermal subdomains (epidermis, PPE, neural crest and neural plate) in normal developing embryos.

Finally, a few transcription factors, including six and eya genes, have been proposed to play a role in inducing PPE specification (Pandur and Moody, 2000; David et al., 2001; Ghanbari et al. 2001; Schlosser and Ahrens, 2004). These transcription factors are expressed in the characteristic region surrounding the anterior neural plate that corresponds to the PPE region during the proper developmental time window. For example, Six1 was proposed to induce PPE fate by promoting other PPE genes and repressing genes required for adjacent ectodermal domains (Schlosser and Ahrens, 2004).

Various studies have confirmed that the initiation of PPE requires a complex interaction between inductive signals and PPE regulatory genes. We are still far from completely understanding the pathway that regulates PPE specification. An important goal is to identify a series of genes that play a role in different steps, and to determine the interaction among them that drives PPE development.

3. Placode differentiation
Once PPE is established as a separate domain in the embryonic ectoderm, it undergoes several steps to further differentiate, and gives rise to individual placodes. The conserved localization of individual placode is the result of local inductive interactions during placodal differentiation. For example, the trigeminal placode is, at least in part, induced by a signal from the midbrain regions (Baker et al., 1999), while the otic placode is induced by the interaction between FGFs and Wnt-8c signals from the central hindbrain territory and its underlying mesoderm (Ladher et al., 2000; Maroon et al., 2002).

Besides signals that induce the formation of individual placodes, there is a cascade of genes that are required to promote the production of neuronal cells from the placodes. As previously described, transcription factors of Six and eya family members (Six1 and eya1) are expressed in pre-placodal ectoderm and also in undifferentiated placode precursor cells in nearly all placodes at a later stage (Cheyette et al., 1994; Serikaku and O’Tousa, 1994; Ruf et al., 2004; Brugmann et al., 2004). Next, Sox2 and Sox3 genes are expressed in many of the individual placodes and parts of PPE, defined as neural stem cells (reviewed by Schlosser and Northcutt, 2000). Sox2 and Sox3 are believed to play a role in initial specification and consolidation of neural fate of placode (reviewed by Wegner, 1999; Moody and Je, 2002). Subsequently, the expression of Pax gene family members in local regions of PPE and later in specific placodes are believed to define the identities of distinct placodes (Baker and Bronner-Fraser, 2001; Schlosser and Ahrens). For example, Pax6 is expressed in the olfactory
placodes, while Pax2/5/8 are expressed in otic placode. As the differentiation program proceeds, several basic-Helix-loop-Helix (bHLH) genes are expressed in a portion of cells in the neurogenic placodes to make these cells neural progenitors. In particular, The Neurogenin-type genes define the progenitor cells of different neural cell types (Reviewed in Schlosser and Northcutt, 2000; Schlosser and Aherns, 2004). In mice, placodal progenitor cells that give rise to neurons in the trigeminal and vestibuloacoustic ganglia require Neurogenin1 (Ngn1), while neurogenin-2 (Ngn2) is required for neurons in the distal geniculate (facial), petrosal and nodose ganglia derived from epibranchial placodes (Ma et al., 1998; Fode et al., 1998).

There are additional transcription factors that act downstream of Ngn and are involved in neuronal differentiation, such as Brn-3a, Phox2a and Phox2b (Fedtsova and Turner, 1995; McEvilly et al., 1996; Xiang et al., 1996, Morin et al., 1997; Pattyn et al., 1999). Interestingly, Begbie et al. reported that the expression patterns of different Neurogenins in the chick embryo placodes were opposite when compared with the mouse, while Brn-3a, Phox-2a and Phox-2b are expressed in the same domain in the chick and the mouse embryos (Begbie, et al., 2002).

4. Migration of placodal neurons

After regional specification, the next developmental challenge is to integrate different embryonic fields in a highly coordinated manner, such that
subsequent anatomy and function can be established. This is particularly important for development of the cranial sensory system, a system with neurons derived from two distinct origins: neural crest and placode. Begbie et al. studied how epibranchial placode-derived neurons migrate internally to the site of ganglion formation (Begbie and Graham, 2001). Using lipophilic dye DiI and DiO to label neural crest cells and placodal cells, respectively, they found that migratory epibranchial neural cells are guided by the neural crests which form glia in the epibranchial ganglia. After neural crest ablation, placodal neurons, although generated, failed to move internally but, instead, remain sub-ectodermally (Begbie and Graham, 2001).

**Neural crest cells (NCCs)**

1.**General induction**

   Neural crest cells, the other source of the neurons in the cranial ganglia, are also a tissue found only in vertebrates. The neural crest and placodes are considered to be vertebrate innovations, and their emergence was closely linked to the evolution of the vertebrates. According to Gans and Northcutt’s “New Head” theory, the presence of neural crest cells and ectodermal placodes provided the embryological basis to form a set of a novel, vertebrate-specific structures (Northcutt, 2005; Northcutt & Gans, 1983). However, a migratory cell population resembling neural crest cells was recently described in the ascidian species *Ecteinasacidia Turbinata*, raising the possibility that the neural crest
might not have evolved at the origin of vertebrates but, rather, has a more ancient history (Graham, 2004).

Neural crest cells are a group of multipotent, migratory cells that give rise to the majority of the peripheral nervous system and the distinctive bone, cartilage and connective tissue of the head (Lumsden et al., 1991; Schilling and Kimmel, 1994). Neural crest cells contribute to almost every organ or tissue in the vertebrate body.

2. Induction and specification of neural crest cells

Neural crest cells arise from the lateral margins of the neural primordium, medial to where the placodes originate. Sometimes the neural plate border domain which gives rise to both neural crest cells and placodes has been collectively called lateral neurogenic ectoderm (LNE) (Brugmann and Moody, 2005). As described above regarding the induction of placodes, secreted molecules from the surrounding tissues are also critical for the induction of neural crest cells as well (Liem et al., 1995; Ikeya et al., 1997; Mayor et al., 1997; LaBonne and Bronner-Fraser, 125). Additionally, there are signals that promote neural crest fate over placode fate, such as Wnt and retinoic acid (LaBonne and Bronner-Fraser, 1998; Chang and Hemmati-Brivanlou, 1998; Mayor and Aybar, 2001; Villanueva et al., 2002). To date, several genes have been identified to positively regulate neural crest fate, such as dlx, and zic, which seem to repress at least one PPE-specifying gene (Six1) (Brugmann et al., 2004; reviewed in
The neural crest forms in close proximity to various signals that influence both neural plate and epidermis cell fates. Thus, after neural crest fate is established, there must be some mechanisms to prevent early precursors from changing fates or differentiating prematurely. It has been suggested that the proto-oncogene c-myc and its downstream target Id3 play roles in maintaining the neural crest precursors in the multipotent state (Light et al., 2005; Kee and Bronner-Fraser, 2005). Knock down of Id3 results in depletion of neural crest precursors and their derivatives, while forced expression of Id3 caused a prolonged multipotent progenitor state of neural crest cells, presumably by acting through cell cycle regulation (Light et al., 2005; Kee and Bronner-Fraser, 2005). Recently, a novel extracellular molecule (Noelin-1) has been proposed to maintain the competence of neural epithelial cells to form neural crest (Barembaum et al., 2000). Noelin-1 was initially expressed at the edge of the neural plate and later in migratory neural crest cells. Over-expression of Noelin-1 in the neural tube resulted in prolonged neural crest cell production and migration.

3. Migration of neural crest cells

Most neural crest cells travel a long distance before reaching their final destination. Migration is a critical event and different neural crest derivatives migrate along the different pathways. For example, neural crest cells in the trunk that give rise to melanocytes migrate dorso-laterally, while those give rise to
DRG neurons migrate dorso-ventrally (Kalcheim and Teillet, 1989; Lecoin et al., 1998).

The BMP signaling pathway, which is involved in the initial induction of the neural crest fate, also plays a role in the initiation of migration. Ectopic expression of BMP-4 advances the migration of neural crest cells, whereas ectopic application of Noggin, a BMP antagonist, results in a delay in migration (Sela-Donenfeld and Kalcheim, 1999).

The first step in migration is the delamination from the neural tube. In order to delaminate from neural tube, neural crest cells need to down-regulate the expression of several adhesion molecules, such as E-cadherin and cad6B, while at the same time up-regulating cadherin7 and cadherin11 (Nakagawa and Takeichi, 1995 & 1998; Cano et al., 2000; Inoue et al., 1997). This cadherin switch demonstrates that a regulatory balance of cadherin is required for emigration. Snail/slug transcription factors repress E-cadherin, and play key roles in facilitating emigration of the neural crest cells (Inoue et al., 1997).

After delamination from the dorsal neural tube, neural crest cells migrate into various territories. Except in bony fishes, neural crest cells delaminate and migrate from the neural epithelium before, during or after the fusion of neural folds, depending on the species and the position along the anterior-posterior axis (Hall, 1999). In bony fishes, however, pre-migratory neural crest cell accumulate lateral to the developing neural keel and they begin to migrate from the solid
neural keel prior to the formation of a definitive neural tube (Schilling and Kimmel, 1994).

Neural crest cell migration occurs as a rostral to caudal wave. The exact migratory pathways are different along the anterior-posterior axis and among organisms (Rickmann et al. 1985; Graham et al., 2004; Morriss-Kay and Tucket, 1991; Tosney, 1982). Most populations of neural crest cells become segmented as they encounter signals that attract or repel them along the pathways. Many classic guidance molecules are involved in the guidance of crest cell migration. In the trunk, the neural crest cells always migrate through the anterior part of each somite, and avoid the posterior part (Bronner-Fraser, 1986; Rickman et al., 1985; Serbedzija et al., 1990). There are several pairs of signals that are critical in defining this migratory path. For example, neural crest cells express Neuropilin-2, while the posterior half of somites express Semaphorin 3F, a repulsive ligand for Neuropilin-2 (Gammill et al., 2006). Furthermore, Ephrin-B1 on the posterior half of the trunk somites might be another repulsive signal for the migration of neural crest cells. In vitro studies demonstrated that neural crest cells avoided migrating on stripes of immobilized ephrin-B1, while addition of soluble ephrin-B1 released the inhibition (Krull et al., 1997; Smith et al., 1997; Davy et al., 2004). Another pair of guidance molecules, Robo and Slit, also plays an important role in guiding neural crest migration. Migrating trunk neural crest cells express the receptors Robo1 and Robo2 and the repulsive interaction with Slit ligands expressed in the ventral mesenchyme at the trunk level prevents them from invading the gut (De Bellard et al., 2003).
In the head, the neural crest cells also migrate in streams despite the lack of somites (Farlie et al., 1999). Cranial neural crest cells could be further divided into forebrain- (Diencephalic), midbrain (Mesencephalic)- and hindbrain (Rhomencephalic)-derived populations. Forebrain and midbrain derived neural crest cells migrate as an almost continuous sheet that contribute to the frontonasal mass, periocular mesenchyme and maxillary prominence, whereas crest cells derived from hindbrain migrate in several streams and contribute to different branchial arches (BA) (Trainor and Tam, 1995). During development, the hindbrain was transiently organized into 7 bulges, termed rhombomeres (r). All rhombomeres generate neural crest cells, but crest cells migrate primarily out of even numbered rhombomeres into the periphery and give rise to a variety of tissues (Graham et al., 1993; Sechrist et al., 1993; Birgbauer et al., 1995; Kulesa and Fraser, 2000). This is because most of the neural crest cells generated by rhombomere 3 (r3) and r5 die by apoptosis, while the remaining neural crest cells migrate anteriorly or posteriorly, instead of laterally, and join the streams from nearby even-numbered rhombomeres (Graham et al., 1993; Sechrist et al., 1993; Birgbauer et al., 1995; Kulesa and Fraser, 2000). Interestingly, neural crest cells from r3 and r5 do not cross into r3 or r5, suggesting the existence of inhibitory signals in these segments. Several studies have provided evidence for the existence of such inhibitory signals, such as ErbB4 (Golding et al., 2000; Ota et al., 2004; Moraes et al., 2005). In ErbB4 deficient mice, neural crest cells from r4 became capable of migrating through the dorsal mesenchyme adjacent to r3, a region which is normally free of neural crest cells. This abnormal neural crest cell
migration resulted in fusion of the trigeminal and facio-acoustic ganglia. Wild-type neural crest cells transplanted into ErbB4 mutants also exhibit a similar aberrant migration. Conversely, when ErbB4 mutant neural crest cells are homotopically transplanted into the r4 of wild type embryos, mutant crest cells migrated just like wild-type crest cells into branchial arch 2 (Golding et al., 2002). These results argue against an autonomous effect of ErbB4 on the neural crest cells since ErbB4 is normally expressed in r3 and r5. Instead, these results suggest that ErbB4 functions to create a non-permissive environment to block neural crest cell migration.

Presumably, such segregation of distinct neural crest cell populations is critical to prevent the mixing among crest cells with different anterior-posterior genetic identities. This is particularly important in the cranial region because the neural crest cells give rise to distinct structures in the different branchial arches.

4. Derivatives of neural crest cells

Neural crest cells are generated along the whole neural tube except for a small region immediately anterior to the mid-diencephalon (Couly and Le Douarin, 1987). Along the anterior-posterior axis, neural crest cells could be divided into several different compartments: cranial neural crests, which is further divided into forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon) crest; and trunk crest, also sub-divided into cervical, thoracic
and lumbosacral crest.

Melanocytes arise from the neural crest of all segments, but the various types of ganglion neurons of peripheral nervous system (PNS) and enteric nervous system (ENS) have distinct levels of origins along the neural axis, as do many other crest derivatives. Cranial neural crest cells are unique in their generation of mesencymal tissues in the context of the normal embryos (reviewed by Le Dourain and Kalcheim, 1999). Transplantation analyses have shown that other crest populations can be forced to generate mesenchymne, even though they do not do so in situ.

The most comprehensive analyses of neural crest cell derivatives took advantage of the chick through the preparation and analysis of surgical chimeras comprised of quail tissues grafted into chick host (Le Douarin et al., 1975). Below is the brief summary of neural crest derivatives and segments of their origins (reviewed by Le Dourain and Kalcheim, 1999; Le Douarin, 1982.). The autonomic nervous system include three divisions—the sympathetic, parasympathetic and enteric—all of which derived from the neural crest cells: sympathetic ganglia are generated from the whole trunk neural crest cells, parasympathetic ganglia are generated from the mesencephalic neural crest cells, and enteric ganglia are generated from the vagal (at the level of somite 1-7) and lumbosacral neural crest cells. Endocrine cells are also generated by neural crest cells which are located in somite 2-4, and 18-24. Peripheral sensory ganglia are generated by the mes-metencephalic neural crest and by the crest
from posterior rhombencephalic to lumbosacral levels. Considering the entire length of the neural tube, those crest cells that migrate into branchial arch 2 (corresponding to r3-5) generate the fewest, if any, sensory neurons. Compared to trunk neural crest cells, one characteristic derivative of cranial neural crest cells is that these cells provide the head with mesenchymal cells. These cells were designated as “mesectoderm” to distinguish them from the mesenchyme derived from the mesodermal germ layer. They give rise to the connective tissues of many head structures, such as the facial musculature, blood vessels, and meninges of part of the brain (Etchevers et al., 2001; Kontges and Lumsden, 1996).

I will describe the derivatives of the rhombocephalic neural crest cells in greater detail. Rhombomere1 and 2 produce numerous neural crest cells which migrate as a collective stream along with midbrain-derived neural crest cells and extensively populate the first branchial arch (Lumsden et al., 1991). These neural crest cells give rise to both neuronal and non-neuronal component in the trigeminal ganglion (V). This group of neural crest cells generates most of facial bones, such as the orofacial prominences, the mandible, hyoid, maxilla, malleus, incus and portions of the skull (Lumsden et al., 1991; Schilling and Kimmel, 1994).

The second branchial arch is composed of neural crest cells that originate from the fourth rhombomere, with vanishingly small contributions from r3 and r5. This group of cells generates the non-neuronal component of facial and
vestibuloacoustic ganglia (Sechrist et al., 1993; Knotges and Lumsden, 1996). In
the facial ganglia, less than 5% of the neurons are generated by neural crest
cells while nearby in vestibuloacoustic ganglia, no neurons are generated by
neural crest cells (Yang et al., 2008). Glial cells in both ganglia are generated by
the neural crest cells. Branchial arch 2 crest cells also generated mesenchymal
tissues, including the portion of the hyoid bone, malleus, otic capsule, and all the
stapes (O’Gorman, 2005)

Neural crest cells from r6 migrate into branchial arch 3 and contribute to
the superior (proximal) ganglion of the glossopharyngeal nerve (IX) (Noden,
1983; Couly and Le Douarin, 1988; Kontges and Lumsden, 1996). Crest cells
from r7 migrate into branchial arch 4 and generate neurons in jugular (proximal)
ganglia of the vagus nerve (X). Both of these groups also contribute to
mesenchymal tissues in those arches.

5. Neurogenesis versus gliogenesis

In the cranial ganglia, neural crest derived neurons and glia differentiate in
the same environment although, in general, neurons are generated prior to glial
cells. Therefore, it is puzzling how neural crest cells choose one fate over the
other. Specification is the result of the interplay among several regulatory
pathways, including the Notch/Delta pathway, the BMP pathway, interactions of
transcription factors and cell-cell contact.
The traditional Notch/delta pathway plays a role in cell fate decision in neural crest cells through lateral inhibition (Skeath and Thor, 2003; Riley and Phillips, 2003). Canonically, Notch acts as a receptor for the ligand Serrate (also known as Jagged in vertebrate) and Delta. Activation of Notch by its ligand results in cleavage of the Notch intracellular domain (N\textsuperscript{ICD}) (Selkoe and Kopan, 2003). Subsequently, N\textsuperscript{ICD} translocate into the nucleus where it binds to transcription factor, such as SuH in flies, CBF in vertebrates and RBP-Jk in mammals, and stimulates the transcription of HES family members (Hairy/Enhancer of Spit). These transcription factors then interfere with the activity of proneural transcription factors related to the fly Achaete-Scute and Atonal families (Mash and Neurogenin family transcription factors in vertebrate, respectively). Therefore the activation of Notch signaling represses neurogenesis. Meanwhile, Notch activation through interaction with Delta-expressing neural precursors promotes gliogenesis (Wakamatsu et al., 2000). Soluble Delta can promote Schwann cell differentiation and suppress neurogenesis in cultured neural crest stem cells. Conversely, inhibition of Notch signaling leads to an increase in the number of cells in the peripheral ganglia expressing early neuronal markers (Morrison et al., 2000).

Although many proneural genes are involved in promoting neurogenesis of neural crest cells, different transcription factors are capable of promoting differentiation of distinct subtypes of neurons, which will be discussed below.

In cultures, BMPs are able to promote neurogenesis of neural crest cells
while neuregulins promote gliogenesis (Shah et al., 1994). However, when neural crest cells are exposed to a saturating concentration of both factors, BMP appears to play a dominant role (Shah and Anderson, 1997). BMP2 induces expression of a proneural gene *Mash1*, a vertebrate homolog to *Drosophila* achaete-scute basic helix-loop-helix transcription factor. *Mash1* is able to maintain the responsiveness of neural progenitors to BMP2, therefore establishing a positive feedback regulation of BMP mediated neurogenesis.

Neurogenin-1 and -2 (Ngn-1, Ngn-2), vertebrate homologs of *Drosophila atonal*, are also required for the generation of neurons (Ma et al., 1998&1999; Fode et al., 1998; Perez et al., 1999). However, Mash1 and Ngn1/2 specify PNS neurons into autonomic and sensory lineage, respectively.

*Mash* regulates the generation of sympathetic neurons, a component of the autonomic nervous system. *Mash1* expression is induced by BMPs in neural crest cultures and is reduced in sympathetic ganglia upon inhibition of BMP pathway *in vivo* (Schneider et al., 1999; Shah et al., 1996). Loss of Mash1 function *in vivo* results in loss of adrenergic neurons (Guillemot et al., 1993; Hirsch et al., 1998). Other effectors critical to the development of the sympathetic neuronal lineage include *Phox2a, Phox2b, eHand* and *dHand* transcription factors (Stanke et al., 1999; Pattyn et al., 1999; Cserjesi et al., 1995). *Phox2a* is up-regulated by *Mash1* and has been shown to be sufficient to induce the formation of sympathetic neurons *in vivo* (Hirsch et al., 1998; Stanke et al., 1999). *Phox2b* expression appears to depend on BMP signaling. Interestingly, *Phox2b* is required for maintaining a stable *Mash1* expression as
well as the subsequent induction of Phox2a. Studies in Phox2b mutant mice indicated that Phox2b is essential for the generation of all autonomic ganglia (Schneider et al., 1999; Pattyn et al., 1999). The bHLH transcription factors eHand and dHand are expressed in the peripheral sympathetic neurons (Cserjesi et al., 1995) and act downstream of BMP pathway. The expression of dHand in developing sympathetic ganglia appears later than Phox2b and Mash 1 (Howard et al., 2000). Furthermore, overexpression of Phox2 genes induces dHand and eHand expression. Additionally, it is shown that eHand expression is lost in Mash1 mutant mice (Ma et al., 1997). Together, these results indicated that eHand and dHand are downstream targets of BMP and Mash1 pathway that specify the sympathetic neurons.

Ngn1 and Ngn2 are critical neurogenic transcription factors expressed in neural crest precursors of sensory neurons. Ectopic expression of Ngns in chick embryos was sufficient to activate the expression of several sensory-specific markers (Perez et al., 1999). Ngn1 and Ngn2 appear to control the differentiation of two distinct sensory precursor populations that generate different complements of sensory neuron subtypes or subgroups. In dorsal root ganglia, for example, Ngn2 is required for an early-differentiating lineage that gives rise to large diameter neurons, whereas Ngn1 is required by a later-differentiating lineage that gives rise to small diameter sensory neurons (Ma et al., 1999). Consistent with the idea that Ngn1 and Ngn2 are employed by different subclasses of neurons in the DRG, the differentiation of sensory neurons in different cranial ganglia also require different Ngns. In Ngn1-/- embryos, the
trigeminal and vestibuloacoustic ganglia were completely absent at E10.5. And at a later stage, the accessory, jugular and superior ganglia were also fail to develop, while the nodose, petrosal and geniculate ganglia were spared (Ma et al., 1998). By contrast, the latter group of ganglia is completely or largely eliminated in Ngn2-/- embryos, except for the nodose ganglia where both Ngn1 and Ngn2 are expressed (Ma et al., 1997; Fode et al., 1998). Downstream of the Ngn genes are atonal–related genes NeuroD/Math3 and the bHLH factors NSCL-1 and NSCL-2 (homologs of the lymphoid determination factor SCL/TAL-1) (Begley et al., 1992; Gobel et al., 1992). Interestingly, Ngn1 is also known to positively regulates the Delta homolog DLL1 (Delta like ligand 1) and can be negatively regulated by Notch signaling, which might play a role in inhibiting gliogenesis (Ma et al., 1998).

Neuregulin and its receptor ErbBs, members of epidermal growth factor receptor (EGFR) sub-family of receptor tyrosine kinases, play an indispensable role in gliogenesis. Upon binding of Neuregulin, ErbB2 forms heterodimers with ErbB3 or ErbB4, leading to the phosphorylation of ErbB3 or ErbB4. The subsequent intracellular signalling cascades through PI3K pathway promote the survival and proliferation of Schwann cells and oligodendrocytes (Li et al., 2001). During development, early migrating neural crest cells express ErbB2 and respond to Neuregulin in vitro, leading to glia differentiation at the expense of neurons. Furthermore mice lacking functional ErbB3 lack Schwann cells (Shah et al., 1994; Reithmacher et al., 1997).
6. The differences between neurons derived from placodes and from neural crest cells

As described above, both placodes and neural crest cells give rise to neurons in the cranial ganglia. As previously described, the relative numbers of neurons generated from each source varies among different ganglia. Furthermore, within specific ganglia, the two populations generate two different neuronal subtypes.

In branchial arch 1, both placode and neural crest cells contribute to the sensory neurons in the trigeminal ganglion. In the facial ganglion in branchial arch 2, the majority of the neurons are derived from placode, while neural crest cells contribute to less than 5% of the neurons. In vestibuloacoustic ganglion, neural crest cells only contribute to glia, whereas neurons are solely derived from the otic placode. In branchial 3 and 4, neural crest cells give rise to the neurons that reside in the proximal ganglia (superior and jugular ganglia), while placode give rise to neurons in the distal ganglia (petrosal and nodose ganglia) of the same arch. The glia cells in all of the ganglia are neural crest derived.

There are several differences between the neuronal subtypes that arise from the two sources, although both neural crest cells and placode give rise to neurons. In general, placodal-derived neurons are generated earlier than crest-derived neurons (D’amico-Martel and Noden, 1980). For example, in chick embryos, both neural crest cells and placode contributed neurons to the trigeminal ganglia. Placode-derived neuroblasts cease dividing, exhibit
cytological properties of neurons and established peripheral projections before the end of the first week of development. These cells are situated in the more distal aspects of the ganglion (Gaik and Farbman, 1973; Noden 1980). In contrast, crest-derived neurons in the trigeminal ganglion do not stop dividing until the seventh day of embryonic development. And through most of the second week of development, these cells are small, densely packed and limited to the more proximal parts of the ganglion (D’amico-Martel and Noden, 1980).

Another important difference between placode-derived neurons and crest-derived neurons is that they convey different senses to the central nervous system. In trigeminal ganglia, placode-derived neurons convey the proprioception sense afferents from the muscles associated with jaw movement, whereas crest-derived cutaneous sensory neurons relay somato-sensory stimuli (touch, pain and temperature) from the face, jaws and teeth (Knouff 1927; Noden, 1980; Narayanan and Narayanan, 1978).

Another difference between crest- and placode-derived neurons is that they respond to different neurotrophic factors. Neurotrophic factors constitute a class of protein molecules that are critical for the development, maintenance and regeneration of the nervous system (Hamburger 1952; reviewed by Lindsay et al., 1994). Nerve growth factor (NGF) is the first such factor to be identified. Neurons that respond to NGF were exclusively neural crest originated (Davies and Lindsay 1985). Correspondingly, TrkA, the receptor for NGF, is only
expressed in dorsal root ganglia and other neural crest-derived ganglia.

In contrast to sensory neurons of crest origins, neurons originating from placodes appear to be unresponsive to NGF (Davies and Lindsay, 1985; Lindsay and Rohrer, 1985). In fact, the lack of responsiveness of these neurons to NGF was a major driving force to search other neurotrophic factors. Sequentially, brain–derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotriphin-4/5 (NT-4/5, also known as NT-4, or NT-5) were isolated (Barde et al., 1982; Hohn et al., 1990; Maisonpierre et al., 1990, Berkemeier et al., 1990). Both BDNF and NT-4/5 bind to Trk B receptors, and are important for the development of placode-derived sensory neurons. In BDNF-/- mice, there are virtually no vestibular sensory neurons that are derived from placodes after two weeks of embryonic development (Conover et al., 1995). In BDNF+/– heterozygote mice, the number of placode-derived vestibular neurons is reduced to half of that in wild-type mice demonstrating a dosage dependent relationship between the BDNF level and the number of placodal neurons. Analysis of the neurons in the nodose ganglia showed a similar loss of neurons in both BDNF and NT4/5 (neurotrophin 4/5) null mutants. Interestingly, BDNF/NT-4/5 double mutants had virtually no nodose ganglia neurons, reflecting an additive effect of the two factors (Conover et al., 1995, Bianchi et al., 1996, Lindsay et al., 1985).

7. The neurogenic potential of neural crest cells is modulated by environment
It is still unclear why neural crest cells contribute to neurons and glia in some ganglia, but generate only glia in others. One possible explanation could be that neural crest cells that migrate out of a certain segment have a limited capability to generate neurons, reflecting intrinsic differences in developmental potential of crest cells themselves. Alternatively, the neurogenic potential of neural crest cells could be modulated by their surrounding environment.

Both surgical ablation experiments in chick and genetic knock-out analysis in mice have indicated that neural crest cells that normally do not give rise to neurons could generate neurons under some circumstances, supporting the idea that the environment plays an important role in modulating the differentiation of neural crest cells (Ayer-LeLievre and LeDouarin, 1982; Fode et al., 1998; Ma et al., 1998). For example, early quail-chick chimera studies showed that cranial neural crest cells in the nodose ganglion, which do not normally form neurons, can generate neurons upon transplantation to appropriate environments (Ayer-LeLievre and LeDouarin, 1982). In one such study, the nodose ganglion was dissected out from 5-9 day old quail and grafted into a 2-day old chick host embryos at the adrenomedullary level of the neural axis. The presumptive glial presursors in the transplanted nodose ganglion were shown to be able to migrate again in younger host, and differentiate into autonomic structures (sympathetic ganglion cell, adrenomedullary cells and enteric ganglia), which is the identity consistent with their new localization in the host embryos (Ayer-LeLievre and LeDouarin, 1982). Thus, the working hypothesis is that the neurogenic potential of neural crest cells which invade the placodal primordium of the nodose
ganglion are suppressed through cell-cell interactions occurring between placodal and crest cells.

This hypothesis is supported by observations after ablation of the nodose placode (Harrison et al., 1995). Manipulations that remove placode-derived neurons have shown that cranial neural crest cells generate additional neurons when placode-derived neurons are missing (Fode et al., 1998; Harrison et al., 1995). Ablation of nodose placode in stage 9 chick embryos resulted in an absence of nodose ganglia that was evident at 6 days after surgery. Most embryos surviving to day 12, however, did form identifiable nodose ganglia, although these ganglia had significantly reduced volume (Harrison et al., 1995). To investigate the source of the neuron population in the regenerated ganglion, Harrison et al. bilaterally replaced the chick nodose placodes with quail "cardiac" neural crest cell from mid-otic to somite 3. Although crest cells normally do not generate neurons in the nodose ganglion, quail-derived neurons were identified in the chimeric embryos 9 days after surgery. This result indicated that the neurogenic potential of cardiac neural crest cells, and possibly other crest cells, is suppressed by placode-derived neurons in the nodose ganglion (Harrison et al., 1995).

Genetic manipulations in mice also confirmed that neural crest cells in distal ganglia are capable of generating neurons though they normally only give rise to non-neuronal tissues in those ganglia. Neurogenin2 is required by neurons derived from epibranchial placodes (Fode et al., 1998). In Neurogenin2
deficient mice epibranchial placodes could still been identified, but there was no evidence of precursor cell delamination resulting in a lack of geniculate (facial) ganglionic development at E9.5. However, by E12.5, the geniculate ganglia were re-established as evidenced by the nearly normal expression of SCG10 and Ret, a gene encoding a GDNF (Glial cell derived neurotrophic factor) receptor subunit that was expressed highly in wild type ganglia (Fode et al., 1998). Therefore, the lack of placode-derived neurons in mutant geniculate ganglia at E9.5-10.5 is subsequently compensated for, presumptively by neural crest cells, resulting in the presence of geniculate ganglia in E12.5 mutant embryos (Fode et al., 1998).

A similar regulation of neurogenic potential has been observed between early- and late-migrating populations of neural crest cells in zebrafish dorsal root ganglia (Raible and Eisen, 1996). In the developing fish DRG, the late migrating crest cells, which normally produce only non-neural derivatives, gave rise to neurons in DRG after ablation of early migrating crest. This observation is consistent with the hypothesis that the presence of placode-derived neurons normally represses neurogenesis by cranial neural crest cells (Ayer-LeLievre and LeDouarin, 1982).

Differences in the numbers of crest-derived neurons may, additionally or alternatively, reflect differences in the developmental potentials of crest cells themselves. Many non-neuronal fates adapted by cranial neural crest cells are modulated by the activities of HOX genes (Couly et al., 2002). In addition, our
observations in Hoxa2 deficient mice suggest that Hoxa2 might be required to repress the neuronal differentiation of neural crest cells in the facial ganglia.

**HOX genes:**

**1. General introduction**

HOX genes play critical roles in defining the segmental identity along the anterior-posterior (A-P) body axis. Mutations in HOX genes result in homeotic transformations which are transformations of structures of one body segment into the corresponding structures of another segment (Bateson, 1894). The first studies on the mechanisms underlying the regionalization of A-P body axis were done in Drosophila. A family of transcription factors called Hom-C genes was found to be responsible for homeotic transformations in flies. There are eight Hom-C genes organized in two clusters, the Antennapedia (Ant-C) and Bithorax (Bx-C) complexes, which are located in distinct loci on the third chromosome (Lewis, 1978; Kaufman et al., 1990).

HOM-C genes are highly conserved and share a 183 base pair DNA sequence called the homeobox or homeodomain (HD) (Gehring, 1987). The homeodomain is a DNA binding domain that recognizes specific DNA sequences that contain a TAAT core sequence (Gehring et al., 1994; Ekker et al., 1994). The early vertebrate homeobox-containing genes (Hox) were identified in mouse and Xenopus by low stringency screening using Drosophila homeobox probes
(Carrasco et al., 1984; McGinnis et al., 1984). Since then, many additional genes have been identified, both in Drosophila and vertebrates. In mammals, a total of 39 HOX genes have been identified. They are organized in four clusters (Hox A, B, C, and D) that are located on four different chromosomes, each cluster about 120kb long in mouse (Duboule et al., 1989; McGinnis and Krumlauf, 1992). Based on homologies between themselves and their Drosophila counterparts, these genes have been subdivided into thirteen homology groups called paralogues (Scott, 1992). Paralogous genes are located at the same relative position in their respective cluster, and therefore can be aligned vertically in a schematic figure. However, not every cluster contains a full spectrum of all the paralogues. Each of the Drosophila HOM-C genes is more closely related to one or several groups of paralogous HOX genes, suggesting that the four mammalian complexes are evolutionally derived from large scale duplications (Kappen et al., 1989; Krumlauf, 1994).

One of the most interesting aspects of the Hox gene clusters is that these genes are arranged in a sequence that reflects their order of expression during embryogenesis and their domain of expression along the AP axis. The former correlation has been defined as "temporal colinearity", while the latter has been defined as "spatial colinearity" (Gaunt, SJ., 1988; Dolle I., et al., 1989; Graham, A., et al., 1989). In greater detail, temporal colinearity means that genes located in the 3’ end of a given Hox cluster will be expressed at earlier time points than those located in the more 5’ end. Spatial colinearity refers to the fact that in general genes located in the 3’ end of a cluster will be expressed in more
anterior regions while genes in the 5’ end are expressed in more posterior regions. It is important to note that the domains of Hox expression refer to their anterior boundaries due to the fact that they have sharp anterior expression borders, but diffusive posterior expression border that usually extend to the tail or tapers non-specifically (Lufkin, 1996). Thus, there are usually several HOX genes co-expressed in the posterior regions of the body at the same time. In any given embryonic segment, the most posterior HOX genes impose dominant effects over those co-expressed Hox gene that have more anterior expression domains. This phenomenon is known as posterior prevalence (Duboule, D., 1991; Duboule & Morata 1994). Posterior prevalence is the underlying mechanism by which homeotic mutations always transform body segments to take on a more anterior identity.

2. The function of Hox cofactors

HOX proteins bind DNA weakly and without great specificity through a highly conserved homeodomain (Hoey and Levine, 1988). This raises the question of how the high levels of functional specificity observed in vivo are achieved. We now understand that Hox DNA-binding specificity is modified through interactions with other DNA-binding proteins, which include PBC and Meis classes of TALE (Three Amino acid Loop Extension) homeodomain proteins (van Dijk and Murre, 1994; Chang et al., 1995; Knoepfler and Kamps, 1995; Shen et al., 1997; Shanmugam et al. 1999). The PBC class comprises Extradenticle (Exd) in Drosophila and multiple vertebrate Pbx homeoproteins in
vertebrates, whereas the Meis class includes Homothorax (Hth) in *Drosophila* and Meis and Prep homeoproteins in vertebrates.

Pbx (pre-B cell leukemia transcription factors) proteins were identified through analysis of the t(1:19) chromosomal translocation that causes pre-B cell acute lymphoblastic leukemia (Kamps *et al.*, 1990; Nourse *et al.*, 1990; Rauskolb *et al.*, 1993). Four Pbx proteins have been identified that have high amino acid sequence homology with each other (Popperl *et al.*, 2000). In *Drosophila*, the loss of the single Pbx homologue, Extradenticle, is phenotypically equivalent to the loss of multiple Hox gene activities, even though the Hox proteins are expressed at normal levels in appropriate domains (Peifer and Wieschaus, 1990). The situation in mammals is complicated because of the functional redundancy and overlapping expression patterns of different Pbx family members. Studies in the zebrafish indicated that the functional differences between Pbx genes are likely to be due to differences in their expression patterns rather than in their biochemical activities (Popperl *et al.*, 2000).

Meis proteins, additional members of the TALE homeobox protein family, are also important cofactors for the transcriptional regulation of HOX genes. The *Meis1* gene (Myeloid ecotropic rival integration site) was identified as a site of viral integration that is common to 15% of murine myeloid tumors (Moskow *et al.*, 1995). Four Meis proteins have been identified in vertebrates. Hth, the *Drosophila* homolog of Meis, is required for Exd function because it is responsible for the nuclear transport of the protein (Abu-Shaar *et al.*, 1999). In
vertebrates, Meis proteins do not seem to regulate the nuclear localization of Pbx proteins, but rather increase their stability (Longobardi and Blasi, 2003; Waskiewicz et al., 2001).

Pbx proteins interact with Meis and HOX proteins via non-overlapping domains and this allows the formation of Meis-Pbx-HOX heterotrimers. The primary interaction between Pbx/Exd and Hox is via a specific region carboxy terminal to the homeodomain in the Exd/Pbx partner and a tryptophan-containing hexapeptide motif (N-Y/F-P/D-W-M-K/R) amino-terminal to the homeodomain in all Hox proteins except those of paralog group 11-13 (Chang et al., 1995; Knoepfler and Kamps, 1995; Neuteboom et al., 1995). Point mutation analysis demonstrated that the tryptophan of the pentapeptide is critical for the cooperative binding between Hox and Pbx (Knoepfler & Kamps, 1995). Substitution of tryptophan with phenylalaine abolished the ability of Hox protein to bind with Pbx1. On the other hand, Pbx proteins interact with Meis proteins via the PBC-A domain N-terminal to the homeodomain of Pbx proteins. In some cases, only the HOX and Pbx components need to contact DNA in order to form a DNA-bound complex and Meis only contribute to the transcription complex through protein-protein interactions with Pbx proteins that stabilize the complex (Berthelsen et al., 1998; Shanmugam et al., 1999).

McGinnis and colleagues proposed a model in which the interaction of co-factor proteins could convert HOX proteins from repressor (or neutral regulators) to activators via conformational changes of HOX proteins to expose a
transcriptional activation domain (Pinsonneault et al., 1997). The study of the Exd/Dfd complex (Deformed, a Drosophila Hox gene) represents one way Hox co-factors regulate Hox protein function. Exd relieves an inhibitory effect of the Dfd homeodomain on the activation function of the Dfd N-terminal activation domain (Li et al., 1999). The Dfd activation domain and a Dfd-VP16 fusion protein are more active in the presence of Exd, in a manner that does not increase the binding affinity of Dfd on a reporter construct. Li et al. confirmed that there is a covert transcriptional activator domain in the N-terminal of Dfd that is normally masked by the Dfd homeodomain through intramolecular contact or via an intermediary masking factor (Li et al., 1999). After Exd binding, the Dfd activator domain is liberated. This Exd switch model is further supported by another study, although not between classical HOX proteins and Exd. Engrailed, a homeodomain containing transcription factor that can also bind to Exd via a tryptophan-containing motif, acts as a potent repressor in many contexts. The transcriptional activation functions of Engrailed are dependent on Exd in many conditions (Peifer and Wieschaus, 1990; Pinsonneault et al., 1997).

PBC binding can also change transcriptional activity through chromatin reorganization that renders DNA more accessible to the activators (Krasnow et al., 1989; Appel and Sakonju, 1993; Capovilla and Batos, 1998; Galant et al., 2002). HOX proteins can form monomers binding to the natural regulator elements bearing clusters of HOX monomer binding sites over several hundreds of base pairs (Galant et al., 2002). It is proposed that HOX proteins interact with each other to restructure chromatin, thereby rendering activator binding sites
inaccessible (Biggin and McGinnis, 1997). The interaction with the cofactors masks one of the homomeric interaction surfaces on the HOX proteins, leads to the reduced binding between monomers at some lower affinity sites, and changes the overall structure of the promoter region to an active configuration so that the transcription activator proteins can bind to DNA and initiate active target gene transcription.

Another dimension of regulation is through recruiting other transcription activators or repressors into the transcriptional complex via protein-protein interactions. In this way, transcriptional activity is not determined by the presence of the cofactor, but instead is determined by the presence of the additional activators or repressors in the transcription complexes. For example, a Hoxb1-Pbx complex repressed the expression of a reporter construct driven by a Hoxb1 autoregulatory element that comprises three Hox-Pbx binding sites in retinoic acid-treated P19 cell monolayer, while it activated the same element after cell aggregation in addition to retinoic acid (Saleh et al., 2000).

The mechanistic basis of this repression is by recruiting corepressors in the transcription complex, including histone deacetylases (HDACs) 1 and 3, and corepressor N-CoR/SMRT (N-CoR, nuclear receptor corepressor; SMRT, silencing mediator for retinoic and thyroid receptors). The corepressors were recruited to the transcription complex via a protein-protein interaction between the N-terminal of Pbx protein and HDAC1, indicating the Pbx partner is required for the repressive activity of the complex. Treatment with a deacetylase inhibitor
trichostatin A not only relieved the repression but also converted the Hox-Pbx complex into a net activator of transcription. Saleh et al. showed that the activation function is mediated by the recruitment of the coactivator CREB by the Hox partner. In response to protein kinase a signaling or cell aggregation, Hox-Pbx complexes switched from transcriptional repressors to activators, indicating the cofactors recruited into the transcription complex could be modulated by the extracellular signalings (Saleh et al., 2000). Together, these results suggest a model whereby Hox-Pbx complexes can act as both repressors and activators of transcription via association with corepressors and coactivators, and the final transcriptional effect could also be influenced by cell signaling.

Interaction with HOX protein function not the only function of Pbx proteins. Pbx1 mutant mice not only have skeletal defects that are likely to reflect their role in modulating the activities of genes in the classical HOX complexes, but show other defects, notably asplenogenesis (DiMartino et al., 2001; Selleri et al., 2001). Splenogenesis is controlled by one of the so-called "orphan" Hox genes (Hox11) that does not belong to the classical HOX genes clusters (Brooke et al., 1998). The binding between Pbx and Engrailed is a example of interaction between Pbx and non-HOX homeodomain proteins (Kobayashi et al., 2003; Peltenburg and Murre, 1996). The midbrain phenotype of Pbx4 mutant in zebrafish resembles that of the En2 and En3 mutant (Scholpp et al., 2003). Pbx can also bind non-homeodomain proteins such as MyoD, a myogenic protein, and plays critical role in the generation of a subset of muscle fibers (Berkes et al, 2004).
The studies of the functions of Meis proteins are far behind those of Pbx proteins. Although not absolutely required for trimer formation, Meis proteins are critical for many enhancer functions. For example, the activity of the r4 enhancer of the Hoxb2 gene is dependent of HOX, Pbx and Meis/Prep binding sites (Jacobs et al., 1999; Ferretti et al. 2000). Studies indicate that Meis can contribute to the transcription complexes through several mechanisms. Firstly, Meis proteins could increase the DNA-binding stability of transcription complexes on sub-optimal sites (Jacobs et al., 1999; Ryoo et al., 1999; Ferretti et al., 2000); secondly, Meis proteins can contribute activation and/or repression function of its own that complement those of Pbx and Hox proteins (Berthelsen et al., 1998; Shanmugam et al, 1999).

The interaction between HOX proteins and Pbx/Meis cofactors are complex. The transcriptional effects of such complexes are hard to predict, and are highly dependent on the context of the cell. Additionally, both HOX proteins and Pbx cofactors have relatively independent functions. Thus, it is important to determine the function of the individual components in the transcription complex and also the whole transcription complexes under various conditions.

3. Modulation of derivatives of cranial neural crest cells by HOX genes

Various studies have demonstrated that HOX genes play critical roles in modulating derivatives of neural crest cells. According to the expression of HOX genes, cranial neural crest cells could be divided into 2 groups: an anterior group
(from mid-diencephalon down to r3) where HOX genes are not expressed, and a posterior group including r4 – r8 where HOX genes of the first four paralogues are expressed (Hox1-4) (Prince and Lumsden, 1994; Couly et al., 1994). The neural crest cells that form much of the facial skeleton are derived from the anterior Hox-negative group of the first branchial arch (BA1) (Couly et al., 1996; Kontges and Lumsden, 1996). This rostral domain of the crest is designated as facial skeletogenic neural crest (FSNC). R3 participates in both domains, as it provides a few neural crest cells to both BA1 and BA2. Those r3 crest cells that migrate rostrally lose their Hox2 expression (HOX gene expressed most anteriorly) as they reach BA1, while those migrating caudally into BA2 maintain Hoxa2 expression (Creuze et al., 2004). Neural crest cells from these two groups respond differently to inductive cues that promote the generation of skeletal components. Neural crest cells that do not express HOX genes respond to patterning cues produced regionally in the anterior endoderm to yield distinct skeletal components of the upper face and jaws. However, HOX-expressing neural crest cells do not respond to these cues (Couly et al., 1998, 2002).

Complete excision of the FSNC results in a complete absence of face in the operated embryos. Interestingly, even small fragments of the HOX-negative neural crest cells are able to regenerate the complete facial skeleton while the HOX-positive neural crest cells cannot, showing that the HOX-positive Neural crest cells cannot replace the Hox-negative domain for facial skeletogenesis (Couly et al., 1998& 2002; Noden, 1983; Trainor et al., 2002). This idea was further supported by the phenotype of heterotopic transplantation experiments in
chick embryos. For example, experimental transposition of the neural fold from the Hox-positive posterior domain to the anterior Hox-negative domain resulted in a failure of facial structure formation. On the contrast, when presumptive second arch neural crest cells (HOX gene positive) were heterotopically replaced by first branchial arch (HOX gene negative) crest cells, the host developed a complete, duplicated BA1 skeletal system in BA2 region (Noden, 1983). A subsequent study demonstrated that fibroblast growth factor 8 (FGF8) expression by the transplanted Hox-negative Neural crest cells were able to suppress the Hoxa2 expression in the BA2 region and transform it into BA1 like structure (Trainor et al., 2002). These observations are consistent with the hypothesis that HOX genes have an inhibitory effect on the differentiation of facial cartilages and bones.

4. Hoxa2, a selector gene for branchial arch 2 identity

A striking example of the regulation of neural crest cell fates by HOX genes is the requirement for Hoxa2 function for normal differentiation of the mesenchymal derivatives in the second branchial arch (Rijli et al., 1993; Creuzet et al., 2002). Hoxa2 is the Hox gene expressed most anteriorly and the only Hox gene expressed up to the r2 (Krumlauf, 1993; Wilkinson, 1993). However, its expression in r2-derived neural crest cells is down-regulated after neural crest cells migrate out of the neural tube, whereas r4-derived neural crest cells maintain high levels of Hoxa2 expression. Thus, in the periphery, the
mesenchyme of branchial arch 1 is \textit{Hoxa2} negative, while that of branchial arch 2 is \textit{Hoxa2} positive (Krumlauf, 1993; Prince and Lumsden, 1994).

\textit{Hoxa2} is believed to play a deterministic role for branchial arch 2 (BA2) identity. Loss of \textit{Hoxa2} function results in neonatal lethality due to bilateral cleft palate and the transformation of some BA2 skeletal elements towards fates normally assumed by BA1 neural crest cells (Gendron-Maguire \textit{et al}., 1993; Rijli \textit{et al}., 1993). Detail characterizations revealed that homozygous mutant animals have multiple cranial skeletal defects. Many of the normal complement of second arch cartilages were either absent or appeared to have been transformed into structures bearing the identities of the first branchial arch. One example of the transformation was a duplication of ossification centers of the bones of the middle ear. The transformation resulted in a duplication of Meckel’s cartilage adjacent to the otic capsules, derivatives of first branchial arch. The skeletal alteration of the \textit{Hoxa2} mutant stand in contrast to apparently normal structure of the there is no obvious defects in the structure of hindbrain, even though most of the rhombomeres normally express \textit{Hoxa2}. The expression pattern of \textit{Krox}-20, a marker for r3 and r5, appeared normal compared to the wild-type embryos (Gendron-Maguire \textit{et al}, 1993).

The peripheral nervous system is also affected by \textit{Hoxa2} deficiency, although slightly different phenotypes were reported by the two groups that originally generated \textit{Hoxa2}\textasciitilde/- mice. Rijli \textit{et al} reported that the caliber of the
facial nerve is reduced while the facial sensory ganglia appeared normal in size and position (Rijli et al., 1993). Gendron-Maguire et al found a reduced size of the glossopharyngeal nerve and the superior ganglia, while the other ganglia appeared normal (Gendron-Maguire, et al., 1993). However, it is not clear from their observations whether Hoxa2 plays any direct role in regulating the neural crest derivatives in peripheral nervous system or whether the cranial ganglia defects are a result of secondary effects from the peripheral mesenchymal defects.

The studies in which Hoxa2 was over-expressed in first branchial arch of chick embryos further confirmed that Hoxa2 actively promoted the development of second arch skeletal mesenchymal elements (Creuzet et al., 2002; Grammatopoulos et al., 2000). Ectopic expression of Hoxa2 in the first branchial arch leads to alterations of crest cell fate that is consistent with a loss of first arch identity. The most obvious effect after electroporation of either viral or plasmid DNA encoding Hoxa2 into BA1 neural crest was a drastic reduction of first arch cartilages, such as Meckel’s cartilage and the quadrate bone, the avian homologue of mammalian incus which is derived from Hoxa2-negative r2 neural crest cells. Additionally, the extent of mesenchymal tissue loss correlated closely with the level of Hoxa2 expression (Creuzet et al., 2002; Grammatopoulos et al., 2000). Again, these studies were focused on the mesenchymal derivatives of the neural crests. It is not known whether Hoxa2, or indeed any Hox genes, are involved in regulating neurogenesis of neural crest cells within developing cranial ganglia.
5. **HOX genes may play a role in neurogenesis**

Studies of *Hox3* paralogues (*Hoxa3, Hoxb3* and *Hoxd3*) imply that HOX gene function may have some role in regulating neurogenesis (Manley and Capecchi, 1997). In addition to abnormalities in the development of the mesenchymal derivatives, the *Hox3* paralog mutant mice exhibited some defects in the ninth and tenth cranial ganglia (Manley and Capecchi, 1997). In *Hoxa3-/-* mice, for example, 25% of the embryos had an apparent deletion of the proximal portion of the IXth cranial ganglion (superior), while neurofilament-positive neurons of the distal portion of the ganglion (petrosal) were present in their normal position, but these did not extend projections into the hindbrain through their normal nerve root. Manley *et al.* speculated that the lack of proximal IXth ganglion might represent a deletion or suppression of neural crest-derived cells that would normally contribute to the formation of the superior ganglion, while the remaining neurofilament-positive cells represent the inferior petrosal ganglion that is derived from placode. Another phenotype seen in *Hoxa3* mutant embryos was a fusion of cranial ganglia associated with glossopharyngeal and vagal nerve which occurred in 50% of embryos examined. This may be a result of the same deletion of proximal structures described above.

Deletion of another member of *Hox3* paralogues, *Hoxb3*, resulted in a similar phenotype but with a much lower penetrance (14%) (Manley and Capecchi, 1997). Though *Hoxd3-/-* embryos had normal cranial ganglia,
*Hoxb3/d3* double mutants had an increased penetrance of the IXth cranial ganglion defects, compared to *Hoxb3* single mutant. These results suggested that *Hox3* genes also have important functions in regulating the neuronal derivatives of neural crest cells.

However, without further detailed studies, it is hard to confirm whether HOX genes are directly involved in the neurogenesis of neural crest cells. The combination of gene deletion and fate mapping techniques has provided great opportunities for analyzing whether any HOX gene regulates neuronal differentiation of neural crest cells.

Most of the relevant fate mapping analyses have been done in avian systems, particularly the quail-chick chimeras system (D’Amico-Martel and Noen, 1980; Creuzet *et al.*, 2002). Many of the fate mapping results are yet to be repeated in mammalian systems, where it is technically challenging to conduct similar fate mapping experiments. Lineage tracing by focal dye labeling allows individual subpopulations of cells to be traced to determine their contribution to specific structure. However, such lineage tracing is mostly carried out in mouse embryos cultured *in vitro*, and the length of experiments where cells can be followed is limited to approximately 2-3 days. The use of cre/loxP technology to fate map murine neural crest cells has overcome the limitations of embryo culture and allows us to track neural crest cell migration and differentiation over long time period. Combinations of rhombomere specific cis-regulatory sequences with cre/loxP technology will further allow lineage tracing of neural crest cells.
from an individual rhombomere. This technique will enable us to not only study precisely the derivatives of a specific group of cells, but also to determine any factors that cause a change of the fate of those cells.

6. if Hoxa2 regulates neurogenesis of r4 neural crest cells

Neural crest cells from the trunk spinal cord generate sensory neurons in dorsal root ganglia, and crest cells from hindbrain rhombomeres generate sensory neurons in cranial ganglia located in different branchial arches (D'Amico-Martel and Noden, 1980; Ayer-Le Lievre and Le Douarin, 1982). However, there is one exception which is that the neural crest cells in the second branchial arch give rise to only a few neurons in facial ganglia and none in vestibuloacoustic ganglia. The paucity of neurons generated by second arch crest suggests that there may be inhibitory mechanisms that are unique to this population.

The principle recognized difference between neural crest cells in branchial arch 2 and crest cells in other branchial arches is that neural crest in branchial arch 2 express Hoxa2, but no other Hox proteins (Krumlauf, 1993; Wilkinson, 1993). Crest cells in more posterior regions also express Hoxa2, but additionally express a combination of other Hox proteins, such as the Hox3 paralogous (Chisaka and Capecchi, 1991; Condie and Capecchi, 1993). Additionally, Hoxa2 plays a critical role in regulating the mesenchymal derivatives of r4 neural crest cells in the second branchial arch. It thus seemed reasonable to speculate that the lack of neurogenesis by BA2 crest cells might be specified by the isolated
action of Hoxa2, and that the combinatorial activity of multiple HOX genes in more posterior arches might result in quite a different neurogenic fate. We therefore decided to do fate-mapping analyses of r4-derived crest cells in mice that lacked Hoxa2 function.

To fate-map r4-derived neural crest cells, we generated a recombinant allele of Hoxb1 (B1\textsuperscript{Cre}) in which the first exon was replaced by the coding sequence for Cre recombinase. The expression pattern of Hoxb1 has been well studied, and is very dynamic during early development (Frohman \textit{et al.}, 1990; Murphy \textit{et al.}, 1989). At embryonic day 7.5 (E7.5), Hoxb1 is expressed in the posterior primitive streak and flanking mesoderm. By E8.5 its expression is observed in hindbrain up to the presumptive r3/4 boundary in the hindbrain, and is elevated in r4 itself. Between E8.5 and E9.5, r4 expresses a high level of Hoxb1, but expression is diminished elsewhere. Based on the expression pattern of Hoxb1, the B1\textsuperscript{Cre} allele, in combination with an appropriate Cre-conditional reporter allele (Soriano, 1999) could potentially label all cells derived from rhombomere 4, including the r4-derived neural crest cells that enter the second branchial arch. Through this method, we will be able to determine precisely the derivatives of the r4 neural crest cells in the facial ganglia, and determine if there are any alterations in the neuronal components in the facial ganglia in Hoxa2 deficient mice.

Hoxa2 is expressed not only in the neural crest cells which generate glia in the facial ganglion, but also in cells that generate the surrounding
mesenchymal tissues in the second branchial arch (Hunt et al. 1991; Prince and Lumsden, 1994). Any inhibition of neurogenesis by Hoxa2 could be mediated by either a cell autonomous or non-autonomous mechanism. Therefore, we ectopically expressed Hoxa2 in the trigeminal ganglion in first branchial arch by in ovo electroporation.

Neural crest cells from r2, which down-regulate Hoxa2 expression after delaminating from the neural tube (Krumlauf, 1993; Prince and Lumsden, 1994), contribute to mesenchymal tissues in the first branchial arch, and generate both neurons and glia in the trigeminal ganglia (D'Amico-Martel and Noden, 1983). Thus, the first branchial arch territory is Hoxa2 negative. To determine if a cell autonomous mechanism influenced neurogenesis, it was important to restrict the expression of exogenous Hoxa2 to the neural crest cells that contribute to the trigeminal ganglion, not in the surrounding mesenchymal tissue in the first branchial arch. To do this, we took advantage of the fact that cells contributing to the ganglion are the last to emerge from the neural tube (Tosney, 1982; Baker et al., 1997; Serbedzija et al.1992), and could be specifically targeted by electroporation of expression constructs at appropriate stage. The first stream of neural crest cells starts to leave the mesencephalon at the stage 6-somite stage, and can be clearly seen under the dissecting microscope on both sides of the neural tube in embryos of 7- to 8- somite stage (Tosney, 1982). This group of early-migrating neural crest cells forms melanocytes and mesenchymal tissues in the first branchial arch, such as jaw cartilage, bone and connective tissues (Baker et al., 1997). Neural crest cells continue to emigrate from the neural tube.
until about the 10-somite stage. These late-migrating neural crest cells form neurons in the cranial ganglion but not cartilage in the first branchial arch (Tosney, 1982; Baker et al., 1997; Serbedzija et al., 1992). To selectively express Hoxa2 in the trigeminal ganglion, we performed the electroporation at stage 10 to transfec the late-migrating neural crest cells before they emigrate from the neural tube. With this technique, we determined the functions of Hoxa2 in regulating neurogenesis of neural crest cells in vivo by analyzing the fate of the neural crest cells that expressed ectopic Hoxa2 within surrounding tissues that did not express.

**P19 cell, a developmentally multipotent cell line**

The regulation of neuronal differentiation involves a cascade of gene activities that are up-regulated or down-regulated during the process. To study the interaction of different factors, cell culture systems are often used because they are easily manipulated. The P19 mouse embryonal carcinoma cell line is a good model system with which to analyze regulation of neuronal differentiation (Jones-Villeneuve et al., 1983; Farah et al., 2000). These multipotent cells can be easily maintained and propagated in tissue culture in an undifferentiated state. In addition, conditions that promote their differentiation have been well studied.

P19 cells were derived from a terato-carcinoma formed following transplantation of a 7.5 day embryo into the adult testis (McBurney and Rogers, 1982). Like other embryonal carcinoma, P19 cells are developmentally
multipotent. Under different conditions, P19 cells can differentiate in vitro into derivatives of all three germ layers – endoderm, mesoderm and ectoderm. For example, dimethyl sulfoxide (DMSO) treatment will induce P19 to develop into mesodermal cell type (McBurney et al., 1982).

There are several ways to promote P19 cells to differentiate into neurons. The first method used was to culture the cells at high density or as aggregates. However, this spontaneous neuronal differentiation is not very effective. Exposure of aggregated P19 cells to retinoid acid (RA) induced neuronal differentiation dramatically after as little as 2-4 h exposure (Jones-Villeneuve, et al., 1982). At the dose of RA normally used (3x10^{-7}), neurons are the first and most abundant cell type generated. By 6 days up to 85% of the cells express neuronal markers. By day 10 astroglial cells mature and can be recognized by their intermediate filaments which are comprised of glial fibrillar acidic proteins (GFAP).

After exposure to RA, gene expression profiles of P19 cells change. The expression of many transcription factors is up-regulated, including several HOX genes (a1 and b1), Pbx1/2/3, Meis, and Prep1 (Pratt et al., 1993; Knoepfler and Kamps, 1997; Qin et al., 2004; Aulad-Abdelghani et al., 1997). Although the meaning of this up-regulation is unclear, it could potentially interfere with our efforts to determine the function of Hoxa2 in regulating neuronal differentiation. Thus, we decided to culture P19 cells at high density without any additional
inductive chemicals, and study the function of Hoxa2 in regulating spontaneous neuronal differentiation.

If HOX genes regulate neurogenesis, how do they interact with other factors that are known to play critical roles to regulate neurogenesis? At which level do they function among the cascade of factors along the neurogenesis pathway? To answer these questions, we utilized the system set up by Farah et al. They promoted P19 neuronal differentiation by transiently expressing some of the bHLH transcription factors, such as neuroD2, MASH1 and Ngn1 (Farah et al., 2000). Those bHLH transcription factors are well known to play critical roles in promoting neurogenesis during early development. They showed that they could induce P19 neuronal differentiation in the absence of RA induction or aggregation. Four days after transient transfection with any of these factors, cell bodies of P19 cells rounded up, elaborated long processes like neurons, and expressed several neuronal markers (TuJ1 and PGP9.5). With the help of this system, we could determine how Hoxa2 interact with the factors, such as Ngns, to regulate neurogenesis of multipotent cells.

Research Goal:

The principal question addressed by the work presented here is whether Hoxa2 plays any role in regulating neurogenesis by neural crest cells. In this study, we used a lineage marking paradigm based on the transient expression of a site-specific Cre recombinase (O'Gorman et al., 1991) to heritably label the
neural crest cell lineage of the second branchial arch. As previously described by others (Arenkiel et al., 2003), we found that in wild-type embryos the neural crest population that arises from Hoxb1-expressing progenitors in r4 preferentially formed glial cells in the facial ganglion, but we also found that it formed a small number of sensory neurons in the facial ganglion and all of the parasympathetic neurons of the ptergopalatine ganglion. Unexpectedly, we found that in Hoxa2-/mutant embryos, the r4-derived neural crest cells generated a large, supernumerary population of facial ganglion neurons, suggesting that Hoxa2 function normally suppresses neurogenesis in this population of neural crest cells.

This hypothesis was tested by over-expressing Hoxa2 in high-density cultures of P19 embryonal carcinoma cells (Jones-Villeneuve et al., 1982; Rudniki and McBurney, 1987). P19 cells are an embryonic carcinoma cell line that can differentiate into neurons, glia or muscle cells under different conditions. After ectopic expression of Hoxa2 with or without its cofactors, we asked if there were any changes in the probability of neuronal differentiation of expressing cells compared to non-expressing cells in control transfections. Co-expression of Hoxa2, along with Pbx and Meis cofactors, reduced the probability of neuronal differentiation in these experiments. To determine whether this inhibition represented a cell autonomous activity of Hoxa2, we carried out a co-culture experiment in which we cultured two groups of P19 cells transfected with different plasmids in close contact, and analyzed the ability of neurogenesis of the Hoxa2 negative cells surrounded by the Hoxa2 positive cells. We further tried
to determine whether Hoxa2 functions downstream of a proneural gene (Ngn1) to inhibit neurogenesis by co-transfecting Hoxa2 with Ngn1.

The results suggested that Hoxa2 could repress neurogenesis of neural crest cells, but they also raise the question of how the neural crest cells that arise from r6, which are also Hoxa2 positive, are able to form neurons of the proximal (superior) ganglion of the vagal nerve. To answer this question, we tested whether the coexpression of Hox3 paralogues, that are also expressed in r6-derived crest cells, were able to relieve the inhibition of neurogenesis caused by Hoxa2.

Finally, to clarify Hoxa2 function in regulating neurogenesis in vivo, we expressed Hoxa2 and its cofactors in neural crest cells that enter the trigeminal ganglion by in ovo electroporation in chick embryos. This group of neural crest cells normally generates both neurons and glia in the trigeminal ganglion. We analyzed the fate of the transfected neural crest cells, and found that the frequency with which transfected cells differentiated as neurons was significantly reduced. Collectively, these data demonstrate that the commitment of pluripotent cells to neuronal and non-neuronal fates may be influenced by the activity of HOX genes and their cofactors.
Chapter 2: Altered neuronal lineages in the facial ganglia of Hoxa2 mutant mice

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ABSTRACT

Neurons of cranial sensory ganglia are derived from the neural crest and the ectodermal placodes, but the mechanisms that control the relative contributions of each are not well understood. Crest cells of the second branchial arch generate few facial ganglion neurons and no vestibuloacoustic ganglion neurons, but crest cells in other branchial arches generate many sensory neurons. Here we report that the facial ganglia of Hoxa2 mutant mice contain a large population of crest-derived neurons, despite the presence of a normal complement of placode-derived neurons. This suggests that Hoxa2 function normally represses, directly or indirectly, the neurogenic potential of second branchial arch crest cells. The phenotype may be an anterior transformation of second arch neural crest cells in the facial ganglion towards a fate resembling that of first arch neural crest cells, which normally do not express Hox genes. We additionally found that overexpressing Hoxa2 in P19 embryonal carcinoma cells reduced the frequency of neuronal differentiation. This reduction required the simultaneous overexpression of Pbx and Meis cofactors, and was antagonized by co-expression of Hoxd3. These data suggest an unanticipated role for Hox gene function in controlling the neurogenic potential of at least some cranial neural crest cells.

Key words: Hoxa2, cranial ganglia, lineage marking, neural crest, neurogenesis, Hox genes, P19 cells, Pbx, Meis.
INTRODUCTION

The sensory neurons of cranial nerve ganglia are mosaic populations that include neurons generated by ectodermal placodes and by neural crest cells in successive stages (Ayer-LeLievre and LeDouarin, 1982; d'Amico-Martel and Noden, 1983). There are large and reproducible variations in the relative numbers and positions of placode-derived and crest-derived neurons in specific cranial ganglia. Whereas the trigeminal ganglion contains many crest-derived neurons, the vestibuloacoustic ganglion contains none and the facial ganglion contains very few. Placode- and crest-derived neurons associated with the glossopharyngeal and vagal nerves are physically segregated into distal (petrosal and nodose) and proximal (jugular and superior) ganglia. The mechanisms that lead to these variations have not been identified.

Manipulations that remove placode-derived neurons have shown that cranial neural crest cells generate additional neurons when placode-derived neurons are missing (Fode et al., 1998; Harrison et al., 1995). A similar regulation of neurogenic potential has been observed between early- and late-migrating populations of neural crest cells in zebrafish dorsal root ganglia (Raible and Eisen, 1996), and between Neurogenin2- (Ngn2) and Neurogenin1- (Ngn1) dependent neurons of mammalian dorsal root ganglia (Ma et al., 1999). Cranial neural crest cells that do not normally form neurons, such as those of the nodose ganglion, can generate neurons upon transplantation to appropriate environments (Ayer-LeLievre and LeDouarin, 1982). Such observations have led
to the suggestion that the presence of placode-derived neurons normally represses neurogenesis by cranial neural crest cells (Ayer-LeLievre and LeDouarin, 1982).

Differences in the numbers of crest-derived neurons may, additionally or alternatively, reflect differences in developmental potential of crest cells themselves. Many non-neuronal fates of cranial neural crest cells are modulated by the activities of Hox genes (Couly et al., 2002). A striking example is the requirement for Hoxa2 function in neural crest cells that arise from the fourth rhombomere (r4) of the hindbrain and form the mesenchyme of the second branchial arch. Loss of Hoxa2 function results in neonatal lethality and the transformation of some second arch skeletal elements towards fates normally assumed by first arch neural crest cells (Gendron-Maguire et al., 1993; Rijli et al., 1993). The phenotype is similar to that observed after heterotopic transplantation of the neuroepithelium that normally gives rise to first arch neural crest into sites that give rise to second arch neural crest (Noden, 1983). Additionally, ectopic expression of Hoxa2 in the first branchial arch leads to alterations of crest cell fate that are consistent with a loss of first arch identity (Creuzet et al., 2002; Grammatopoulos et al., 2000). Hoxa2 thus appears to actively promote development of second arch skeletal elements and connective tissues from crest-derived mesenchyme. Whether Hoxa2, or indeed any Hox gene, contributes to the control of neurogenesis by neural crest cells within developing cranial ganglia is not known.
In this study we have used a lineage marking paradigm based on the transient expression of a site-specific recombinase (O'Gorman et al., 1991) to heritably label the second arch neural crest cell lineage. As previously described by others (Arenkiel et al., 2003), we found that in wild-type embryos the neural crest population that arises from *Hoxb-1*-expressing progenitors in r4 preferentially formed glial cells in the facial ganglion, but we also found that it formed a small number of sensory neurons in the facial ganglion and all of the parasympathetic neurons of the ptergopalatine ganglion. Unexpectedly, we found that in mutant embryos lacking *Hoxa2* function, the r4-derived neural crest cells generated a large, supernumerary population of facial ganglion neurons, suggesting that *Hoxa2* function normally represses neurogenesis in this population of neural crest cells. To test this hypothesis we examined the effect of *Hoxa2* overexpression on the frequency of neuronal differentiation in high density cultures of the P19 embryonal carcinoma cell line (Jones-Villeneuve et al., 1982; Rudniki and McBurney, 1987). In these experiments, coexpression of *Hoxa2* and the *Pbx* and *Meis* transcriptional cofactors reduced the probability of neuronal differentiation. We additionally found that this effect could be antagonized by simultaneous expression of a Hox3 paralogue. Thus, both the cranial ganglia phenotype of *Hoxa2* mutant embryos and neurogenesis assays with P19 cells suggest that during normal embryogenesis and in vitro, the commitment of pluripotent cells to neuronal and non-neuronal fates may be influenced by the activity of Hox genes and their cofactors.
MATERIALS AND METHODS

Mice.

The *Hoxb1* locus was isolated from a 129 strain genomic lambda phage library (Stratagene) using a cDNA probe (Frohman *et al*., 1990). A targeting vector (Fig. 1A) was prepared that contained a Cre recombinase coding sequence from pOG231 (O'Gorman *et al*., 1997), a neomycin resistance cassette from pMC1neo-polyA (Thomas and Capecchi, 1987) and a HSV thymidine kinase cassette from pNT (Tybulewicz *et al*., 1991). Linearized vector was transfected into CCE embryonic stem cells (Robertson *et al*., 1986) and recombinant clones were isolated by selection with G418 and ganciclovir and identified by Southern blotting (Fig. 1B). Germline transmission of two independent clones was obtained; both recombinant alleles had phenotypes that were the same as reported null alleles (Goddard *et al*., 1996; Studer *et al*., 1996) and behaved as simple Mendelian recessives. The mutant alleles were backcrossed onto a B6D2F1 background. Mice with a null allele of *Hoxa2* (*Hoxa2*tm1Grid (Gendron-Maguire *et al*., 1993)) were obtained from Jackson Laboratories. Mice bearing the Cre-conditional *R26R* reporter allele (Soriano, 1999) were obtained from Philippe Soriano. Mice bearing a null allele of the *Bdnf* locus (Conover *et al*., 1995) were obtained from David Katz. Each strain was maintained by backcrossing to B6D2F1 hybrids. For timed pregnancies the day of vaginal plugging was designated E0.5. Embryos were harvested into cold PBS and fixed in 4% phosphate-buffered formaldehyde for 1-4 hrs, washed in PBS, and either stored in methanol for whole mount in situ hybridization or equilibrated
with 30% sucrose in PBS for frozen sectioning for immunohistochemistry or in situ hybridization.

Expression constructs.

Expression plasmids for GFP (pUS2-GFP), Neurogenin1 (pCS2-Ngn1), NeuroD2 (pUS2-NeuroD2) (McCormick et al., 1996), and puromycin (pUS2-puro) were generously provided by Dr. David Turner. Expression plasmids for Hoxa2 (pCMV-Hoxa2), Pbx1 (pCMV-Pbx1) and Meis1 (pCMV-Meis1) were prepared by amplifying the respective coding sequences from a mouse cDNA library (Clonetech) and cloning the products into pCDNA3.1 (Invitrogen). The pCMV-Hoxd3 expression plasmid was generously provided by Nancy Boudreau (Boudreau et al., 1997). pUS2-Ngn1 was made by shuttling the Ngn1 sequence from pCS2-Ngn1 into pUS2. An plasmid expressing a mutant Hoxa2 (W99A) protein unable to heterodimerize with Pbx proteins was prepared by using a mutagenesis kit (Quikchange, Stratagene) to change the conserved tryptophan residue of the pentapeptide sequence (Knoepfler and Kamps, 1995) to alanine. The resulting plasmid was fully sequenced to confirm that only the desired change occurred.

Neuronal differentiation assays using P19 cells.

P19 cells were maintained at subconfluent densities in alpha MEM with 7.5% calf serum and 2.5% fetal bovine serum (FBS, Gibco) (Rudniki and McBurney, 1987). Twenty-four hours before transfection aliquots of 2x10^5 cells
were plated in 6cm dishes. In the first series of experiments P19 cells were transfected with pUS2-GFP and various combinations of expression plasmids for *Hoxa2, Hoxd3, Pbx1, Meis1,* and *Neurog1.* Each transfection included 2 µg of one to four expression plasmids, 2µg of pUS2-GFP, and enough neutral vector (pUS2-puro) to bring the total amount of plasmid DNA to 10 µg in FuGene6 transfection reagent (*Roche*). After overnight incubation, cells were resuspended and seeded on gelatin-coated cover-slips in 24-well plates (2x10⁴ cells per well) and 24 hours later the medium was changed to Opti-MEM with 1% FBS to promote differentiation. The medium was changed after a further two days and the cells were fixed and stained with antibodies to PGP9.5 (*Wilkinson et al.*, 1989) after a total of four days in differentiation medium. Given the low frequencies of neuronal differentiation seen in most of these transfections, neuronal differentiation was scored as the fraction of PGP9.5-positive neurons that arose from transfected (GFP-positive) cells. Because neuronal differentiation was efficiently induced by Neurog1, neurogenesis in transfections that included the Neurog1 expression plasmid was scored as the fraction of GFP-positive cells that were also PGP9.5 positive.

In a second series of experiments a test population of P19 cells was seeded onto feeder layers of P19 cells that had been transfected with a combination of *Hoxa2, Pbx1,* and *Meis1* expression plasmids (see timeline in Figure 8). The feeder cells were transfected on day 1 as described above (omitting the GFP expression plasmid), mitotically inactivated 24 hours later
using MitomycinC, and then seeded at a density of $6 \times 10^5$ cells per well on gelatinized coverslips in 24-well plates (day 2). The test population of P19 cells was transfected with pUS2-GFP on day 2, and was resuspended and plated on the feeder cells at a density of 3000 cells per well in Opti-MEM with 1% FBS on day 3. The cultures were maintained with daily changes of medium for a further four days before being fixed and labeled for scoring. In these experiments neuronal differentiation was scored as the fraction of GFP-positive cells that had differentiated as neurons (PGP9.5-positive).

**In ovo electroporation**

In ovo electroporations were done on HH stage 10 (Hamburger and Hamilton, 1952) chick embryos. Expression plasmids were mixed in PBS containing Trypan blue and pressure-injected into the lumen of the neural tube just posterior to the midbrain vesicles. All injection mixes contained $4 \mu g/\mu l$ of a CMV-GFP expression plasmid, various combinations of Hoxa2, Pbx1, and Meis1 expression plasmids (each at $1.5 \mu g/\mu l$), and enough empty CMV expression vector to bring the total plasmid concentration to $8.5 \mu g/\mu l$ for each mix. Electroporation was performed using a BTX electroporator (ECM 830) delivering five 50ms square pulses of 18 V through platinum electrodes placed on either side of the neural tube at the midbrain level. Transfected embryos were allowed to develop for 4 days and then examined under fluorescent illumination using a Zeiss dissecting microscope. Embryos expressing GFP in trigeminal ganglia were fixed and processed for immunohistochemistry.
**Immunohistochemistry.**

Sections or coverslips were blocked with 5% normal donkey serum in PBST (PBS plus 0.3% triton) and primary antibodies were applied overnight at 4°C. Primary antibodies included: goat anti β-galactosidase (Biogenesis, 1:3000); rabbit anti β-galactosidase (Cappel, 1:5000); mouse anti β-tubulin (Tuj1) (Babco, 1:1000) (Lee et al., 1990); rabbit anti neurofilament 200 (Sigma, 1:3000); rabbit anti PGP 9.5 (Accurate, 1:20,000) (Wilkinson et al., 1989); mouse anti-Islet1/2 (Developmental Studies Hybridoma Bank, 1:100) (Ericson et al., 1992); rabbit anti TrkA (Chemicon, 1:500); rabbit anti phosphohistone H3 (Upstate, 1:800); mouse anti Sox10 (Lo et al., 2002). Secondary antibodies (Jackson Immunochemicals) conjugated to either Cy3 or FITC were applied for two hours. Histological material generated from mouse embryos was examined and photographed with a Zeiss Axioskop microscope equipped with a Hamamatsu digital camera, while sections of electroporated chick embryos were photographed using a Zeiss LSM410 confocal microscope.

**In situ hybridization.**

The probes for mouse Delta-like1 (Dll1, (Bettenhausen et al., 1995), mouse Delta-like3 (Dll3, (Dunwoodie et al., 1997), and Notch1 (Conlon et al., 1995) were provided by Ronald Conlon. The second exon of Hoxa2 and the entire Neurog1 and Neurogenin2 (Neurog2) coding sequences were amplified by PCR from genomic DNA, cloned into pCR4-TOPO (Invitrogen), and their
identities were confirmed by sequencing. Digoxigenin-labeled sense and antisense probes were synthesized using a kit (*Roche*) according to the manufacturer’s instructions. Hybridization was performed as described by Schaeren-Wiemers and Gerfin-Moser (Schaeren-Wiemers and Gerfin-Moser, 1993).

**Quantification.**

For sectioned material, cell counts were made on every fourth, 15 µ thick frozen section from serial sets through the facial and vestibuloacoustic ganglia of E14.5 embryos. Singly PGP9.5-positive cellular profiles and doubly PGP9.5- and β-galactosidase marker-positive profiles were counted separately without correction for nuclear size. The volumes of ganglia were determined using Openlab software. The data presented represent counts from both ganglia of three wild-type and three mutant embryos that were then averaged. For the first series of P19 cell transfections, coverslips were scanned in a predetermined, orthogonal pattern. All fields containing PGP9.5-positive neuronal profiles were photographed until at least 200 cells had been sampled, and the number of PGP9.5-positive cells that coexpressed GFP was determined. For the feeder cell experiment and the *Neurogenin1* transfections, coverslips were scanned in the same manner, all fields containing GFP-positive cells were photographed until a total of 200 cells had been sampled, and the number of GFP-positive cells that coexpressed PGP9.5 was determined. All transfection assays were repeated three times. For quantification of neurogenesis in chick embryos, approximately
100 GFP-positive cells were scored for nuclear Islet1/2 expression in at least three embryos for each plasmid combination.
RESULTS

Labeling of r4 and r4-derived neural crest by the $B1^{Cre}$ allele.

To mark the neural crest that populates the second branchial arch for lineage analysis, we prepared a recombinant allele of $Hoxb1$ ($B1^{Cre}$) in which the first exon was replaced by the coding sequence for Cre recombinase (Materials and Methods, Fig. 1A, B). This allele was null for $Hoxb1$ expression (not shown), and was predicted to express Cre in tissues that normally express $Hoxb1$. Cre-mediated recombination of a Cre-conditional reporter allele would be expected to heritably label the lineages that descend from $Hoxb1$-expressing progenitors. The expression pattern of $Hoxb1$ is highly dynamic (Frohman et al., 1990; Murphy et al., 1989). At early stages (embryonic day 7.5 (E7.5)), it is expressed in the posterior primitive streak and flanking mesoderm. By E8.5 expression is observed in the neural tube up to the presumptive r3/4 boundary in the hindbrain, and is elevated in r4 itself. Between E8.5 and E9.5, $Hoxb1$ expression is increases in r4 but rapidly decreases in more caudal regions of the hindbrain. Based on this expression pattern, the $B1^{Cre}$ allele could potentially label all cells derived from rhombomere 4, including the r4-derived neural crest cells that enter the second branchial arch.

In $+/B1^{Cre}$ embryos the patterns of Hoxb1 protein expression (from the wild-type allele) and Cre recombinase expression (from the B1Cre allele) were identical (Fig. 1F, H, and data not shown). At E9.5, cells in r4 expressed high
levels of both proteins. By contrast, both Hoxb1 and Cre proteins were nearly undetectable in neural crest cells that had emerged from r4. This finding is consistent with reports that Hoxb1 mRNA is not expressed in r4-derived neural crest (Frohman et al., 1990; Murphy and Hill, 1991). We were unable to detect Hoxb1 or Cre protein in more distal cells of the second branchial arch at E9.5 or E10.5, or in any craniofacial tissues or the hindbrain at E11.5, E12.5, E15.5 or P0 (not shown). We never observed either Hoxb1 or Cre expression in the neural tube rostral to r4.

In embryos containing the \( B1^{Cre} \) allele and the Cre-conditional R26R ß-galactosidase marker allele (Soriano, 1999) (+/\( B1^{Cre} \); +/R26R ), ß-galactosidase expression from the recombined marker allele could be observed in the presumptive r4 territory as early as E8.75 (Fig. 1C). By E9.5, all cells in r4 expressed the marker (Fig. 1D, G, I). By contrast, no marker expression was observed in neural tissues anterior to r4, and only low levels of marker activation were seen in more posterior rhombomeres. The labeling of the posterior hindbrain is likely to result from the transient expression of Hoxb1 and Cre prior to approximately E9. Considerably more recombination in r5 and the posterior hindbrain was seen in embryos homozygous for the \( B1^{Cre} \) allele (Fig. 1E). In these embryos r4 was still completely labeled, despite the fact that Hoxb1 positively regulates its own expression (Popperl et al., 1995).
At E10.5, the territory expressing the marker extended from the root of the facial nerve to the distal extremity of the second branchial arch and included virtually all second arch mesenchymal cells (Fig. 1J, 2A). Temporally, neural crest cells populate the branchial arches in a distal to proximal order, suggesting that the marker was recombined in the first neural crest cells to emerge from r4. The central mesodermal core, which could be identified by the expression of either MyoD (not shown) or Islet1/2 proteins (Fig. 2A), did not express the marker. The surface ectoderm, including the neurogenic ectodermal placode, was also negative for marker expression (Fig. 2B). Unlabeled, Islet1/2-positive cells were positioned between the placode and the developing facial ganglion; these were likely to be neuroblasts that had recently delaminated from the placode that were migrating towards the ganglion (Fig. 2B).

In summary, Cre expression from the $B1^{Cre}$ allele led to recombination of the R26R allele and ß-galactosidase expression in all cells of r4 by E9.5, and resulted in marker expression by the r4-derived neural crest cell lineage, including at least most of the earliest r4-derived crest to emerge from the hindbrain. Our immunohistochemical data confirmed observations made by others using in situ hybridization that suggest $Hoxb1$ expression is extinguished in craniofacial tissues after approximately E10.5. We have additionally been unable to detect Cre expression from the $B1^{Cre}$ allele after that stage, and have not observed novel sites of marker expression that cannot be easily traced to the migration of second arch cells in a staged embryo series. We conclude that
virtually all of the cells within the second branchial arch territory that express the R26R marker in +/B1<sup>Cre</sup>; +/R26R embryos arise from r4-derived neural crest cells, and that populations that do not express the marker do not arise from r4-derived crest cells.

**Labeling of anterior facial nerve branches.**

The boundary between the mesenchyme of the first and second branchial arches was sharply defined, and through E15 the only marker-expressing cells consistently found anterior to this boundary were associated with branches of the facial nerve (Fig. 2C-O). The greater petrosal nerve branches from the facial nerve trunk immediately distal to the facial ganglion, runs anteriorly above the oropharynx to innervate the ptergopalatine ganglion, and is comprised of preganglionic parasympathetic axons. At E10.5, axons of the nerve had emerged from the second arch territory and were invested with cells that expressed the marker; some of these also expressed Sox10, which labels glial lineages (Kuhlbrodt et al., 1998) (Fig. 2C). At E13.5 the ptergopalatine ganglion was populated by neuronal and non-neuronal cells that expressed the second arch lineage marker (Fig. 2M, N).

A second division of the facial nerve is the chorda tympani, which branches from the facial nerve trunk ventral to the first pharyngeal pouch and contains sensory axons of facial ganglion neurons that innervate lingual taste buds. In some E10.5 specimens, the initial axons of the chorda tympani were
observed to cross into first arch mesenchyme without being accompanied by second arch cells (Fig. 2F), but in most E10.5 specimens and at all later stages the nerve was already invested with Sox10-positive cells that co-expressed the marker (Fig. 2C-E, G (inset), L). The axons of the chorda tympani, like their parental cell bodies (see below) did not express the lineage marker (Fig. 2G-I). By contrast, axons of the third major facial nerve branch, its motor root, did express the marker (Fig. 2G, J, K). The motor root exited the second arch territory between E11.5 and E12.5 and was also accompanied by many marker positive second arch cells (Fig. 2L). Labeled second arch cells were also associated with the distal sensory axons of the eighth cranial nerve (Fig. 2O).

**r4 neural crest generates few neurons in the facial ganglion of wild-type embryos.**

At E10.5, only a few of the Islet1/2-positive sensory neurons of the facial ganglia in wild-type embryos expressed the lineage marker (Fig. 3A). Because the ectodermal placode of the second arch did not express the lineage marker while virtually all r4-derived neural crest cells did, and because the placode contributes neurons to the facial ganglion in both birds (Ayer-LeLievre and LeDouarin, 1982; d'Amico-Martel and Noden, 1983) and mice (Fode et al., 1998), we refer to these marker-negative sensory neurons as placodal neurons. Cells expressing the lineage marker and Sox10, a marker of glial precursors (Kuhlbrodt et al., 1998), were scattered throughout the ganglion and ensheathed its external margins (Fig. 3B). At all stages examined (E10.5-E17.5) small
clusters of neurons that expressed the lineage marker were consistently found in the proximal ganglion near or within the facial nerve root, and additional labeled neurons were scattered at the periphery of the ganglion (Fig. 3A, C). At E14.5, approximately 6% of facial ganglion neurons expressed the lineage marker (Table 1). The fraction of crest-derived neurons did not appear change at E17.5 (not shown).

Neural crest-derived neurons in the facial ganglia of Hoxa2 mutant mice.

Embryos heterozygous for the B1Cre allele and either heterozygous or homozygous for the Hoxa2tm1Grid null allele did not show changes in the expression of Hoxb1 or Cre at E9.5, when the expression of both in the hindbrain was confined to r4 (not shown). As in wild-type embryos, only faint immunoreactivity for Hoxb1 protein was observed in the ventral portion of r4 at E10.5, and none could be detected in hindbrain or craniofacial tissues at E11.5 or E12.5. This is consistent with previous reports that the expression of the Hoxb1 allele is not altered in mice lacking Hoxa2 function (Gendron-Maguire et al., 1993; Rijli et al., 1993; Davenne et al., 1999).

At E10.5, the distribution of placode and crest-derived cells within the facial ganglia of embryos homozygous for the Hoxa2tm1Grid null allele was similar to that in controls. In both, the majority of Islet1/2 expressing neurons in did not express the lineage marker (Fig. 3A,D), and both contained small numbers of marker positive neurons in proximal portions of the ganglion (arrows). By
contrast, most Sox10-positive presumptive glial cells did express the marker (Fig. 3B,E), although a few marker-negative glial cells were present in both wild-type and control embryos (arrowheads).

A consistent and striking difference arose by E11.5, when a substantial number of neurons expressing the lineage marker were present in the proximal ganglion of the mutants (Fig. 3C, F). By E14.5, placode- and crest-derived neurons formed abutting, largely distinct portions of the ganglion (Fig. 3G), and the number of crest-derived neurons in Hoxa2 mutant embryos exceeded the total number of neurons present in wild-type embryos (Table 1). The emergence of the crest-derived population was not caused by a marked deficiency of placode-derived neurons, which were present in similar numbers in wild-type and mutant embryos (Table 1). Phosphohistone H3 and Islet1/2 labeling showed comparable numbers of mitotically active neuroblasts in E10.5 control and mutant embryos (Fig. 4A, B). Because there was a slight reduction in the number of placode-derived neurons in the sample of Hoxa2 mutant embryos, and because the complete absence of placode-derived neurons in the facial ganglion has been associated with the delayed generation of sensory neurons, presumably by neural crest, in Neurog2 mutant embryos (Fode et al., 1998), we asked if reductions of facial ganglion cell number necessarily invokes the production of crest-derived neurons. We therefore combined the $B1^{Cre}$ and $R26R$ alleles with a null mutation of the $Bdnf$ locus (Conover et al., 1995). Although the number of facial ganglion neurons in embryos without $Bdnf$ function is reduced
by approximately 60% (Jones et al., 1994), examination of serial sections from two Bdnf mutant embryos revealed no evidence of supernumerary, crest-derived neurons at E14.5 (Fig. 3H).

**Supernumerary, crest-derived neurons express TrkA receptors.**

The survival of cranial sensory neurons requires the activities of neurotrophins and Trk receptors (Huang and Reichardt, 2001; Huang and Reichardt, 2003). Most facial ganglion neurons require either BDNF or NT3 for survival (Liu and Jaenisch, 2000), and express either TrkB or TrkC, while a small number express TrkA (Matsumoto et al., 2001). When the facial ganglia of Hoxa2 mutant embryos were examined by immunohistochemistry at E12.5 and E14.5, the crest-derived neurons were found to express TrkA, whereas few neurons in the distal ganglion of mutant embryos or the entire facial ganglion of wild-type embryos expressed this receptor (Fig. 4C, D). TrkA positive processes were observed to extend distally into the facial nerve and proximally into the CNS in the mutants. Whether these TrkA-positive processes establish functional connections was not determined.

**Hoxb-1 gene dosage does not alter facial ganglion lineages.**

In principle, a reduction of Hoxb1 expression caused by the B1Cre null allele could have altered ganglionic lineages in control and Hoxa2 mutant embryos. Two lines of data suggested this was not the case. Examination of TrkA expression in Hoxa2 mutant embryos that were wild-type at the Hoxb1
locus showed that at both E12.5 and E14.5 the same supernumerary population of TrkA-expressing neurons was present (not shown). We also determined the numbers and distributions of labeled and unlabeled neurons in embryos that were homozygous for the $B1^{Cre}$ allele and wild-type at the Hoxa2 locus. As in $+/B1^{cre}$ embryos, $B1^{Cre}/B1^{Cre}$ embryos showed uniform labeling of the r4-derived neural crest population (Fig. 1E and data not shown). At E14.5 the numbers of crest-derived neurons in $+/B1^{Cre}$ and $B1^{Cre}/B1^{Cre}$ embryos were similar, and the number of unlabeled, placode-derived neurons was, if anything, slightly increased in $B1^{Cre}/B1^{Cre}$ embryos (Table 1). It is therefore unlikely that haploinsufficiency for Hoxb1 function contributed to the phenotype we have ascribed to the loss of Hoxa2 function.

**Expression of Hoxa2 in the facial ganglion and surrounding tissues.**

To determine the potential sites of Hoxa2 gene action we examined its expression at E9.5 and at E11.5. At E9.5 Hoxa-2 was expressed in the hindbrain, the facial nerve root, and in second branchial arch mesenchyme, but not in the central, neuron-rich portions of the facial and vestibuloacoustic ganglia (Fig. 5A). A layer of non-neuronal cells surrounding the ganglion expressed Hoxa2; those surrounding the vestibuloacoustic ganglion did not. We found no evidence of Hoxa2 expression in the ectodermal placode of the second branchial arch (not shown). Expression was observed in the posterior ectoderm of the arch, as has been reported for the chick (Couly *et al.*, 1998). By E11.5, after many crest-derived neurons were generated in Hoxa2 mutants, expression in the nerve root
and in cells surrounding the facial ganglion had diminished (Fig. 5B). Expression increased in the second arch mesenchyme, and low levels were detected in third arch mesenchyme. Thus, the placode-derived neurons of the facial ganglion, their ectodermal precursors, and the mesenchyme surrounding the facial nerve root and ganglion, did not express detectable levels of *Hoxa2* mRNA from E9.5 to E11.5.

**Ectopic gene activation in *Hoxa2* mutant embryos.**

Because the generation of placode- and crest-derived neurons in cranial ganglia is governed by multiple molecular pathways, we sought to determine if some of these were altered by loss of *Hoxa2* function. Neurog1 and Neurog2 are basic helix-loop-helix proteins required for the development of different populations of placode- and crest-derived neurons (Fode *et al.*, 1998; Ma *et al.*, 1998; Ma *et al.*, 1999). *Neurog2* is required for the generation of placode-derived facial ganglion neurons (Fode *et al.*, 1998). We found that *Neurog2* was expressed by the second arch placode of *Hoxa2* mutant embryos at levels comparable to controls (Fig. 5C, D). *Neurog1* is not required for the generation of most facial ganglion neurons, but is essential for the generation of many neural crest-derived neuronal populations (Ma *et al.*, 1998; Ma *et al.*, 1999). *Neurog1* expression could not be detected in the facial ganglia of E11.5 control embryos (Fig. 5E). By contrast, *Neurog1* was strongly expressed in the anterodorsal region of the facial ganglia of *Hoxa2* mutant embryos (Fig. 5F) surrounding and proximal to neuronal cell bodies (Fig. 5G). This corresponds to the position of
marker-expressing neurons at E11.5 (Fig. 3F) and marker- and TrkA-expressing neurons at E12.5 (Fig. 4D and data not shown). Thus, the expression of Neurog1, an early component of a neurogenic molecular cascade, was inappropriately activated in the facial ganglia of Hoxa2 mutants.

Members of the Notch/Delta family of receptors and ligands may regulate neurogenesis and gliogenesis in developing sensory ganglia (Lindsell et al., 1996; Myat et al., 1996; Wakamatsu et al., 2000), although their roles in controlling neurogenesis in cranial ganglia are not established. We were unable to detect Dll1 transcripts in the facial ganglia of wild-type embryos at E11.5 (Fig. 6A), and Notch1 expression was marginally higher than background in the central portion of the facial ganglia and elevated at its periphery (Fig. 6C). By contrast, the expression of Dll1 and Notch1 was markedly elevated in the facial ganglia of E11.5 Hoxa2 mutant embryos (Fig. 6B, D). The highest expression for both genes surrounded the more proximal portions of the ganglion (Fig. 6E and data not shown). The expression of Notch1, Dll1, and Neurog1 in the facial ganglia of mutant embryos was thus clearly different from that observed in controls, and more closely resembled the expression pattern observed in the trigeminal ganglia of control embryos.

Hoxa2, Pbx1, and Meis1 collectively inhibited spontaneous neuronal differentiation of P19 cells.
To test the idea that Hox gene function influences neurogenesis by cell populations capable of adopting both neural and non-neural phenotypes, we overexpressed combinations of Hox proteins and Pbx and Meis transcriptional cofactors in high density cultures of P19 cells. P19 embryonal carcinoma cells form a variety of differentiated cell types, and neurogenesis is efficiently induced by aggregation culture in the presence of retinoic acid (Jones-Villeneuve et al., 1982). Low levels of neurogenesis are also seen in the absence of exogenous retinoic acid in long-term cultures or culture under serum-free conditions (Rudniki and McBurney, 1987). Because retinoic acid exposure induces Hox gene expression in embryonal carcinoma cell lines (Featherstone et al., 1988; Popperl and Featherstone, 1993; Simeone et al., 1990) and the expression of Pbx and Meis proteins in P19 cells (Knoepfler and Kamps, 1997), we examined the effect of transfected Hoxa2, Pbx1, and Meis1 on neuronal differentiation in high-density cultures of P19 cells maintained in low-serum medium.

Under the conditions used, transfection efficiencies were uniformly high (ca. 50%) and the frequency of neuronal differentiation was quite low (<5%) (Fig. 7A-C). We therefore determined the fraction of PGP9.5-positive neurons (Wilkinson et al., 1989) that arose from GFP-positive, transfected cells. We found that when cells were transfected with GFP alone, approximately 44% of the neurons arose from transfected cells (Fig. 7D). In cultures simultaneously transfected with Hoxa2, Pbx1, and Meis1, there was a consistent reduction in the number of neurons that arose from transfected cells, to approximately 32%
(p<0.05, unpaired t-test). The frequency of neurogenesis in cultures transfected with *Hoxa2* by itself, or with *Hoxa2* and either *Pbx1* or *Meis1*, was similar to that observed in the GFP control transfections (Fig. 7D and data not shown).

To ask whether the combined activity of *Hoxa2* and *Pbx1* in these experiments required direct interactions between the proteins, we used a mutation of *Hoxa2* that prevents heterodimer formation. Wild-type *Hoxa2* contains a pentapeptide sequence (consensus Y/F P W M K/R) amino-terminal to the homeodomain that is required for cooperative DNA binding by Hox and Pbx protein dimers (Knoepfler and Kamps, 1995), and changing the tryptophan residue of this sequence prevents the formation of functional dimers (Neuteboom *et al.*, 1995). We therefore prepared a mutant *Hoxa2* expression construct encoding a W99A mutation of the pentapeptide and performed a second set of assays in P19 cells. Cotransfection of the W99A *Hoxa2* mutant together with *Pbx1* and *Meis1* did not alter the base-line level of neuronal differentiation observed in control, GFP-only transfections (Fig. 7E).

We also sought to determine if the inhibition of neurogenesis represented a cell autonomous activity of *Hoxa2*, or if its expression by surrounding cells could inhibit neurogenesis. We therefore prepared a “test” population of GFP-expressing P19 cells and seeded them at low densities on high density “feeder” layers of P19 cells that had been cotransfected with expression plasmids for *Hoxa2* and the cofactors, or with a control plasmid. There was no difference in
the frequency of neuronal differentiation of test cells seeded on either type of feeder layer (Figure 8). This result suggests that the Hoxa2-mediated inhibition of neurogenesis we observed represented a cell autonomous activity of Hoxa2.

Because we observed ectopic Neurog1 expression in the facial ganglia of Hoxa2 mutant embryos (Figure 4E-G), we attempted to determine if Hoxa2 expression could alter neurogenesis caused by the forced expression of Neurog1 in P19 cells. As reported by others (Farah et al., 2000), we found that a high proportion (89%) of P19 cells transfected with a US2-Neurog1 expression vector differentiated as neurons (Figure 9). This frequency was not altered by simultaneous expression of Hoxa2, Pbx1, and Meis1. Thus, while the analysis of Hoxa2 mutant mouse embryos suggests that Hoxa2 may repress, directly or indirectly, the expression of Neurog1 from the endogenous locus in vivo, these experiments suggest that the neurogenesis initiated by high levels of Neurog1 is not affected by Hoxa2.

**Hoxd3 cotransfection relieved Hoxa2-mediated inhibition of neuronal differentiation.**

These data suggest that Hoxa2 can repress neurogenesis in pluripotent cell populations, but they also raise the question of how the neural crest cells that arise from rhombomere 6, that also express Hoxa2, are able to form neurons of the proximal (superior) ganglion of the ninth cranial nerve. One difference between r4- and r6-derived neural crest cells is that r6-derived cells also express
Hox3 paralogues (Manley and Capecchi, 1997), while r4-derived cells do not. To test the possibility that Hox3 paralogues relieve the inhibition of neurogenesis caused by Hoxa2, we examined neuronal differentiation in P19 cells cotransfected with Hoxa2 and Hoxd3. In the presence of Pbx and Meis, significantly higher rates of neurogenesis were observed in cultures cotransfected with Hoxa2 and Hoxd3 relative to cultures transfected with Hoxa2 alone (p<0.01, Fig. 7D). Transfection of Hoxd3 and the cofactors, without Hoxa2, also resulted in a consistent elevation of neurogenesis over that seen in GFP control transfection (p<0.05), and this increase required the cofactors. These results suggest that Hox3 paralogues antagonize the inhibition of neurogenesis caused by Hoxa2 in a manner consistent with the principle of posterior prevalence in Hox gene function (Gonzalez-Reyes and Morata, 1990).

*Hoxa2-mediated inhibition of neurogenesis in vivo.*

To determine if the inhibition of neurogenesis observed in transfected P19 cells was relevant to the differentiation of neural crest cells within intact embryos, we used electroporation in chick embryos to force the expression of various combinations of Hoxa2, Pbx1, and Meis1 in neural crest precursors that normally generate both neurons and glia in the trigeminal ganglion. Neural crest cells that migrate to the trigeminal ganglion are derived from the midbrain and r1 and r2 of the hindbrain, and do not express any Hox gene. Although Hoxa-2 is expressed within the r2 neural tube, its expression is extinguished in the neural crest cells that emerge from r2 (Prince and Lumsden, 1994). We electroporated mixtures of
plasmids encoding GFP and Hoxa2, with or without the Pbx1 and Meis1. The electroporations were targeted to the midbrain-hindbrain junction of HH stage 10 embryos (Hamburger Hamilton, 1952), just before the late-migrating Neural crest cells, that contribute to the trigeminal ganglia emerge from the neural tube. This avoided significant levels of plasmid expression in the crest-derived ectomesenchyme of the first branchial arch, as occurs after slightly earlier electroporations (Creuzet et al., 2002), and did not generate notable alterations in the organization of craniofacial tissues at the time of harvest (stages 27-28).

In control embryos electroporated with a GFP expression plasmid, approximately 70% of the GFP-expressing cells in the trigeminal ganglia also expressed Islet1/2, identifying them as neurons (Figure 10). By contrast, when Hoxa2 and both the Pbx1, and Meis1 cofactors were expressed simultaneously, the frequency of neuron differentiation was significantly reduced, to approximately 40% (p< 0.01). The frequency of neuronal differentiation in a second set of control embryos, electroporated with a plasmid mix that contained Hoxa2 and Pbx1 expression vectors, but omitted the Meis1 vector, did not differ from the GFP-only controls. Thus, the forced expression of exogenous Hoxa2, in the presence of both Pbx1 and Meis1, reduces the frequency of neuronal differentiation by precursors of the trigeminal ganglion.
DISCUSSION

Control of peripheral neurogenesis by *Hoxa2*.

A combination of lineage analyses of *Hoxa2* mutant mouse embryos, overexpression experiments in P19 cells, and misexpression studies in chick embryos has shown that the activity of HOX genes can influence neurogenesis by cranial neural crest cells and pluripotent embryonal carcinoma cells. We found that the facial ganglia of *Hoxa2* mutant embryos contained a population of neurons derived from neural crest cells that was not present in wild-type embryos. The generation of these neurons occurred despite the presence of a grossly normal population of placode-derived neurons. The phenotype suggests that *Hoxa2* expression in r4-derived neural crest cells normally inhibits their ability to differentiate into sensory neurons, and that this inhibition is relieved in the mutants. Additional data consistent with this hypothesis were obtained in transfections of P19 cells, in which *Hoxa2* overexpression caused a reduction in neuronal differentiation, and in experiments in which forcing the expression of Hoxa2 in cells that populate the trigeminal ganglion reduced the probability of neuronal differentiation. In both P19 cells and chick embryos, the reduction of neurogenesis required the presence of Pbx and Meis cofactors. In P19 cells, Hoxa2-mediated inhibition of neurogenesis was abolished by mutation of the Hox protein domain required for dimerization of Hox and Pbx proteins, and could be antagonized by a member of the Hox3 paralogous group.
In mouse embryos, the absence of *Hoxa2* expression clearly affected the generation of facial ganglion sensory neurons by r4-derived neural crest cells, but did not appear to alter the generation of parasympathetic neurons of the ptergopalatine ganglion by the same crest population. The signaling cascades that control sensory and parasympathetic neuron differentiation are distinct from one another. The apparently normal generation of r4-derived parasympathetic neurons in the ptergopalatine ganglion suggests that *Hoxa2* does not influence the signaling pathways, including *Mash1* and *Phox2a* among other factors, that regulate parasympathetic neurogenesis (Hirsch et al., 1998; Lo et al., 1998; Morin et al., 1997). By contrast, the appearance of crest-derived sensory neurons in the facial ganglia of the mutants was accompanied by a striking upregulation of *Neurogenin1* expression, and increases in *Notch1* and *Dll1* expression as well. *Neurogenin1* is required for the generation of sensory neurons by cranial neural crest cells (Ma, 1999), and in a number of systems *Neurogenin1* overexpression, by itself, is sufficient to induce ectopic neurogenesis (Ma, 1996). In transfections of P19 cells we found that *Hoxa2* expression could not antagonize neurogenesis promoted by forced *Neurog1* overexpression. This result does not address the question of whether *Hoxa2* normally represses, directly or indirectly, the expression of the endogenous mouse *Neurogenin1* locus in vivo. It does, however, suggest that *Hoxa2* cannot significantly inhibit neurogenic mechanisms that are initiated by Neurog1 expression. Our results are most consistent with the idea that *Hoxa2* acts
upstream of *Neurogenin1* in neural crest cells to inhibit neurogenesis, and that the mutant phenotype reflects a loss of this inhibition.

The generation of neurons by r4-derived neural crest cells in *Hoxa2* mutant embryos is unlikely to substantially alter the generation of glial cells by the same lineage. Cranial neural crest cell populations are capable of remarkable levels of regulative migration, proliferation, and differentiation, including the complete reconstitution of neuronal and glial populations of cranial ganglia following surgical manipulations (Couly *et al*., 1996). Additionally, the survival and proliferation of glial cell precursors is regulated by factors in the local environment, including many that are produced by differentiating neurons (Anderson, 1997; Dong *et al*., 1995; Wakamatsu *et al*., 2000). We therefore speculate that the generation of crest-derived neurons in the proximal portion of the facial ganglion of *Hoxa2* mutant embryos is likely to result in a local increase in the survival and/or proliferation of glial precursors within the same r4-derived neural crest cell population. We think it is unlikely that the generation of these neurons has any effect on the proliferation or differentiation of the ectomesenchymal derivatives of the r4 neural crest, which have migrated beyond the ganglionic anlagen by the time the supernumerary crest-derived neurons begin to differentiate.

**Fidelity of lineage marking by the *B*1<sup>Cre</sup> allele.**
The labeling of peripheral neurons and glia by the combination of the $B1^{Cre}$ and $R26R$ alleles appeared to be virtually complete and highly selective. Neural crest cells populate the branchial arches in a distal to proximal order (Serbedzija et al., 1992), so the first cells to emerge from the hindbrain form distal ectomesenchyme. Although there is only a narrow temporal window between the onset of Hoxb-1 expression (Frohman et al., 1990) and the generation of neural crest (Nichols, 1986; Serbedzija et al., 1992), we found that the most distal ectomesenchyme in the second arch was labeled, indicating that the marker was recombined in the earliest neural crest cells to migrate out of r4. Cell populations known to arise from non-crest lineages were not labeled, including the ectoderm, the mesodermal core, and the vascular endothelium (Le Douarin, 1983). In addition to r4-derived neural crest, the second branchial arch is thought to contain small contributions from r3- and r5-derived crest (Lumsden et al., 1991; Sechrist et al., 1993; Serbedzija et al., 1992). We did not observe recombination in r3, and only low levels of recombination in r5, and yet we were unable to find more than a rare unlabeled ectomesenchymal cell in the second arch. We did observe a small population of unmarked glial cells in the facial ganglion in wild-type embryos that could have been derived from r3 or r5 neural crest cells. The small number of unlabeled cells in distal portions of the second branchial arch suggests that r3- and r5-derived cells do not proliferate extensively, that they do not survive, or that they are restricted to the margins of the labeled territories, where they would not be recognized as part of the second arch in these preparations.
It is unlikely that the phenotype we observed in Hoxa2 mutant embryos was caused by the presence of the null, B1\textsuperscript{Cre} allele used to mark the r4-derived lineages. Supernumerary, TrkA-positive neurons were present in the ganglia of Hoxa2-mutant embryos that were wild-type at the Hoxb1 locus (Hoxa2-/-:Hoxb1+/+), and, conversely, were absent in embryos that lacked all Hoxb1 function and were wild-type at the Hoxa2 locus (Hoxa2+/+:Hoxb1\textsuperscript{Cre}/Hoxb1\textsuperscript{Cre}). It is also unlikely that the phenotype is due to alterations in the expression of adjacent Hoxa family members, similar to the alterations shown for recombinant alleles of some other HOX genes (Aubin et al., 1998; Barrow and Capecchi, 1996; Ren et al., 2002; Rijli et al., 1994), because the expression of Hoxa1 and Hoxa3 is reported to be unaltered in Hoxa2 mutant embryos (Gendron-Maguire et al., 1993; Rijli et al., 1993).

The results reported here differ from those of an earlier report that also described the fates of neural crest cells derived from Hoxb-1-expressing neuroepithelium (Arenkiel et al., 2003). Both studies agree on the predominantly glial fates of second arch cells in the facial ganglion. Whereas Arenkiel et al. did not comment on the lineage of the ptergopalatine ganglion in their report (Arenkiel et al., 2003), here we found that the neurons of this ganglion arose from second arch neural crest cells. Significantly, this establishes that neither Hoxb1 or Hoxa2 expression in the r4 neuroepithelium or in the neural crest derived from r4, by themselves, preclude neurogenesis. Although both Hoxa2
and Hoxb1 are expressed in the r4 neuroepithelium, we (Fig.1F) and others (Frohman et al., 1990; Murphy et al., 1989) have found that Hoxb1 expression is extinguished as crest cells emerge from r4, while Hoxa2 expression is maintained at high levels in the same cells. The generation of facial ganglion neurons by r4-derived crest in Hoxa2 mutants and the lack of such neurogenesis in Hoxb1 mutants, strongly argue that the inhibition of sensory neuron fates within the facial ganglion during normal embryogenesis is a consequence of Hoxa2, and not Hoxb1, gene function.

We additionally found that r4-derived glial cells were associated with both motor and sensory components of the facial nerve. Although Arenkiel et al. did not illustrate the finding, they reported that r4-derived crest associated exclusively with motor components, and did not associate with sensory axons (Arenkiel et al., 2003). Here, r4-derived neural crest cells were present in the most distal portions of the chorda tympani, associated with sensory axons that innervate lingual taste buds. r4-derived cells also formed the glia associated with the axons running between the acoustic ganglion and the otic vesicle.

There are several possible explanations for this difference between the reports. Cre may be more efficiently expressed from the fusion allele used here because it would not require re-initiation of translation from a bicistronic message, as would be the case (Gorski and Jones, 1999) for the IRES-Cre cassette used by Arenkiel et al. (Arenkiel et al., 2003). We favor this explanation because
Arenkiel et al. illustrated incomplete activation of reporter expression in the motor neuron axons and glia of a purely motor branch of the facial nerve, suggesting that marker activation may be incomplete elsewhere as well. Alternatively, it could also be argued that we see labeling of glia associated with sensory axons because the $B1^{Cre}$ allele ectopically activated the reporter in cells that do not normally express $Hoxb1$. Given that the timing and distribution of Cre expression duplicated that of $Hoxb1$, and that reporter expression closely resembled the cumulative pattern of $Hoxb1$ expression, we feel this explanation is unlikely. Finally, it is possible that the difference between the sets of findings is due to unrecognized differences in genetic background among the mouse strains used, though we again think this is not likely to be the explanation.

**Site of $Hoxa2$ gene action.**

The repression of sensory neurogenesis in the facial ganglion of wild-type embryos could result from a cell autonomous activity of $Hoxa2$, or from cell-cell interactions between crest cells in the ganglionic anlagen and those forming the ectomesenchyme of the second arch, which also express $Hoxa2$. Both the pattern of $Hoxa2$ expression in wild-type embryos and our P19 cell transfections suggest that the inhibition is mediated by cell autonomous mechanisms. In mutant embryos, neurogenesis by crest cells began between E10.5 and E11.5. In wild-type embryos, the mesenchymal cells expressing high levels of $Hoxa2$ mRNA had migrated past the facial ganglion by E9.5, and the mesenchymal cells surrounding the ganglion did not express $Hoxa2$ mRNA. In contrast, high levels
of Hoxa2 mRNA were observed in cells within the proximal nerve root, the region where crest-derived neurons first appeared in mutant embryos. Thus, in wild-type embryos, Hoxa2 was expressed in the population of cells that generated the supernumerary neurons in the mutants, and was not expressed at appreciable levels in surrounding tissues.

The argument that Hoxa2 acts in a cell autonomous manner to repress neurogenesis was strengthened by experiments in which Hoxa2, Pbx1, and Meis1 were over-expressed in P19 cells and first branchial arch neural crest cells in chick embryos. In both cases the misexpression led to reproducible reductions in the frequency of neuronal differentiation. In P19 cells, the frequency of neuronal differentiation in a test population was not influenced by expression of Hoxa2, Pbx1 and Meis1 in feeder cell layers. In the chick embryo electroporations, the GFP-expressing cells were dispersed throughout the trigeminal ganglia, making it unlikely that the transfected cells expressing Hoxa2 affected one another through cell interactions. Collectively, these experiments suggest that the reduction of neuronal differentiation in P19 cells, the reduction in trigeminal neurogenesis in electroporated chick embryos, and the small number of crest-derived neurons present in the facial ganglia of wild-type embryos, are each due to a cell autonomous activity of Hoxa2.

The transfection data also add to accumulating evidence (Knoepfler and Kamps, 1997) that the Pbx and Meis proteins, probably through their interactions
with Hox proteins, are key players in the control of neuronal differentiation in P19 cells (Knoepfler and Kamps, 1997; Qin et al., 2004a; Qin et al., 2004b). It has long been recognized that the induction of neuronal differentiation by retinoic acid in embryonal carcinoma cells, including P19 cells, is associated with increased expression of multiple HOX genes (Pratt et al., 1993). More recently it has been found that retinoic acid also leads to increases in the levels of Pbx and Meis proteins (Knoepfler and Kamps, 1997; Qin et al., 2004a), and that reducing Pbx protein levels, through the use of antisense or short interfering RNA's, reduces neuronal differentiation in retinoic acid treated P19 cells (Qin et al., 2004b).

Pbx and Meis proteins are TALE (three amino acid loop extension) homeodomain transcription factors that modulate the activity of many members of the Hox gene family (Moens and Selleri, 2006). The association of Hox and Pbx proteins in DNA-bound complexes requires a pentapeptide sequence (F/YPWMR/K) amino-terminal to the homeodomain of the Hox partner (Knoepfler et al., 1999). The cooperative binding of Hox and Pbx proteins alters the binding affinity and specificity of the Hox partner, and typically increases transcription of target genes, although more complex interactions that depend on cellular context have also been reported (Saleh et al., 2000). We are not aware of prior reports describing alterations in the frequency of neuronal differentiation of P19 cells that are caused by misexpression of Hox family members. Here, we found that P19 cells overexpressing Hoxa2 or Hoxd3 exhibited reproducible changes in the
frequency of neurogenesis, that these changes were only observed in the presence of the Pbx and Meis cofactors, and that the Hoxa2 mediated inhibition was not observed when the critical tryptophan residue of the peptide (F/YPWMr/K) required for interaction with Pbx proteins (Knoepfler and Kamps, 1995) was mutated.

**HOX genes and peripheral neurogenesis.**

We suggest that phenotype of the facial ganglion in Hoxa2 mutant embryos represents an anterior transformation of the developmental potential of a set of cranial neural crest cells, similar to that seen in mesenchymal tissues of Hoxa2 mutants (Gendron-Maguire et al., 1993; Rijli et al., 1993), and in a variety of skeletal and CNS tissues of animals lacking various single Hox gene functions (Duboule and Morata, 1994; Gonzalez-Reyes et al., 1990). The transformation appears to be limited to the generation of ectopic neurons, and does not extend to the formation of connections between these neurons and the targets normally innervated by trigeminal ganglion neurons (data not shown). Differences in the terminal fates of neural crest cells generated at different levels of the body axis, and in particular the differentiation of sensory and autonomic neurons, are strongly influenced by the tissues through which crest cells migrate and in which they settle (Baker et al., 1997; Le Douarin and Smith, 1988). This applies to the generation of neuronal and non-neuronal cells by cranial neural crest cells as well. For example, the normally gliogenic crest cells of the nodose ganglion form neurons when they are transplanted to the trunk (Ayer-LeLievre and LeDouarin,
Additionally, the removal of placode-derived neurons through surgical ablation (Harrison et al., 1995) or by genetic means (Fode et al., 1998) can induce neural crest cells that normally do not form neurons to generate large numbers of neurons.

An exception to the dominant role of surrounding tissues in the determination of neural crest cell fates is the control of ectomesenchymal fates of first and second branchial arch neural crest cells by Hox gene function. Transplantation of the Hox-negative frontonasal, mesencephalic, or metencephalic neural folds into more posterior levels results in the ectopic formation of bones of the lower jaw (Noden, 1983), which likely results from the repression of Hoxa2 expression by FGF8 signaling from the transplanted tissue (Trainor et al., 2002). Conversely, Hox-expressing neural crest cells from the hindbrain do not form appropriate facial skeletal elements when transplanted into the first branchial arch domain (Couly et al., 1998), and the misexpression of HOX genes in first branchial arch neural crest cells also prevents the formation of facial skeletal structures normally derived from these cells (Creuzet et al., 2002).

In these paradigms, a key feature of the transposed crest cell populations is the presence or absence of the expression of any of several HOX genes. By contrast, the repression of neurogenesis reported here is likely to be specific to Hoxa2, as HOX genes are expressed in other cranial crest cell populations that
do generate neurons. The idea that Hoxa2 function represses neurogenesis may explain phenotypic attributes of embryos with reduced Hox3 paralogous group function. In embryos lacking function of any two Hox3 group genes, neurons of the proximal portion of the ninth cranial ganglion, normally derived from neural crest cells that arise from the posterior hindbrain, are often missing (Manley and Capecchi, 1997). As both Hoxa2 and members of the Hox3 paralogous group are expressed in these neural crest cells, it is possible that, in the absence of sufficient Hox3 function, the normally inapparent inhibition of neurogenesis by Hoxa2 becomes apparent. Our observation that the inhibition of neurogenesis by Hoxa2 was antagonized by the simultaneous expression of Hoxd3 in P19 cell cultures is consistent with this mechanism. Both the mutant phenotypes and the results of the P19 cell transfections are also consistent with the posterior prevalence of Hox gene functions (Duboule, 1991; Gonzalez-Reyes and Morata, 1990). By this argument, Hoxa2 activity in second branchial neural crest cells arch suppresses the capacity for neurogenesis shown by the Hox-negative first branchial arch neural crest, and the function of Hox paralogous group 3 gene in the third branchial arch antagonizes this repression of neurogenesis by Hoxa2.

**Two stages of neurogenesis.**

Biphasic patterns of neurogenesis occur in sensory ganglia at all levels of the neuraxis. Placode-derived neurons are born before crest-derived neurons in cranial ganglia (Altman and Bayer, 1982; d'Amico-Martel and Noden, 1980). In
spinal dorsal root ganglia, Neurog2-dependent neurons arise before Neurog1-dependent neurons (Ma et al., 1999). Furthermore, the two phases are largely independent of one another. Placode-derived neurons of the seventh, ninth and tenth ganglia are generated in the absence of Neurogenin-1 function, as are the Neurog2-dependent neurons of spinal dorsal root ganglia (Ma et al., 1998; Ma et al., 1999). Loss of Neurog2 function leads to a slight delay in the onset of Neurogenin1-dependent neurogenesis in spinal dorsal root ganglia, but the Neurogenin1-dependent neurons eventually form substantial populations (Ma et al., 1999).

In Hoxa2 mutant embryos, crest-derived neurons arose after placode-derived neurons had assembled in the ganglionic anlagen. The onset of crest-derived neurogenesis may mark the point at which second branchial arch crest cells become competent to differentiate as neurons. This would be consistent with the timing of neurogenesis, presumably by neural crest, in the facial ganglia of Neurogenin2 mutant embryos (Fode et al., 1998). Placode-derived facial ganglion neurons are not generated in these embryos, there are no neurons present at E10.5, but a substantial population is present at E12.5. If the initial failure of crest to generate neurons represents an active repression of neurogenic potential, then the timing of neurogenesis in these two mutants suggests that this early repression is independent of the presence of placode-derived neurons, which are present in the Hoxa2 mutants, or Hoxa2 function, which is present in the Neurogenin2 mutants. Alternatively, if it represents a
delayed acquisition of neurogenic potential, then this potential is normally subject to further inhibition by Hoxa2, and this inhibition can be overridden by the absence of placode-derived neurons.

Conclusion:

A large body of experimentation has established that neural crest cells are endowed with broad developmental potential (Le Douarin, 1983), that their differentiation is strongly influenced by signals generated by surrounding tissues, and that under appropriate conditions even fates traditionally considered incompatible with one another can be adopted (McGonnell and Graham, 2002). Nonetheless, in the intact embryo particular subsets of the potential fates are adopted by cohorts of neural crest cells that arise at specific times and at particular axial levels. Although Hox gene expression is a major determinant of axial patterning in the embryo, differences in peripheral neurogenic fates of neural crest cells have not typically been associated with temporal or spatial differences in Hox gene activity. The results presented here suggest that a closer examination of the relationship between Hox gene expression and the neuronal fates of neural crest cells is warranted.

Acknowledgements.

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### Table 1.

Neuronal number in the facial ganglion of wild-type and *Hoxa2* and *Hoxb1* mutant mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>A2+/+:B1&lt;sup&gt;Cre&lt;/sup&gt;+/−</th>
<th>A2−/−:B1&lt;sup&gt;Cre&lt;/sup&gt;+/−</th>
<th>A2+/+:B1&lt;sup&gt;Cre&lt;/sup&gt;−/−</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± S.E.</td>
<td>%</td>
<td>Mean ± S.E.</td>
</tr>
<tr>
<td>total</td>
<td>991±59</td>
<td>100</td>
<td>3622±253**</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td></td>
<td>(n=6)</td>
</tr>
<tr>
<td>placode</td>
<td>933±56</td>
<td>100</td>
<td>828±78&lt;sup&gt;NS&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td></td>
<td>(n=6)</td>
</tr>
<tr>
<td>crest</td>
<td>58±11</td>
<td>100</td>
<td>2803±228**</td>
</tr>
<tr>
<td></td>
<td>(n=5)</td>
<td></td>
<td>(n=6)</td>
</tr>
</tbody>
</table>

Counts of total neurons, neurons that did not express the lineage marker (placode), and neurons that did express the lineage marker (crest) in the facial ganglia of E14.5 embryos.

%, percent of A2+/+; B1<sup>Cre</sup>+/− value; n, number of ganglia counted; NS: no significant difference from A2+/+; B1<sup>Cre</sup>+/− by unpaired Student’s t-test; *, 0.01<P<0.05; **, P<0.0001.
Figure 1.
Figure 1. Structure and activity of B1Cre allele.

(A) Vector (V), native Hoxb1 locus (N), and recombinant B1Cre allele (R). The targeting vector contained (5’ to 3’) an HSV thymidine kinase cassette (tk), 1.8 kb of Hoxb1 genomic sequence, a Cre coding sequence (c), a neomycin cassette (n), and 10.5 kb of genomic sequence. Hoxb1 exons are shown as black rectangles, BamH1 (b) endonuclease sites and the sizes (kb) of the fragments generated are indicated. (B) Southern blots of BamH1-digested DNA from wild-type (cce) embryonic stem cells and three recombinant clones (54, 56, 89) probed with an external probe (hatched box in panel A). Other digestions and probes confirmed the organization of the recombinant clones (not shown). (C) Histochemical staining of +/B1Cre, +/R26R (Hoxa2 +/+ ) embryo at E8.5 showed marker expression in the presumptive r4 domain (arrow). (D, E) Staining of E9.5 +/B1Cre (D) and B1Cre/B1Cre (E) embryos (also +/R26R, Hoxa2 +/+ ) showing marker expression in r4 and the second branchial arch (b2) and the absence of product in the first arch (b1). B1Cre/B1Cre embryos showed increased labeling of posterior hindbrain and third arch. (F–I) Horizontal sections (anterior to the top, midline to the right) of an E9.5 +/B1Cre, +/R26R (Hoxa2 +/+ ) embryo. (F, H) Adjacent sections showing Hoxb1 (F) and Cre (H) expression in r4, lack of expression in r3 and r5, and sharply reduced expression in r4-derived neural crest cells (nc) (iv, fourth ventricle). (G, I) Sustained marker expression in the neural crest in the sections shown in F and H, respectively. (J) Sagittal section (dorsal to right and anterior at top) of an E10.5 Hoxa2 +/+ , +/B1Cre, and +/R26R embryo showing labeling of second arch mesenchyme, including cells dorsal to the pharyngeal cavity (*), lack of first arch labeling, and low level of labeling in
the third arch (b3). (ct: chorda tympani, gp: greater petrosal nerve) Scale bars: I, 50 µm for panels panels F–I; J, 100 µm.
Figure 2.
Figure 2. Marking of second arch tissues by the B1Cre allele in +/B1Cre, +/R26R (Hoxa2 +/+ ) embryos.

(A) Sagittal section (dorsal at top and anterior to left) through r4, the facial nerve (nvii) and facial (gvii) and vestibuloacoustic (gviii) ganglia, and the first and second branchial arch (b1, b2) of an E10.5 embryo labeled with antibodies to β-galactosidase (red) and Islet1/2 proteins (green). The mesoderm (mes) and neurons of the ganglia were Islet1/2 positive and marker negative and the ectodermal placode (pl) was faintly positive for Islet proteins. The arrow indicates a cluster of neurons that expressed β-galactosidase. (B) Islet1/2 positive cells (arrow) located between the placode and facial ganglion. (C) Sagittal section of an E10.5 embryo labeled with antibodies to Sox10 (green) and β-galactosidase (red). The early outgrowth of the greater petrosal (ngp) and chorda tympani (nct) nerves included cells that expressed the marker and Sox10. Dotted line outlines pharyngeal cavity. The signals for each antibody in the chorda tympani are shown separately in panels D and E. (F) Earliest outgrowth of chorda tympani in another E10.5 embryo showed isolated axons (arrowheads) labeled with β-tubulin antibody (Tuj1, green). (G) Horizontal section of an E11.5 embryo through the chorda tympani and motor root (mvii) of the facial nerve labeled with antibodies to β-tubulin (green) and β-galactosidase (red). Inset shows an anterior portion of the chorda tympani within the first arch associated with marker-positive cells. (H–K) DAPI stain of cell nuclei (H, J) and immunostaining of β-galactosidase expression (I, K) within the boxes outlined in panel G showing that cells were largely excluded from the axon fascicles of the chorda tympani (H).
and motor root (*, J), that the axons of the chorda tympani did not express the marker (I), and that axons of the motor root (*, K) did express the marker. (L) Sagittal section of E14.5 embryo showing that three principal branches of the facial nerve extended from the second arch into the first arch and were accompanied by labeled cells. Arrow indicates axons of a trigeminal nerve branch not associated with labeled cells (nvii, mixed trunk of facial nerve). (M) Histochemical staining of the roof of the oral cavity at E14.5 showing marker expression in the ptergopalatine ganglion (g) and greater petrosal nerve. (N) Sagittal section through the ptergopalatine ganglion of an E13.5 embryo labeled with antibodies to β-galactosidase (red) and PGP9.5 (green) to show double labeling of neurons and marker only labeling of peripheral glial cells. Arrowhead points to trigeminal axons not associated with second arch glial cells. (O) Sensory axons (*) passing from the vestibuloacoustic ganglion to the otic vesicle (ov) were associated with cells that expressed the lineage marker. Inset shows the marker signal alone. Scale bars: A–C, G, 100 µm; L, 200 µm; N, O, 50 µm.
Figure 3.
**Figure 3. Facial ganglia of wild-type and Hoxa2 mutant embryos.**

β-galactosidase is shown in red in all panels, colabeling with other markers is shown in green. (A–F) Sagittal sections of E10.5 (A, B, D, E) and E11.5 (C, F) wild-type (A–C) and Hoxa2 mutant (D–F) embryos. (A, D) Islet1/2 colabeling shows the similar organization of facial ganglia in wild-type and mutant embryos at E10.5, both of which contained small numbers of crest-derived neurons in the proximal portion of the ganglion (arrows). (B, E) Sox10 labeling shows that most cells in the glial lineage coexpressed the lineage marker in both mutant and wild-type embryos, although a few cells in each did not (e.g., arrowheads). (C, F) At E11.5, Islet1/2 colabeling shows a few doubly labeled neurons in a wild-type ganglion (arrows, C) and a substantial population of crest-derived neurons (gviinc, F) in the mutant. (G) PGP9.5 colabeling at E14.5 shows a large population of crest-derived neurons (gviinc) in a Hoxa2 mutant embryo that express both Islet1/2 and the lineage marker: this population was not present in wild-type embryos (see Figs.4 C, D). (H) PGP9.5 colabeling of the facial ganglion of an E14.5 BDNF mutant embryo showed that the neural crest formed few neurons. Scale bars: 50 µm.
Figure 4.
Figure 4. Neuronal proliferation and TrkA expression in facial ganglia.

(A, B) Facial (gvii) and vestibuloacoustic (gviii) ganglia of E10.5 wild-type (A) and Hoxa2 mutant (B) embryos labeled with antibodies to Islet1/2 (red) to label neurons and phosphohistone H3 (green) to label dividing cells. Arrows indicate doubly labeled cells shown in insets. (C, D) Trigeminal (gv), facial, and vestibuloacoustic ganglia of wild-type (C) and mutant (D) E14.5 embryos labeled with antibodies to the lineage marker (red) and TrkA (green). The facial ganglia of wild-type embryos contained few TrkA-positive neurons, while the trigeminal ganglia contained many. Inset in panel C shows a section of the facial ganglion from another embryo that contains two placode-derived neurons (arrowheads) and one crest-derived neuron (arrow) that expressed TrkA. Most neurons of the crest-derived portion of the facial ganglion in mutant embryos expressed TrkA (D) and TrkA-positive axons exited the ganglion (arrowhead, inset). Scale bars: 50 µm.
Figure 5.
Figure 5. Expression of molecular markers in wild-type and Hoxa2 mutant embryos.

(A) Horizontal section (anterior to the top and midline to the left) of an E9.5 wild-type embryo labeled with a Hoxa2 probe. Strong expression was seen in hindbrain rhombomeres 4 and 5 (r4, r5), in the neural crest of the seventh cranial nerve root (nc), and weaker expression was seen in the mesenchyme of the second branchial arch (b2). Expression was not observed in the facial ganglion (gvii). (hv, head vein; gvi, vestibuloacoustic ganglion; ov, otic vesicle). (B) Sagittal section (anterior to the left and dorsal to the top) of an E11.5 embryo. The hindbrain (hb) and mesenchyme of the second branchial arch expressed high levels of Hoxa2, and low level expression was seen in rootlets of the seventh and eighth nerves (arrows) and in the mesenchyme of the third branchial arch (b3). Expression in the seventh and eighth ganglia was similar to the background signal observed in the trigeminal ganglion (gv). (C, D) Neurog2 expression in E9.5 wild-type (C) and Hoxa2 mutant (D) embryos. The placode for the facial ganglion (F) of the mutant embryo showed apparently normal levels of expression (T, P, N; trigeminal, petrosal, nodose placodes). (E–G) Neurog1 expression in sagittal sections of E11.5 wild-type (E) and mutant (F, G) embryos. Expression was not seen in the region of the facial ganglion (*, E) in the wild-type embryo, but was seen in cells of the trigeminal ganglion (gv) and in neurons migrating from the otic vesicle (arrowheads) of both. In the mutant embryo, high levels of Neurog1 were seen in the anterodorsal region of the facial ganglion (*,
F). In panel G, Islet1/2 colabeling (green) shows that some of the cells in the region of high Neurog1 expression (*) were neurons. Scale bars: A, B, E, F, 100 μm; G, 50 μm.
Figure 6.
Figure 6. Notch1 and Dll1 expression in wild-type and Hoxa2 mutant embryos.

(A–F) Sagittal sections through the facial ganglia of E11.5 wild-type (A, C, E) and mutant (B, D, F) embryos labeled with probes for Dll1 (A, B) and Notch1 (C, D). Panels E and F show the distribution of Islet 1/2 immunoreactivity in the sections shown in panels C and D. Anterior is to the left and dorsal is up in all panels. Dll1 expression was not observed in the facial ganglia (gvii) of wild-type embryos, and Notch1 expression was observed only at the periphery of the ganglion, but the expression of both was elevated in the facial ganglia of mutant embryos. Overlap of Notch1 and Islet1/2 expression in anterodorsal portion of the facial ganglion in the mutant is indicated by arrows in panels D and F. The asterisks in panels C and D indicate expression in cells migrating from the otic vesicle to the vestibuloacoustic ganglion. gv, trigeminal ganglion; gvii, facial ganglion; gviii, vestibuloacoustic ganglion; hb, hindbrain; ov, otic vesicle. Scale bar: F, 200 µm for all panels.
Figure 7.

D

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Figure 7. Neuronal differentiation of P19 cells overexpressing Hox and cofactor proteins 4 days after transfection.

(A–C) Immunofluorescence micrographs of a single field showing (A) all transfected cells (GFP, green), (B) cells that have differentiated as neurons (PGP9.5, red), and (C) a merged image. Yellow cells in panel C are transfected cells that differentiated as neurons. Arrowheads in panels A and C indicate transfected cells that did not become neurons, and arrows in panels B and C indicate neurons that differentiated from untransfected cells. Scale bar in panel A indicates 50 µm for panels A–C. (D) Quantification of three independent experiments showing the percentage of neurons (PGP9.5 positive cells) derived from cells transfected with expression plasmids for different combinations of Hox and Hox cofactor proteins (*p < 0.05 compared to GFP alone, **p < 0.01 compared to GFP alone or Hoxa2/Pbx1/Meis1). (E) Quantification of three separate experiments using wild-type or mutant Hoxa2 (W99A) (**p < 0.01 compared to control or Hoxa2 (W99A) transfection).
Figure 8.

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**A**

GFP

**B**

PGP9.5

**C**

DAPI

**D**

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<tr>
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Figure 8. Neuronal differentiation of GFP-transfected P19 cells cultured for four days on feeder layers transfected either with Hoxa2, Pbx1, and Meis1, or with a control expression plasmid.

A schematic showing the time line of the experiments is shown at top. **A-C:** Fluorescence micrographs of a single field showing (A) all GFP-positive test cells and (B) PGP9.5 labeling of neurons. Arrows indicate two test cells that differentiated as neurons. **C:** DAPI staining of all cells present, the majority of which are feeder cells. **D:** Quantification of neuronal differentiation showing the percentage of GFP-positive test cells that differentiated as neurons. Scale bar in (A) = 50m for all A-C.
Figure 9.
Figure 9. *Ngn1*-induced neuronal differentiation of P19 cells was not inhibited by *Hoxa2* expression.

Cells were transfected with expression plasmids for either *Ngn1* alone, or for *Ngn1* and *Hoxa2, Pbx1* and *Meis1* (*Ngn1/A2/P1/M1*). **A-C**: Fluorescence photomicrographs of a representative field from a culture transfected with *Ngn1, Hoxa2, Pbx1*, and *Meis1* expression plasmids showing that all transfected, GFP-positive cells (A) could also be labeled with antibodies to PGP9.5 (B), indicating they had differentiated as neurons. **C**: DAPI staining showing all cells present in the field. **D**: Quantification of neuronal differentiation showing the percentage of transfected cells that differentiated as neurons. Note that there was no difference of the neurogenesis between cells transfected with *Ngn1* alone and cells transfected with *Ngn1, Hoxa2, Pbx1* and *Meis1* (*Ngn1/A2/P1/M1*). Scale bar in (= ~ m) for A-C.
Figure 10. Neuronal differentiation of neural crest cells in the trigeminal ganglia after in vivo electroporation.

(A, B) Lateral views of an electroporated embryo (rostral at top, dorsal at left) under conventional (A) or fluorescence (B) illumination. In panel B, GFP expression is visible in the hindbrain (hb) adjacent to the fourth ventrical (VI) and in the trigeminal ganglion (gV). (mb: midbrain). (C) Coronal section through the trigeminal ganglion of a stage 27 embryo that was electroporated at stage 10 with expression plasmids for GFP, Hoxa2, Pbx1, and Meis1, that was stained with antibodies to GFP (green) and Islet 1/2 (red). The inset at lower right shows three GFP-positive neurons with Islet1/2-positive nuclei (arrowheads); the inset at upper left shows two cells whose nuclei did not express Islet1/2 (arrows). (D) Quantification of neuronal differentiation showing the percentage of GFP-positive cell bodies that contained Islet1/2-positive nuclei after electroporation with the indicated expression plasmids. GFP-positive cells in the trigeminal ganglia of embryos transfected with Hoxa2/Pbx1/Meis1 were less likely to differentiate as neurons than those in embryos electroporated with either GFP alone or with Hox2/PBX1 (**p < 0.01).
REFERENCES


Chapter 3. Discussion
Discussion:

In this thesis I summarized the observations in Hoxa2 deficient mice, the over-expression experiments in P19 cells and the mis-expression studies in chick trigeminal ganglion. All the results are consistent with our hypothesis that Hoxa2 can suppress the neurogenesis of multipotent cells, such as neural crest cells. We found that in Hoxa2/- mice, there was an extra population of neurons derived from neural crest cells, which was not present in the facial ganglion of wild-type embryos. However, the placode-derived neurons in the same ganglion were present with no detectable defects in the mutant embryos. The facial ganglia appeared approximately normal at E10.5 stage, while from then on, there was a great increase in the crest–derived neurons. These neurons were mostly aggregated in the proximal region of the facial ganglia, closer to the neural tube than the other parts of the ganglia. In addition, the expression of Ngn1 and Notch/Dll1 was inappropriately up-regulated in the facial ganglia in Hoxa2 mutant embryos, with the strongest signaling in the same proximal portion of the ganglia, corresponding to the position where the crest-derived neurons were generated. These finding suggests that Hoxa2 is required for the inhibition of neural crest cells to generate sensory neurons in the facial ganglion. To test this hypothesis, we over-expressed Hoxa2 in P19 cells and in chick neural crest cells that contribute to the trigeminal ganglion. In both experiments, we saw a reduction of neuronal differentiation of these multipotent cells. This inhibitory function of
Hoxa2 required the presence of both of its cofactors, Pbx and Meis. A member of Hox3 paralogous group, Hoxd3, could antagonize this inhibitory effect of Hoxa2 in P19 cells.


Neural crest cells, which delaminate from the neural tube, are a group of heterogeneous cells; some are in a multipotent state while some might be restricted to certain lineages. They migrate along specific paths, and as they migrate, and when they reach their specific destinations, they receive various signals that will strongly influence their differentiation. Ngn1 is the critical transcription factor required by neural crest cells for the generation of sensory neurons in the cranial ganglia. Many pathways that are involved in modulating neurogenesis of neural crest cells are connected with each others through their regulation of Ngns, such as Wnt pathways and Notch/Delta pathways. I propose that Hoxa2 interacts with the signals that induce neural crest cells to generate sensory neurons and modulate responses of neural crest to those signals through regulation of expression of Ngn1 (Figure 1).

Many aspects of the behavior of second branchial arch crest cells appeared normal in Hoxa2 mutant mice. The organization of hindbrain rhombomeres was not disturbed. The basic organization of the second branchial arch appeared to be normal; the physical relationships between surface ectoderm, pharyngeal endoderm, ectodermal placode, mesodermal core and crest-derived ectomesenchyme were not altered from that seen in wild-type
embryos. The most prominent defects in Hoxa2 mutant mice are duplications of some of the first arch skeletal elements within the second arch territory (Gendron-Maguire et al., 1993; Rijli et al. 1993). However, these defects are not because r4 neural crest cells do not generate skeletal tissue or because they migrate into the wrong territories. The capability of r4 crest cells to generate bone and cartilage does not change in the mutant embryos. With the aid of the same lineage tracing system used here, O’Gorman confirmed that each duplicated structure is at the site where first and second arch cartilages meet in wild-type embryos, and illustrated that they are generated by the neural crest cells that are normally recruited to form skeletal elements within the second branchial arch (O’Gorman, 2005). It is been proposed that inductive signals from the pharyngeal cleft at the border of the branchial arch one and two spread both rostrally (to the first arch mesenchyme) and caudally (to the second arch mesenchyme) in a symmetrical fashion (Rijli, et al., 1993). Hoxa2 modulates the response of r4 neural crest cells to these signals, and generates branchial arch two specific structures. In Hoxa2 mutant embryos r4 neural crest seems to migrate along the normal path, indicated by the observations that both wild-type and mutant embryos, the boundary between the first and second branchial arches was sharply delineated. There is little cell-mixing at the border in the malleus where the two group of neural crest cells meet each other, and r4 crest cells never migrate deeply into the first branchial arch territory (O’Gorman, 2005). Thus, without Hoxa2 function, branchial arch two neural crest responds to
the inductive signals and generate branchial arch one structures, forming a mirror image of the these structures.

As neural crest cells emigrate from the neural tube, they receive various signals to promote differentiation of neural crest cells into various lineages. The mechanisms that control multipotent maintenance versus progenitor generation are unclear. Kleber et al. proposed a model in which neural crest cells emerge from the neural tube with equal competence. For some cells a combination of Wnt and BMP signaling maintains multipotency and suppresses their neuronal specification (Kleber et al., 2005). Other emigrating neural crest cells are not (or at least not continuously) exposed to the synergistic activity of Wnt plus BMP and adopt a Wnt-dependent sensory fate. Wnt/β- catenin signals have been proposed to promote the generation of neural crest cells from early dorsal neuroepithelial cells (Garcia-Castro et al., 2002). Recent studies indicate that at a later stage this signal also induces sensory neurogenesis by the neural crest cells that emigrate from the neural tube (LeDouarin and Dupin, 2003; Kleber and Sommer, 2004). Neural crest cells without β-catenin emigrate and proliferate normally but are unable to acquire a sensory neuronal fate (Harada et al., 1999). Lee et al. generated mice expressing a constitutively active form of beta-catenin specifically in neural crest cells (Lee et al. 2004). At E10.5, mutant neural crest cells aggregated in prominent cranial ganglion-like structures dorsal to the normal site of branchial arch formation, while the branchial arch structures were mostly absent. Ganglia with sensory neuronal features in the mutant were formed instead of pharyngeal and vagal nerve, and at the location of the normal
superior cervical ganglia. The Wnts are expressed in the dorsal neural tube at the time of neural crest emigration (Parr et al., 1993; Lee et al., 2003). The restricted expression of Wnts and their short-range activity likely explains the relatively low percentage of sensory neurons found in neural crest cells and also explained the reason for their generation in close proximity to the neural tube. As a matter of fact, sensory neurons derived from the neural crest cells are always located in either the proximal portion of the cranial ganglia, such as in the trigeminal ganglion, or in the proximal group of ganglia associated with a cranial nerve, such as in superior and jugular ganglia. It is interesting to notice that in Hoxa2 mutant mice, the extra group of neurons of the facial ganglion are also generated in the place close to the neural tube. Therefore, Hoxa2 might function to interfere with the Wnt signal so that neural crest cells do not give rise to sensory neurons in the facial ganglia.

Hoxa2 might function through down-regulating the expression of Ngn1, the critical player in the Wnt pathway to prevent sensory neurogenesis. Sustained β-catenin activity could result in increasing Ngn1/2-positive cells, both in the trunk and at ectopic cranial locations (Lee et al., 2004). In P19 cell culture system, overexpression of Wnt-1 can promote cells differentiate into neuron-like cells in the absence of retinoic acid (Tang et al., 2002). In addition, RT-PCR revealed that Ngn1 was up-regulated during differentiation stage. This evidence indicates that Ngn1 is the downstream effector in Wnt pathway to promote sensory neurogenesis. During normal development, Ngn1 expression could not be detected in the facial ganglia (Yang et al., 2008). In Hoxa2 mutant embryos,
the generation of facial sensory neurons which requires the expression of Ngn1 was affected, while the generation of r4 derived parasympathetic neurons in the ptergopalatine ganglion which requires Mash1, another bHLH transcription factor, appeared approximately normal. These observations, together with the fact that Hoxa2 could not inhibit the neuronal differentiation promoted by Ngn1 in P19 cells, indicated that Hoxa2 could function upstream of Ngn1 and inhibits sensory neurogenesis through downregulating the expression of Ngn1.

In Hoxa2 mutant mice, we also found the extopic expression of Notch and Dll1 in the facial ganglia with the strongest signal at the position where neurons derived from the crest were generated. Notch/Delta pathway is known to regulate neurogenesis in the dorsal root ganglia. The cells with high Delta expression will eventually differentiate into neurons, while the neighboring cells whose Notch pathway is activated will differentiate into glia. Our observation in Hoxa2 mutant mice indicated that this pathway could also regulate neurogenesis in the cranial sensory ganglia. In addition, Notch/Delta pathway regulates the expression of some proneural genes, including Ngns. Ngns upregulate the expression of Delta in the same cells, while Ngns expression is downregulated by activated Notch signaling (Heitzler et al., 1996; Hinz et al., 1994; Kunisch et al., 1994).

Thus, the two pathways which are critical to regulate sensory neurogenesis, Wnt pathway and Notch/Delta pathway, interact with each other through their regulation of the expression of Ngn1 whose expression may also be regulated by Hoxa2.

2. HOX genes and peripheral neurogenesis.
Our data suggest that Hoxa2 inhibits neurogenesis both in vitro and in vivo, and does so by a cell autonomous mechanism. Although the function of Hoxa2 and many other HOX genes in embryonic development has been extensively studied, our study of Hoxa2 function is the first to provide direct evidence that a Hox gene participates in the control of the peripheral neurogenesis. One immediate question is whether other HOX genes play a similar role during neurogenesis?

Our data significantly amplify the data of Manlay and Capecchi (Manlay and Capecchi, 1997) who found that large reductions in Hox3 group function could lead to alterations in the organization of the glossopharyngeal and vagal ganglia, but were unable to determine if the changes were due to a cell autonomous function of these genes. We found that transient expression of Hoxd3 with its co-factors in P19 cells led to an increased rate of spontaneous neuronal differentiation in high density cultures. Therefore, in contrast to Hoxa2’s role in the inhibition of neurogenesis, Hoxd3 might serve as a positive regulator to promote neurogenesis. This conclusion awaits further confirmation, for example, by Hoxd3 mis-expression in non-neurogenic neural crest cells in transgenic mice.

Finally, our data in P19 cells establish that Hoxd3 function can antagonize the inhibition of neuronal differentiation caused by Hoxa2 expression. This is consistant with the model of posterior prevalence (Duboule, 1991; Duboule and Morata, 1994), whereby the HOX genes expressed in more posterior domains of
the embryo (in this case Hoxd3) can inhibit the activities of HOX genes that are additionally expressed in more anterior domains (in this case Hoxa2). Both Hoxa2 and Hox3 paralogues a3, b3 and d3 are expressed in neural crest cells that emerge from r6 to neurons of the superior ganglion. Reducing Hox3 function by removing the function of any two of the three Hox3 paralogues expressed in r6-derived neural crest resulted in a significant reduction in the number of crest-derived neurons (Manley and Capecchi, 1997). We observed effects similar to this in P19 cell transfections. While transfection of Hoxa2 alone inhibits neuron formation, the simultaneous cotransfection of Hoxd3 relieves this inhibition. The frequencies of neurogenesis are similar between cells co-expressing Hoxd3 and Hoxa2 versus cells expressing Hoxd3 alone, suggesting that the effects of Hoxd3 and Hoxa2 on neurogenesis are not simply additive. Instead, Hoxa2’s inhibitory function on P19 neurogenesis is completely suppressed by the presence of Hoxd3, which is consistent with the principle of posterior prevalence and the phenotype of mice with losses of Hox3 group function.

The interplay of Hoxa2 and Hox3 genes in regulating neurogenesis could be further elucidated by in vivo electroporation experiments in chick embryos. We have shown that ectopic expression of Hoxa2 in the trigeminal ganglion of developing chick embryos resulted in a decreased neurogenesis as shown by the reduced percentage of Islet1/2 positive neurons. Similar future experiments could be performed to ectopically express Hox3 paralogues in the facial ganglion to test whether Hox3 genes could promote neurogenesis. During normal development, a population of Hoxa2 expressing neural crest cells, which
contributes to the facial ganglion, gives rise mostly to glial cells and less than 5% of the neurons in the facial ganglion, and none in the vestibuloacoustic ganglion. If Hox3 paralogues could indeed promote neurogenesis in vivo and suppress the inhibition of neurogenesis by Hoxa2, we would expect to see that more neural crest cells give rise to neurons in the facial ganglion and/or vestibulo-acoustic ganglion after ectopic expression of Hoxd3 in those neural crest cells.

Does the regulation of peripheral neurogenesis by Hoxa2 and/or Hoxd3 represent a general function of HOX genes? Although our study is the first to report a regulatory function of HOX genes in sensory neurogenesis, various studies have suggested a role of HOX genes in the generation of distinct motor neuron identities in the spinal cord (Dasen et al., 2003 & 2005; Shah et al., 2004; Liu et al., 2001). Spinal motor neurons have different columnar identities at distinct locations along mediolateral and rostrocaudal axes of the neural tube. Lateral motor column (LMC) neurons, which innervate the limbs, are found only at the brachial (forelimb) and lumbar (hindlimb) level, while at thoracic levels, the column of Terni (CT) is present, whose neurons project to the sympathetic ganglion. Along the mediolateral axis, LMC neurons are located laterally within the ventral horn, while CT neurons occupy more medial and dorsal positions. Two members of the Hox-c paralogous group, Hoxc6 and Hoxc9, are critical in defining the identity of these two groups of neurons. Hoxc9 expression is restricted to the thoracic level, and Hoxc6 expression is confined to the brachial level, correlating with the position of CT and LMC neurons respectively (Dasen et al., 2003). When Hoxc9 was mis-expressed at brachial levels, LMC
differentiation is disrupted. LMC neurons switched their identity towards a thoracic identity as demonstrated by their position and their expression of the CT neuronal marker BMP5, instead of the LMC marker Islet-2/Lim-1. Conversely, expression of Hoxc6 in thoracic regions resulted in switch of CT neuronal identity toward a lumbar identity (Dasen et al., 2003). Similar roles for Hox proteins have been revealed during hindbrain motor neuron specifications. Hindbrain motor neurons are organized into clusters or nuclei rather than columns. For example, Hoxb1 is critical for facial motor neuron differentiation, migration and axon projection (Studer et al., 1996; Goddard et al., 1996; Bell et al., 1999). Loss of both Hoxa3 and Hoxb3 leads to impaired development of somatic motoneuron progenitors (pSMN) and ectopic V2 interneurons along the dorsal-ventral axis of r5 (Gaufo et al., 2003). Thus, HOX genes convey the identities of special groups of motor neurons in the spinal cord along the rostrocaudal axis.

Further upstream, the expression of HOX genes in the spinal cord is modulated by factors emanating from adjacent mesodermal tissues, including members of the fibroblast growth factor (FGFs) family from the presomitic mesoderm and RA from the somites (Ensini et al., 1998; Liu et al., 2001). How HOX genes interact with those signals and lead to the final neuron identities is not known. Recently, an “opposing signal” model has been proposed to demonstrate how the expression of some HOX genes is modulated along the rostrocaudal axis by antagonism between the RA and FGF signals from the mesoderm (Diez del Corral, et al., 2003). Retinoic acid promotes the expression of multiple HOX genes. The promoters of vertebrate HOX genes contain
conserved RA response elements (RAREs) in their 5’ enhancer regions (Simeone et al., 1991; Marshall et al., 1994; Frasch et al., 1995). The HOX genes that are located in 3’ end of the Hox complex are more sensitive to RA than those at the 5’end (Boncinelli et al., 1991; Simeone et al., 1991). Conversely, Fgf8 signalling is required for the spinal cord expression of more 5’ HOX genes (Liu et al., 2001; Pownall et al., 1998).

These two mutually repressing signals define the expression of HOX genes during the elongation of the spinal cord. It is proposed that as the body axis extends, caudal FGF signaling from the primitive streak is attenuated by the retinoic acid signaling from emerging somatic mesoderm. The Hox expression in the emerging segment is stabilized by the retinoic acid signaling newly present in that segment, while in more caudal regions FGF level increases and promotes the expression of Hox genes which are located in more 5’ end within the clusters (Diez del Corral and Storey, 2004). Thus, opposition of the FGF and RA pathways coordinately determine the specific subtype identities of neurons along the rostrocaudal axis via regulating the expression of HOX genes.

As with motor neuron columns occupying different locations along the rostrocaudal axis, the origins of NCC derivatives are also organized in segments. Neural crest cells give rise to peripheral sensory neurons in cranial ganglia, dorsal root ganglia, sympathetic, parasympathetic and enteric neurons in autonomic systems. Distinct groups of neurons are generated by neural crest cells from distinct levels of neural tube along the rostrocaudal axis. For example,
parasympathetic neurons are generated from the mesencephalic neural crest, and enteric ganglia are generated from the vagal and lumbosacral neural crest cells (Le Douarin et al., 2004). So is it possible that HOX genes define the derivatives of the neural crest cells using a mechanism similar as controlling the identities of the motor neuron pool?

It will be valuable to determine whether HOX genes also define the identities of neural crest cells that originate from different segments along the neural tube and control their ability to generate subtypes of neurons in the peripheral nervous system. To test this hypothesis, it will be necessary to first determine whether the expression patterns of HOX genes correspond to the segmental organizations of different derivatives of neural crest cells. Genetic manipulation of candidate HOX genes, such as Cre/LoxP mediated conditional ablation of certain HOX genes in specific groups of migrating neural crest cells, will provide insights into whether some HOX genes are required for the neurogenesis of specific subtypes of sensory neurons.

3. Potential downstream mechanisms of Hoxa2 in regulating neurogenesis.

To determine whether Hoxa2 regulates neurogenesis by modulating the transcription of Ngn1, I performed a reporter assay in P19 cells by co-expressing Hoxa2/Pbx1/Meis1 and a Ngn1-LacZ promoter reporter construct (Murray et al., 2000). Ngn1-LacZ contains a 2.7 kb DNA fragment encoding the first exon of Ngn1 and its upstream sequence. This DNA sequence has been shown to exhibit Neurogenin1 basal promoter activity as it drives the expression of lacZ
reporter gene in transgenic mice in most of the endogenous Ngn1 expressing tissues such as trigeminal ganglion, and perrosal ganglion. This 2.7 kb fragment has also been shown to promote the expression of the reporter lacZ gene in P19 cells under conditions in which the expression of endogenous Neurogenin1 is induced, such as exposure to retinoic acid (RA) (Murray et al., 2000; McCormick et al., 1996).

However, exposure to retinoic acid will cause dramatic changes in the expression profiles of many additional transcription factors including members of HOX genes. Therefore, RA exposure might interfere with our purpose of determining the function of Hoxa2 in regulating Ngn1 transcription. Thus, instead of adding RA to promote neurogenesis, I cultured P19 cells in aggregation for 24 hours after transfection in order to promote spontaneous neurogenesis, and measured the reporter activity after 48 hours of differentiation. My preliminary data did not support a direct role of Hoxa2 in regulating the transcription of Ngn1. Ngn1-LacZ reporter expression was indeed induced during spontaneous neurogenesis of P19 cells cultured in aggregation, and this induction of reporter expression was not affected by the co-expression of Hoxa2 and its cofactors, Pbx1 and Meis1. However, we could not exclude the possibility that Hoxa2 may bind to a regulatory element that is outside of the 2.7kb promoter region. In addition it is important to remember that an in vitro reporter assay may not completely mimic the transcription regulation in vivo. Future experiments such as chromosome IP and gel mobility shift assays should be employed to further address whether Hoxa2 can directly regulate the transcription of Ngn1. These
experiments will also help to determine whether other proneural genes are direct
targets of Hoxa2 or any other HOX genes.

In Hoxa2 deficient mice, we also noticed that the Notch/Delta pathway
was activated. Both Notch (the receptor) and Dll1 (Delta like ligand) were up-
regulated in the Hoxa2 mutant facial ganglion compared to wild-type. This
canonical pathway is believed to play a critical role in regulating neurogenesis
through lateral inhibition (Skeath and Thor, 2003; Riley and Phillips, 2003). Upon
binding to its ligand, Notch receptor is cleaved intracellularly and translocates into
the nucleus. This leads to a down-regulation of proneural genes such as
Neurogenins and a subsequent up-regulation of Delta (the ligand) in the same
cells. The cells that express high levels of Delta will eventually differentiate into
neurons, while the neighboring cells in which the Notch signaling is activated and
have a low Delta expression on the membrane will turn into glia. Is it possible
that Hoxa2 regulates neurogenesis by regulating the transcription of Notch, Delta
or other players in the pathway? Our conclusion that both Notch1 and Dll1
expression was up-regulated in Hoxa2/-/- embryos was based on in situ
hybridization of their RNA transcripts. The relatively low resolution of RNA in situ
prevented us from asking whether these cells correlate exactly with the
supernumerous Islet1/2 positive neurons in the same ganglion. Future studies
utilizing reporter transgenes that express GFP under the control of Notch
promoter (Lewis et al, 1998) or LacZ transgene under the control of Dll1
promoter (Beckers et al, 2000) could be carried out by crossing these reporter
transgenes into Hoxa2 null background to determine whether Hoxa2 controls the
transcription of Notch and/or Delta. Furthermore, similar promoter reporter experiments in P19 cells, chromosome IP and gel mobility shift assays could also be performed to determine whether Hoxa2 directly regulates the transcription of Notch/Delta or other factors in the pathway.

Many of the identities of Hox downstream targets in vivo remain to be identified. This is perhaps the primary reason that despite extensive genetic and biochemical studies, the molecular mechanisms of HOX genes are still not fully understood. For a long time, tremendous effort has been spent trying to identify the direct downstream transcriptional targets of HOX genes only to find that HOX genes themselves are the targets through auto- or cross-regulation. For example, among the Hox paralogue group 1 genes, Hoxa1 is required for the initial expression of Hoxb1, while Hoxb1 is required for its own maintenance (Popperl et al., 1995; Studer et al., 1998; Nonchev et al., 1997). To identify Hox target genes, indirect approaches such as screening the promoter regions of known developmental genes for homeodomain binding motifs have been carried out. Among the genes identified by this approach are cell adhesion molecules (such as N-CAM), insulin, bone morphogenic proteins (Bmp-2 and Bmp-7), and endothelin receptor genes, etc (Edelman & Jones, 1993; Franciswest, et al., 1995; Hirsch et al., 1991; Jones et al., 1992; Kapur et al., 1995). However, whether these genes are actual targets of the HOX genes in vivo or not remains to be verified. Molecular and biochemical methods such as purification of Hox protein-DNA complexes directly from the nuclear chromatin, and gel mobility shift assays will provide strong evidence to confirm whether the putative target genes
are directly regulated by Hox proteins and its co-factors (Gould et al., 1990 & 1992; Tomotsune et al. 1993, Safaei, 1997).

In summary, the presumptive downstream targets of HOX genes identified so far are involved in numerous cellular processes, including cell adhesion and migration (N-CAM and EphB4, etc), cell cycle (p53 and p21), organogenesis and differentiation (SP13, Six2 etc). (Jones et al., 1992; Bruhl et al., 2004; Kutejova et al., 2005; Raman et al., 2000; Bromleigh and Freeman, 2000; Safaei, 1997). The experimental procedures used typically have made it impossible to conclusively decide if they are direct targets and even so, the Hox binding sites responsible for the transcriptional regulation may not have been confirmed. For example, in one putative target, SP13, a serine protease inhibitor involved in neurogenesis, the Hox binding sites in the promoter are yet to be defined (Safaei et al., 1997).

Future studies to discover additional direct targets of HOX genes will need to rely on a combination of large scale screening techniques followed by biochemical binding assays. Chromatin immunoprecipitation (ChIP) assays together with microarray technology will significantly accelerate the identifications of large numbers of putative genes regulated by specific Hox proteins (Lei et al., 2005; Williams et al., 2005).

In order to further understand Hoxa2’s role in neurogenesis, several future experiments can be carried out. For example, r4-derived neural crest cells can be purified after they migrate out of the neural tube in explant culture. Isolated
RNA transcripts from these cells could then be subjected to microarray analysis to compare the expression profiles between cells harvested from Hoxa2-/− and Hoxa2+/− mice. Candidate genes identified in this assay would be further selected for the existence of putative Hoxa2 binding motifs in their promoter regions. Those candidates that are positive for both screening standards will be further tested by ChIP assays to confirm whether they are the direct Hoxa2 targets.

4. The function of Hox co-factors in neurogenesis.

Our data in both P19 cells and the chick trigeminal ganglion have demonstrated that Hoxa2 requires its cofactors Pbx1 and Meis1 to regulate neurogenesis. First, over-expression of Hoxa2 by itself had no effect on the P19 spontaneous neurogenesis or on the neurogenesis in chick trigeminal ganglion. Instead, co-expression of Hoxa2 with both Pbx1 and Meis1 resulted in an inhibition of neurogenesis in both systems. Second, when tryptophan, an indispensable amino acid residue required for Hoxa2’s binding to Pbx co-factors (Neuteboon et al., 1995), was mutated, the mutant Hoxa2 protein failed to show any inhibitory effects on P19 neurogenesis compared to wild type protein. These observations are not surprising since Hox co-factors are known to form transcription complexes with Hox and increase the DNA binding affinities of some Hox proteins (LaRonde-LeBlanc and Wolberger, 2003). And numerous studies have confirmed the requirement of co-factors for Hox activity (Moens and Selleri, 2006).
Interestingly, a recent study has suggested that Pbx1 is required for the neuronal differentiation of P19 cells (Qin et al., 2004). These experiments showed that Pbx1/2/3 mRNA transcript levels were significantly increased in P19 cells during retinoic acid induced neuronal differentiation. Retinoic acid treatment has also been shown to induce the expression of Meis2 (Oulad-Abdelghani et al., 1997). The co-incidental induction of Pbx and Meis co-factors with neuronal differentiation implies a potential role of these factors in regulating neurogenesis.

Second, P19 cell lines that either stably express an antisense RNA against Pbx1b (a splice isoform of Pbx1) or transiently express siRNA against Pbx1 failed to differentiate into neuronal or endodermal cells upon retinoic acid induction (Qin et al, 2004). These results clearly suggest an indispensable role of Pbx family of transcription factors during neuronal and endodermal differentiation. We do not think that these results are contradictory to our observation that Pbx1 together with Hoxa2 and Meis1 could inhibit the neuronal differentiation of P19 cells. Alternatively, the Pbx family of transcription factors might promote the neurogenesis of P19 cells via binding to other Hox proteins or even independent of Hox proteins. Supporting this idea, recent studies have shown that Pbx proteins could bind to partners other than Hox, such as bHLH transcription factors (Knopfler et al., 1999; Maves et al., 2007).

Additional recent studies, mostly in Drosophila, have shown that some of functions of co-factors are Hox-independent, suggesting that these proteins function more broadly than to modulate the activities of Hox transcription factor complexes. The generation of antenna requires exd and hth (Casares and Mann,
1998). *Exd and Hth* control antennal development without interacting with any of the classically defined HOX genes. In addition, Exd proteins interact with more divergent homeodomain proteins. For example, Exd is required for some function of Engrailed, a non-Hox homeodomain protein that contains a divergent tryptophan hexapeptide motif (Alexandre and Vincent, 2003; Kobayashi *et al.*, 2003; Serrano and Maschat, 1998).

Pbx proteins may also bind to non-homeodomain transcription factors as well. Knoepfler *et al.* (1999) showed that Pbx bound DNA cooperatively with the myogenic bHLH proteins MyoD, myf5 and others. As for Pbx-Hox interactions, these Pbx-bHLH interactions occurred via a conserved tryptophan motif N-terminal to the bHLH DNA-binding domain. By inhibiting Pbx function in zebrafish embryos, Maves *et al.* (2007) show that Pbx proteins are required in order for MyoD to induce the expression of a subset of muscle genes in the somites. In the absence of Pbx function, expression of myogenin (myog) and of fast-muscle genes is inhibited, whereas slow-muscle gene expression appears normal. Thus, Pbx also play an important role in regulating generation of fast-muscles. Studying the mechanisms that promote P19 cells differentiation could be a good system to study the function of Pbx as a transcription co-factor and identifying the binding partners in a different pathway.

5. Conclusion:

Neurogenesis is a complex process. There are many intrinsic factors, such as proneural transcription factors, and extrinsic signals, such as
neurotrophic factors, FGFs and RA, that interact with each other to promote or inhibit neurogenesis. HOX genes have been extensively studied, and their functions have been defined in many tissues, including the definition of segmental identity along the A-P axis, and cell adhesive and migratory functions in expressing cells. With the help of new techniques, such as gene knock-out and lineage tracing techniques, we studied the function of HOX genes in greater detail. We found that HOX genes also play a role in regulating peripheral neurogenesis of multipotent cells, a novel function that had not been appreciated. In contrast to the HOX genes, experimental analyses of Pbx and Meis gene functions has barely begun. The results reported ther discovered unanticipated functions of HOX genes and the cofactors. These functions should be further confirmed in a broader set of peripheral neurons, and the mechanisms by which HOX genes and cofactors interact with intrinsic factors and extrinsic signalings to regulate neurogenesis should be studied in more detail.
Figure 1

A

- Wnt/BMP
- Wnt

Stem cell maintenance
Sensory neurogenesis

B

Frizzled

Notch
Delta
Ngn1

Notch
Delta
Ngn1

Hoxa2/P/M
Other

Neural crest
Neural crest

Neural crest stem cell
Sensory progenitor
Figure 1. *Hoxa2 inhibit neurogenesis of neural crest cells.*

A. Wnt in synergy with BMP signals maintains neural crest stem cell in a multipotent state, while Wnt signals on its own induce sensory neurogenesis. B. Some cells that are not exposed to synergistic activity would become sensory neurons (on the right), whereas the expression of Hoxa2 and the cofactors, Pbx (P) and Meis (M) in the same cells inhibits the neurogenesis of the crest cells. Wnt signals from the neural tube bind to the receptor Frizzled on the neural crest cells. Ngn1 is upregulated by Wnt pathway, and sequentially promotes the expression of Delta on the membrane of the same cells. Hoxa2 together with Pbx and Meis could inhibit the expression of Ngn1 through several different mechanisms which is still unclear (indicated by ?). Notch signals in the same cells also down-regulate the expression of Ngn1. Delta expressed on the membrane binds to the Notch receptors on the neighboring cells (on the left), and activated Notch pathway which inhibit those cells to generate neurons.
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