Abstract

The peripheral nervous system of the head is derived from cranial ectodermal placodes and neural crest cells. Placodes arise from thickenings in the cranial ectoderm that invaginate or ingress to form sensory ganglia and the paired sense organs. We have combined embryological techniques with array technology to identify genes that are expressed as a consequence of placode induction. As a secondary screen, we used whole mount in situ hybridization to determine the expression of candidate genes in various placodal domains. The results reveal 52 genes that are found in one or more placodes, including the olfactory, trigeminal, and otic placodes. Expression of some of these genes is retained in placodal derivatives. Furthermore, several genes are common to both neural crest and ectodermal placodes. This study presents the first array of candidate genes implicated in placode development, providing numerous new molecular markers for various stages of placode formation. Importantly, the results uncover previously unknown commonalities in genes expressed by multiple placodes and shared properties between placodes and other migratory cells, like neural crest cells.

Keywords: Placodes; Trigeminal; Olfactory; Otic; Epibranchial; Subtraction; Induction; Neural crest; Chicken

Introduction

The peripheral nervous system originates from two sources of cells: cranial ectodermal placodes and neural crest. Placodes are discrete regions of thickened epithelium that form in the cranial ectoderm and give rise to portions of the cranial sensory ganglia and to the paired sense organs (rev. Le Douarin, 1986; Webb and Noden, 1993). Some placodes, like the otic, nasal, and lens placodes, form as visible thickenings that subsequently invaginate. Others, like the trigeminal and epibranchial placodes, are not distinguishable morphologically. Cells from these placodes delaminate from the ectoderm over a period of time, migrate, and then condense to contribute to the cranial ganglia. Some of the cranial sensory ganglia arise solely from placodes (e.g., acoustic ganglion from the otic placode), whereas others are derived from both placodes and neural crest (e.g., trigeminal placode). Placodes share several properties with neural crest cells, including the ability to undergo an epithelial to mesenchymal transition, migrate, and differentiate into common progeny including sensory neurons, glia, neuroendocrine cells, and cells that can secrete special extracellular matrices.

Although the mechanisms involved in neural crest induction, migration, and differentiation have been explored extensively, far less is known about placodes. Much of our understanding of placodal development in avian embryos comes from quail/chick chimeras in which pieces of ectoderm have been grafted orthotopically (e.g., D’Amico-Martel and Noden, 1983). Recent studies have examined the competence of ectoderm to form placodes, their time of specification, and commitment, as well as factors controlling their differentiation (Begbie et al., 1999, 2002; Baker and Bronner-Fraser, 2000; Baker et al., 1999, 2002; Groves and Bronner-Fraser, 2000).

Despite the obvious importance of placodes in the development of the peripheral sensory nervous system in the head, the molecular signaling events leading to and resulting from placode induction remain largely unexplored.
A small number of molecular markers have fortuitously been found for various placodes. For example, the paired homeodomain protein Pax3 and the fibroblast growth factor receptor FREK (Stark et al., 1997) have served as good markers for the trigeminal placode, but the lack of additional markers makes studying placode formation and differentiation difficult. To surmount these problems, we have combined classical embryological techniques with a gene profiling strategy to determine new candidates in the process of placode induction. The goals are twofold: (1) to find additional placodal markers to study their development and (2) to find candidate genes involved in various aspects of placodal induction.

Previous work from this laboratory has shown that a signal from the neural tube is required for trigeminal placode induction (Stark et al., 1997). Furthermore, it is possible to recapitulate this process in vitro by combining competent ectoderm with dorsal neural tube to elicit expression of molecular markers for the trigeminal placode (Baker et al., 1999), and similar tissue interactions and/or signals are likely to be involved in induction of other placodes. We have taken advantage of this ability to recapitulate trigeminal placode formation in vitro to compare the profile of genes expressed by placode “induced” versus “noninduced” tissue. “Induced” tissue represents competent ectoderm in combination with dorsal neural tube, whereas “noninduced” tissue is the competent ectoderm or the dorsal neural tube cultured alone. By performing a subtractive, large-scale cDNA library screen (Gammill and Bronner-Fraser, 2002; Rast et al., 2000), we have identified many candidate molecules and markers involved in the process of placode formation. Candidates obtained from the library were confirmed by performing whole mount in situ hybridization to determine their expression in various placodal domains. Using this approach, we have found one clone that appears to be specific for anterior neural placodes, others expressed in multiple placodes, as well as some clones that are expressed exclusively in the ectoderm. In addition, we have identified genes that are common to both neural crest and ectoderm. The results reveal shared properties between placodes and other migratory cells, particularly neural crest cells, and suggest commonalities between multiple placodes, implying that similar genetic pathways may regulate their development.

Methods and materials

Embryos and explants

Fertile chicken eggs (Gallus gallus domesticus) and quail eggs (Coturnix coturnix japonica) were incubated at 38°C until they reached the desired Hamburger and Hamilton stage (St.) or somite stage (ss). Embryos were fixed overnight in 4% paraformaldehyde at 4°C for in situ hybridization. Ectodermal explants were removed from 3- to 4-ss embryos and placed in Ringer solution on ice until needed. For preparation of dorsal neural tubes, a large segment of the neural tube from the trunk was removed by dissection with tungsten needles from a 12-ss embryo. The neural tubes were then treated with 1 mg/ml Dispase (Roche) in DMEM with 20mM HEPES pH 8.0 for 15 min on ice, followed by 10 min at 38°C. Neural tubes were then allowed to recover in F12 media with N2 supplements (Invitrogen) with 0.1% bovine serum albumen (Sigma) for at least 10 min on ice. Using glass and tungsten needles, ectoderm was first removed from the neural tubes, then the dorsal neural tubes were dissected away from the other tissue. Dorsal neural tubes were placed in F12/N2 media on ice until needed. Collagen matrix gels were made as previously described (Artinger and Bronner-Fraser, 1993) except that commercially available collagen was utilized (Collaborative Research). The bottom layer of collagen was allowed to solidify for 20 min at room temperature before tissue was added. Once ectodermal explants were positioned on top of the dorsal neural tubes, a top layer of collagen was added to cover the tissue. The top layer was allowed to set for 10 min at 38°C with 5% CO2. F12/N2 media were then added before culturing for 18 h 38°C with 5% CO2.

RNA isolation and macro-arrayed library preparation

Total mRNA was isolated from the collagen gels using either a total RNA isolation kit (Stratagene) or RNAqueous kit (Ambion). PolyA RNA was isolated using the Micro-Poly(A) purist kit (Ambion).

The macro-arrayed cDNA library was prepared from 4- to 12-ss chicken embryos (Gammill and Bronner-Fraser, 2002). Briefly, 147,456 clones were arrayed into 384-well plates, and colonies were spotted onto eight 20-cm square nylon membranes using the Q-bot (Genetix).

Placode-enriched probes and hybridization

For the noninduced pool, 110 ectoderm explants and 55 dorsal neural tube explants were cultured individually and pooled, and, for the induced tissue, 55 conjugates consisting of 110 ectoderm explants and 55 dorsal neural tubes were cocultured and pooled. cDNA was synthesized from each pool as previously described (Gammill and Bronner-Fraser, 2002). The subtracted cDNA was prepared by hydroxypapatite chromatography using the method of Rast et al. (2000). Macro-arrayed filters were hybridized and analyzed as previously described (Gammill and Bronner-Fraser, 2002; Rast et al., 2000).

DNA sequencing and analysis

All candidates were miniprepped (Qiagen), and DNA sequencing was performed (Davis Sequencing, Macrogen Sequencing). Sequences were then compared to known
genes using tBlastX Version 2.26 (Altschul et al., 1997). In addition, raw sequences were analyzed by the Gene Ontology Annotator (http://udgenome.ags.udel.edu/gofigure). Clones that did not have matches were subsequently compared to the recently released chicken genome sequence using preEnsemble (http://pre.ensembl.org/). If a putative gene was detected within the sequence by Genscans (Burge and Karlin, 1997), then the predicted protein sequence was analyzed by BlastP (Altschul et al., 1997) for homology. Otherwise, the chromosomal location of the unknown gene was recorded, and the BlastN (Altschul et al., 1997) e-value compared to the chicken genome was noted.

**In situ hybridization**

Whole mount in situ hybridizations were performed for all clones to confirm the enrichment after subtraction. St. 10 embryos were chosen to screen the candidate genes because commitment to the trigeminal placode fate has begun by this time, but the cells are still present in the ectoderm (Baker et al., 1999). The distribution of selected genes was examined at later stages using similar protocols. Antisense digoxigenin-labeled RNA probes were made according to manufacturer’s directions (Roche). In situ hybridization was performed as previously described (Henrique et al., 1995) using either BM purple or NBT/BCIP (Roche) for color detection. Whole mount pictures were taken using a Zeiss Stemi SVII microscope with an Olympus DP10 digital camera. Selected embryos were then processed for cryosectioning (20-μm-thick sections) as previously described (Sechrist et al., 1995). Expression of the clones was scored and photographed using a Zeiss Axioskop2 Plus.

**Immunohistochemistry**

Conjugates of ectoderm and dorsal neural tubes in collagen were fixed with 4% paraformaldehyde for 30 min at room temperature and rinsed in PBS. Conjugates were then cryosectioned as described by Sechrist et al. (1995). Gelatin was removed from the slides with PBS for 5 min at 42°C. Primary antibodies were used at the following concentrations in blocking solution (10% horse serum, 0.1% Triton X-100, in 0.1% bovine serum albumen in PBS) at 4°C for overnight: Pax3 hybridoma supernatant (Developmental Studies Hybridoma Bank) 1:100, rabbit neurofilament M (NFM; Chemicon) 1:1000, mouse Hu C/D (Molecular Probes) 1:500, and QCPN hybridoma supernatant (Developmental Studies Hybridoma Bank) 1:1. Sections were then rinsed at least three times for 10 min in PBS at room temperature. Secondary antibodies were utilized at the following concentrations: goat antimouse IgG2a Alexa 488 1:2000, goat antirabbit IgG Alexa 596 1:2000, goat antimouse IgG Alexa 596 IgG2b 1:2000, and goat antimouse IgG1 Alexa 647 1:1000 (Molecular Probes). Images were photographed using a Zeiss Axioplan 2.

For whole mount immunohistochemistry, St.-15 chicken embryos were collected and fixed in 4% paraformaldehyde for 2 h at room temperature and rinsed in PBS. Embryos were then blocked in solution as described above for at least 1 h at room temperature. Pax-3 hybridoma supernatant was used at 1:100 and rabbit antibody Pax2 (Zymed) at 1:200 in blocking solution at 4°C overnight. Embryos were rinsed with PBS at room temperature and then overnight at 4°C. Goat antimouse IgG Alexa 488 and goat antirabbit IgG Alexa 596 (Molecular Probes) were diluted 1:1000 in 0.1% Triton X-100, in 0.1% bovine serum albumen in PBS, and incubated overnight at 4°C. Embryos were rinsed with PBS at room temperature and then overnight at 4°C. Fluorescent embryos were photographed using a Zeiss Axioskop2 Plus.

**Results**

**Production of placode-enriched cDNA**

We took advantage of the ability to recapitulate placode formation in vitro to identify genes that were expressed following placode induction. Our laboratory has previously demonstrated that trunk level neural tube from 10- to 13-somite stage (ss) embryos can induce Pax3, a marker for trigeminal placode cells, in 3- to 7-ss midbrain level ectodermal explants in chicken or quail (Baker et al., 1999). Accordingly, competent midbrain ectodermal explants from 3- to 4-ss chicken embryos and trunk dorsal neural tubes from 12-ss chicken embryos were grown in collagen gels either alone or conjugated together (Fig. 1). We selected only the dorsal portion of the neural tube since the inducing signal appears to be a secreted factor from this domain (Baker et al., 1999; Stark et al., 1997), and this eliminates the need to subtract ventral neural tube genes from the induced population. When midbrain level ectodermal explants were cultured alone, explants did not express Pax3 protein after 18–24 h in culture (Figs. 2A–C). When juxtaposed with dorsal neural tubes, however, greater than 90% of ectodermal explants express Pax3 at 18 h (Figs. 2D–F). To generate cDNA for the induced pool of genes, two ectodermal explants were placed on top of a single dorsal tube explant for 18 h at 38°C (Fig. 1). For the noninduced pool, ectodermal explants and dorsal neural tubes were cultured in collagen gels separately under identical conditions and for the same length of time.

Macro-arrayed filters were sequentially hybridized with induced and noninduced cDNA, and hybridization intensities were compared to determine which clones were up-regulated by the process of placode induction. We selected 84 genes with the highest fold increase in the induced recombinants relative to the uninduced tissue. These genes were sequenced from the 5’ end and analyzed by tBlastX (Altschul et al., 1997) to identify similarities to known genes (Table 1, circle). The identification of the genes was
secondarily verified by evaluating the raw sequence using the Gene Ontology interface.

Clones were separated into 13 different classes based on their sequence similarity and ascribed function (Table 1). Genes that were classified as unknown were subsequently compared to the recently released chicken genome (preEnsemble). If the DNA sequence overlapped a predicted gene (Genscans; Burge and Karlin, 1997), the putative protein sequence was analyzed using BlastP (Altschul et al., 1997; Table 1, diamond); otherwise, the chromosomal location was noted along with BlastN (Altschul et al., 1997) e-value (Table 1, rectangle). The e-value scores for each clone are shown in color, with red having the highest likely similarity (b\(10^{-101}\)), followed by orange (b\(10^{-100}\) to b\(10^{-76}\)), yellow (b\(10^{-75}\) to b\(10^{-51}\)), green (b\(10^{-50}\) to b\(10^{-26}\)), and blue (b\(10^{-25}\) to b\(10^{-10}\)) in descending likeliness of similarity.

Relative developmental stage of induced ectodermal tissue

To determine the embryonic stage equivalent of induced placode tissue, we first characterized the time of appearance of known placodal markers in induced explants relative to expression of the same markers in vivo. Because Pax3 is expressed in both placodal ectoderm and dorsal neural tube, interspecies recombinants of quail 3- to 4-ss midbrain level and chick 12-ss dorsal neural tube were utilized to allow distinction of quail ectoderm using the quail specific antibody, QCPN. Three markers (Pax3, neurofilament M, and HuD) were used to estimate the equivalent embryonic age of the induced placodal ectoderm. Pax3 protein is readily detectable in the embryo in the midbrain level ectoderm by 8 ss (Stark et al., 1997). Neurofilament M (NFM) is first seen in a few cells in the ectoderm and a few delaminating cells at 13 ss, whereas the neuronal differentiation marker HuD is seen in the condensing trigeminal ganglia (J. Sechrist and M. Bronner-Fraser, unpublished data).

In 18-h recombinants of competent ectoderm and dorsal neural tube, Pax3 expression was abundantly expressed in the quail ectoderm (Figs. 2D–F); however, no double NFM/Pax3+ or Hu/Pax3+ cells were detected in the induced ectoderm (n = 0/16 and n = 0/12 conjugates respectively, data not shown). By 24 h of culture, NFM could be seen in a few cells that were Pax3+ (n = 6 of 11 conjugates) (Figs. 2G–J). However, Hu/Pax3+ cells were not detectable until 36 h (n = 5 of 8 conjugates) (Figs. 2K–N). Taken together, these results suggest that the approximate age of the induced ectoderm used for the purposes of our screen corresponds to an embryonic age of 8–12 ss (St. 9–11).

Expression pattern of up-regulated genes

As a secondary screen to verify that the up-regulated genes were expressed in placodes, we performed whole mount in situ hybridization on St.-10 chicken embryos, corresponding to the approximate age of the “induced” cDNA pool. In whole mount, embryos were analyzed for expression of genes in the olfactory and otic placodes, which can clearly be identified in the anterior-most tip of the neural folds and adjacent to rhombomere 5 of the hindbrain, respectively (Table 1). Because trigeminal and epibranchial placode ectoderms are more difficult to
Fig. 2. Placode cells express neuronal markers after 24 h of culture. Ectodermal explants were cultured for 18 h, and ectoderm and dorsal neural tube conjugates were cultured for 18, 24, and 36 h. Pax3 is expressed in both dorsal neural tube and placodal ectoderm. To distinguish, quail ectoderm and chick dorsal neural tubes were utilized. Pax3 is not expressed at 18 h when ectoderm is cultured alone but does express the quail marker QCPN (A–C). When ectoderm (ecto) is cocultured with dorsal neural tube (dnt), Pax3 is abundantly expressed in the induced ectoderm (D). QCPN (E) is only expressed by the quail ectoderm. Double-positive Pax3 and QCPN cells (arrow head) are induced placodal cells (F). The first triple-positive Pax3, NFM, QCPN cells can first be seen at 24 h of culture (arrow, G–J). Hu is expressed later in cultured conjugates; triple-positive Pax3, Hu, QCPN can first be seen at 36 h of culture (arrow, K–N). These results indicate that the approximate age of the induced explants after 18 h of culture is between 8 and 12 ss. Note that Pax3 has nuclear, QPCN has perinuclear, Hu has cytoplasmic, and NFM has cytoplasmic and filamentous staining.
discern in whole mount, embryos were subsequently sectioned and analyzed by microscopy for presence of transcripts at the level of the midbrain for trigeminal placode region (Table 1) and at the level of the branchial arches for epibranchial placodes. In some cases, sections of the olfactory and otic placode levels were used to verify expression observed by whole mount. Of the 84 genes analyzed by the secondary screen, 52 were expressed in midbrain level ectoderm (trigeminal placode), olfactory, or otic placodes, 4 were not expressed in any placodes, and the remainder were not interpretable.

Profile of genes expressed in multiple placodes

It has been hypothesized that all neural placodes share a common early lineage and are likely to express some of the same genes to accomplish similar goals (rev. Baker and Bronner-Fraser, 2001). The in situ hybridization patterns from this screen support this hypothesis since 71% of the genes were common to the trigeminal, olfactory, and/or otic placodes. Shown in Fig. 3 are examples of genes found in various combinations in the trigeminal level ectoderm, olfactory placode, and otic placode. Most were found in all three (37 out 52 clones), with five genes expressed in both the trigeminal and olfactory placodes, and three in a combination of trigeminal and otic placodes. For example, translation initiation factor eIF-2γ chain, which is involved in the early steps of protein synthesis (rev. Sonenberg and Dever, 2003), was found in all three placodes (Figs. 3A, F–H) as well as in the neural crest and dorsal neural tube (Fig. 3G). Kinesin 13A, which transports the mannose-6-phosphate receptor to the plasma membrane (Nakagawa et al., 2000), was found in the olfactory and trigeminal placodes (Fig. 3B), whereas NADH2 dehydrogenase, a gene involved in the respiratory chain and important in normal development (rev. Fantel and Person 2002), was found only in the trigeminal level ectoderm and otic placode (Figs. 3C, I, J). No genes were found to be exclusive to the trigeminal placode. Three genes were expressed in the olfactory and otic placodes and one exclusively in the otic placode. For instance, Chromokinesin, a gene required for mitotic chromosomal positioning and bipolar spindle stabilization (Antonio et al., 2000; Levesque and Compton, 2001), was found in the olfactory and otic placodes (Fig. 3D), and clone 19E17, with very low homology to SoxE and classified as an unknown, was found exclusively in the otic placode (Fig. 3E). That the majority of the genes found by this library screen were expressed in the trigeminal, olfactory, and/or otic placode supports the effectiveness of the screen and also suggests that there are signaling pathways common to multiple placodes.

Not surprisingly, many of these genes identified in our screen are involved in processes associated with changes in a cell’s state. These categories include genes involved in mitosis, cytoskeleton, receptors/ligands/downstream signaling, and transcription factors. Individual examples of genes in these categories are highlighted below.

Cytoskeletal genes

Six genes from this screen are involved in cytoskeletal processes. For example, tubulin β2, one of the major components of microtubules (rev. Oakley, 2000), was expressed in the ectoderm in the midbrain level of St.-10 embryos (Fig. 4A) as well as in the olfactory and otic placodes (Table 1). No expression was observed in the neural crest or dorsal neural tube.

CD 151 (Figs. 5A, B), also called PETA-3/SFA-1, is a member of the family of tetraspanins (rev. Hemler et al., 1996; Wright and Tomlinson, 1994; Maecker et al, 1997) and has a particularly interesting distribution pattern that resembles the expression of Pax3 (Fig. 5C) in the trigeminal placode at St. 10. However, unlike Pax3, CD 151 lacks the neural crest and dorsal neural tube expression components of Pax3. Its expression was first detected at St. 9 correlating with the time of specification of the trigeminal placode (data not shown). At St. 10, midbrain level ectoderm had robust expression of CD 151 (Fig. 5B); in addition, it was expressed in the olfactory but not the otic placode (Fig. 5A). At St. 11, CD 151 expression in the ectoderm (Figs. 5D, E) closely resembled that of Pax3 (Fig. 5F).

Metabolism

Lactate dehydrogenase H subunit is involved in the final step of anaerobic glycolysis (rev. Voet and Voet, 1990) and may have a novel function in the trigeminal placode. It was expressed in the midbrain ectoderm, olfactory, and otic placodes as well as the trigeminal ganglion, but not in the neural crest and the dorsal neural tube (Fig. 4B).

Expression of genes associated with cell proliferation

Only one gene identified in our screen was involved in cell cycle and mitosis and present in the midbrain level ectoderm: MIF2 suppressor. It is a member of the SMT3 family and is similar to ubiquitin (Mannen et al., 1996). It was strongly expressed in the midbrain ectoderm, neural crest, and, to a lesser extent, the dorsal neural tube (Fig. 4C). Similar to tubulin β2 and lactate dehydrogenase H subunit, it was expressed in the olfactory, otic, and trigeminal placodes.

Protein production/degradation

The 60S ribosomal protein L12 is involved in protein production by binding directly to the 26S ribosomal RNA (rev. Gudkov, 1997). It was highly expressed in the midbrain ectoderm, neural crest, and at lower levels in dorsal neural tube (Fig. 4D). In addition to the trigeminal placode level ectoderm, it was also expressed in the olfactory and otic placodes (Table 1). Perhaps the high expression in these areas is indicative of high protein production, as both ectoderm and migrating crest are undergoing many changes.
### Table 1
Genes expressed in the midbrain level ectoderm and other placodes at St. 10

<table>
<thead>
<tr>
<th>Clone / Sequence similarity</th>
<th>Midbrain</th>
<th>Placodes</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Cell Death</td>
<td>Ecto NC dNT</td>
<td>Trig Olf Otic</td>
<td></td>
</tr>
<tr>
<td>132G5 BCL2-associated antagonist 3</td>
<td>+ + +/+</td>
<td>+ + +</td>
<td>Has anti-apoptotic activity</td>
</tr>
<tr>
<td>211H9 Programmed cell death protein 6</td>
<td>+ + +/+</td>
<td>+ + +</td>
<td>Ca2+ binding protein required for T cell receptor, FAS, and glucocorticoid-induced death</td>
</tr>
<tr>
<td>B. Chromatin remodeling/histones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>394K14 Histone acetylase complex subunit MRG15-1 or -2, or MORF-4</td>
<td>+ + +</td>
<td>+ + +</td>
<td>May be a negative or positive regulator of transcription, works in complex with other factors</td>
</tr>
<tr>
<td>C. Cytoskeleton</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67G10 CD151 (PETA-3/SFA-1)</td>
<td>- - +</td>
<td>+ +</td>
<td>Member of tetraspanin family, modulates integrin signaling</td>
</tr>
<tr>
<td>93G16 Fibulin-1, isoform D precursor</td>
<td>+ + +</td>
<td>+ + -</td>
<td>Component of elastic extracellular matrix fibers, basement membranes and blood</td>
</tr>
<tr>
<td>132N10 β actin</td>
<td>+ +</td>
<td>+ +</td>
<td>Cell motility</td>
</tr>
<tr>
<td>150G16 Tubulin α6</td>
<td>+ + +</td>
<td>+ +</td>
<td>Major component of microtubules</td>
</tr>
<tr>
<td>258F4 Tubulin β2 chain, embryonic</td>
<td>+ +</td>
<td>+ +</td>
<td>Major component of microtubules</td>
</tr>
<tr>
<td>315F22 EDT-soluble extracellular protein 130 kDa</td>
<td>+ +</td>
<td>+ +</td>
<td>Involved in the initiation of the epithelial-mesenchymal transformation of cardiac endothelium</td>
</tr>
<tr>
<td>D. Metabolism</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>233O17 Lactate dehydrogenase II subunit</td>
<td>+ -</td>
<td>+ +</td>
<td>Interconverts pyruvate and lactate with concomitant interconversion of NADH and NAD(+)</td>
</tr>
<tr>
<td>240C20 NADH dehydrogenase 24K chain precursor</td>
<td>- + -</td>
<td>+ +</td>
<td>Transfers electrons from NADH to respiratory chain</td>
</tr>
<tr>
<td>242B16 TPA: pantothenate kinase 1β</td>
<td>- +</td>
<td>+ +</td>
<td>TPA is defective in Hallervorden-Spatz syndrome</td>
</tr>
<tr>
<td>E. Mitosis/Cell cycle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46L15 Chromokinesin</td>
<td>- + +/+</td>
<td>+ +</td>
<td>Required for mitotic chromosomal positioning and bipolar spindle stabilization</td>
</tr>
<tr>
<td>226N23 MIF2 suppressor</td>
<td>+ + +</td>
<td>+ +</td>
<td>May be part of function and/or structure kinetochore</td>
</tr>
<tr>
<td>F. Protein production/ degradation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12P12 ADP-ribosylation factor 1 (ARF-1)</td>
<td>+ + +</td>
<td>+ +</td>
<td>Small GTPase involved in protein trafficking</td>
</tr>
<tr>
<td>157K6 60S ribosomal protein L12</td>
<td>+ + +</td>
<td>+ +</td>
<td>Binds directly to 26S ribosomal RNA</td>
</tr>
<tr>
<td>G. Receptors/ligands/ down- stream signaling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23C4 Progesterone binding protein</td>
<td>+ + +/+</td>
<td>+ -</td>
<td>Receptor for progesterone, membrane-bound</td>
</tr>
<tr>
<td>51D9 Calmodulin 1, 2, or 3</td>
<td>+ + +/+</td>
<td>+ +</td>
<td>Modulates a large number of enzymes by Ca2+, including protein kinases and phosphatases</td>
</tr>
<tr>
<td>51L2 MAPK6/ERK3</td>
<td>+ + +/+</td>
<td>+ +</td>
<td>Negatively regulates cell cycle progression</td>
</tr>
<tr>
<td>98M4 GAP SH3 binding protein (G3BP-1)</td>
<td>+ + +</td>
<td>+ +</td>
<td>RAS protein signal transduction</td>
</tr>
<tr>
<td>101L16 Calcitonin gene-related peptide/receptor component protein (CGRP-RCP)</td>
<td>+ + +/+</td>
<td>+ +</td>
<td>Modulates CGRP responsiveness in a variety of tissues</td>
</tr>
<tr>
<td>128B9 β subunit heterotrimeric GTP-binding protein</td>
<td>+ + +/+</td>
<td>+ +</td>
<td>Required for GTPase activity, replacement of GDP by GTP, G protein effector interaction</td>
</tr>
<tr>
<td>145F18 Serine/threonine protein kinase-like protein</td>
<td>+ +</td>
<td>+ +</td>
<td>Putative Ser/Thr kinase</td>
</tr>
<tr>
<td>149F15 Vitamin D receptor-interacting protein (DRIP92)</td>
<td>+ +</td>
<td>+ +</td>
<td>Involved in ligand depend transactivation of transcription by the Vitamin D receptor</td>
</tr>
<tr>
<td>212O3 Presenilin-like protein 4</td>
<td>+ +</td>
<td>+ +</td>
<td>Intermembrane protease</td>
</tr>
<tr>
<td>230A12 Frzb1</td>
<td>+ + +/+</td>
<td>+ +</td>
<td>Soluble Wnt-binding protein that antagonizes Wnt signaling</td>
</tr>
<tr>
<td>242D21 Protein phosphatase 2C</td>
<td>+ + +/+</td>
<td>+ +</td>
<td>Dephosphorylates with broad specificity</td>
</tr>
<tr>
<td>360I22 Short transient receptor potential channel 4 associated protein</td>
<td>+ +</td>
<td>+ +</td>
<td>Regulates cellular Ca2+ homeostasis</td>
</tr>
<tr>
<td>H. RNA binding proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>378N16 Fox-1</td>
<td>+ + +</td>
<td>+ -</td>
<td>Both positive and negative regulation of tissue-specific splicing via GCAUG</td>
</tr>
<tr>
<td>394J15 Transformer-2β</td>
<td>+ + +</td>
<td>+ +</td>
<td>Sequence-specific RNA-binding protein, participates in the control of pre-mRNA splicing</td>
</tr>
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</table>
**I. Transcription Factors**
- 63K13: Enhancer of Zeste homolog 1
- 91E4: Basic leucine zipper and w2 domains 1
- 113B10: HLH-PAS transcription factor NXF
- 151E5: Hairyl
- 158B23: X-box binding protein 1

Sequences were analyzed by tBlastX (circle). If the sequence did not have any hits, they were subsequently analyzed by preEnsemble. If Genscans predicted a gene, the predicted protein then underwent BlastP analysis (diamond). If no gene was predicted, the chromosomal location and BlastN e-value compared to the chicken genome was noted (rectangle). The e-value scores for each clone are shown in color with red having the highest likely similarity (e-101), followed by orange (e-100 to e-76), yellow (e-75 to e-51), green (e-50 to e-26), and blue (e-25 to e-10) in descending likelihood of similarity.

**J. Transcription-General**
- 46L12: Nucleolin
- 125C16: RNA polymerase II polypeptide G

Induces chromatin condensation

**K. Translation**
- 130P4: Translation initiation factor 4AII
- 231O15: Ribosomal protein S6
- 259I5: Translation initiation factor eIF-2γ-chain

Early steps of protein synthesis

May play a role in controlling cell growth and proliferation by selective translation of mRNAs

**L. Transporters**
- 75J3: Kinesin 13A
- 116E1: ATP synthetase α
- 224G16: (Na+,K+)-ATPase-β2

Transports mannose-6-phosphate receptor to plasma membrane

Produces ATP from ADP in proton gradient in mitochondria

Non-catalytic unit of active enzyme that exchanges Na+ and K+ ions across plasma membrane

**M. Unknown**
- 19E17: SoxE; Chromosome Unassigned 85415985-85416451
- 25P14: PRO2963; Chromosome Unassigned 48883605-48884276
- 33M6: Chromosome 23: 3913436-3913945
- 66F17: KIAA0153 protein; Chromosome 1: 65138660-65138920
- 77D24: Chromosome 4: 58655120-58655907
- 151B12: Chromosome 6: 10375192-10375776
- 211G6: Hypothetical protein FLJ11838; Chromosome 21: 6156459-6156860
- 234B20: Chromosome Unassigned: 28039657-28040009
- 240C5: SoxE; Chromosome Unassigned: 85415985-85416665
- 242D5: Chromosome 3: 42795567-42796681

Sequences were analyzed by tBlastX (circle). If the sequence did not have any hits, they were subsequently analyzed by preEnsemble. If Genscans predicted a gene, the predicted protein then underwent BlastP analysis (diamond). If no gene was predicted, the chromosomal location and BlastN e-value compared to the chicken genome was noted (rectangle). The e-value scores for each clone are shown in color with red having the highest likely similarity (e-101), followed by orange (e-100 to e-76), yellow (e-75 to e-51), green (e-50 to e-26), and blue (e-25 to e-10) in descending likelihood of similarity. Each clone was analyzed by whole mount in situ hybridization at St. 10. The presence (+), absence (−), or possibility (+/−) of signal at the midbrain level were analyzed by sections. The otic and olfactory placodes were analyzed by whole mount and sections when available. Ecto indicates ectoderm; NC, neural crest; dNT, dorsal neural tube; Trig, trigeminal placode region; Olf, olfactory placode; Otic, otic placode.

(For interpretation of reference to colour in this table legend, the reader is referred to the web version of this article.)
Receptors/ligand and downstream factors involved in intracellular signaling

We have identified a number of receptors, secreted molecules, and signal transduction mediators that have not previously been linked to placode development. These are excellent candidates to send and receive signals for stimulating migration and/or instructing placodal cell specification. For example, presenilin-like protein 4 is an intermembrane protease that may be involved in the cleavage of Notch receptors promoting Notch signaling (rev. Dewji et al., 2004; Selkoe and Kopan, 2003). Presenilin-like protein 4 was found in the midbrain level ectoderm and neural crest, but at a much lower level in the dorsal neural tube (Fig. 4E). Similarly, it was found in the olfactory and otic placodes (Table 1).

Calmodulin 1/2/3 was distributed in the midbrain level ectoderm and neural crest (Fig. 4F) and was also found in the olfactory and otic placodes (Table 1). This may play a role in altering Ca\(^{2+}\)-regulated signaling since calmodulin 1/2/3 may be able to compete for binding with calmodulin to different cellular substrates (Kortvely et al., 2003).

Another signaling component, the \(\beta1\) subunit of heterotrimeric GTP-binding protein, was expressed in midbrain ectoderm, neural crest, and dorsal neural tube (Fig. 4G). In addition, it appeared to be present in the olfactory but not the otic placode (Table 1). This subunit is important for G protein signaling and is required for GTPase activity, replacement of GDP by GTP, and G protein effector interactions (rev. Neer, 1994).

With respect to secreted factors, we confirmed that Frzb1 was up-regulated in developing placodes. The expression of Fzb1 in the developing chicken embryo has been previously described, but its ectodermal expression was not highlighted (Baranski et al., 2000; Ladher et al., 2000). Frzb1 was expressed as early as St. 8 in the ectoderm just adjacent to the open neural tube before specification of the trigeminal placode (Fig. 6B), with much more prominent staining in the neural folds (Figs. 6A, B). By St. 9, Fzb1 was expressed in the ectoderm directly adjacent to the neural folds where the first trigeminal placode cells are specified (Fig. 6D). By St. 10, larger numbers of trigeminal placode cells are specified but have not yet begun to migrate. At this time, Fzb1 was present in the midbrain level ectoderm (Fig. 6F) as well as the olfactory and otic placodes (Fig. 6E). By St. 12, however, Fzb1 was down-regulated in the midbrain level ectoderm just before ingestion of trigeminal placode cells (data not shown).
Several transcription factors were identified in the screen. Hairy1, a transcriptional repressor of bHLH proteins such as MyoD and ASH1, was expressed in the midbrain ectoderm and dorsal neural tube and at lower levels in the neural crest (Fig. 4I). It was also found in the olfactory and otic placodes (Table 1). Hairy1 plays a role in cell fate and boundary positions (Fisher and Caudy, 1998; Kageyama and Nakashashi, 1997; Palmeirim et al., 1997). Placodal expression was not previously attributed to Hairy1.

Another gene, Enhancer of Zeste homolog 1, has been shown to be involved in gene transcription and chromatin structure in Drosophila (rev. Dorsett, 1999). Expression of Enhancer of Zeste homolog 1 was seen in the midbrain ectoderm, neural crest, neural tube (Fig. 4J), as well as the olfactory and otic placodes (Table 1). Several of the other genes we identified are putative transcription factors, based on characteristic domains. For example, the basic leucine zipper and w2 domains 1 gene is a putative transcription factor (Strausberg et al., 2002) that was expressed in the midbrain ectoderm and neural crest (Fig. 4H) and the olfactory and otic placodes (Table 1). The presence of a variety of known and putative transcription factors in the placodes is consistent with the fact that these newly specified cells are likely to be initiating new cellular programs.

Novel molecules expressed in the placodes

Seven of the genes isolated are potentially novel based on lack of known homologues. For example, clone 234B20 has no known homology in the database. Interestingly, in the midbrain, it was expressed exclusively in the ectoderm (Fig. 4K). It was also expressed in the olfactory and otic placodes (Table 1). Although classified as an unknown, clone 240C5 has very low homology to SocE, which when depleted in Myxococcus xanthus arrests growth and induces sporulation (Crawford and Shimkets, 2000). It was strongly expressed in the midbrain ectoderm and does not appear to be in the neural crest or dorsal neural tube (Fig. 4L). It also had expression in the olfactory and otic placodes (Table 1).

Analysis of gene expression at trigeminal ganglion formation

Most of clones discovered by this subtractive library screen are expressed in regions where placodes have been induced and are present in multiple placodes. To examine whether their expression extends beyond the time of placode induction, we examined the expression pattern of several genes at a later time. Expression patterns were compared with those of known placodal markers, Pax2 and Pax3, at St. 15. Pax2 is an early marker of the otic and epibranchial placodes (Groves and Bronner-Fraser, 2000; Hidalgo-Sánchez et al., 2000), whereas Pax3 is a marker of the ophthalmic lobe of the trigeminal placode (Stark et al., 1997). Pax3 is present in the condensing ophthalmic lobe of the trigeminal ganglia and somites at St. 15 (Fig. 7A), but absent from the maxillary or mandibular lobes of the trigeminal ganglia at this stage. Pax2
is restricted to the otic cup and the first two epibranchial placodes at St. 15 (Fig. 7B).

The expression patterns of CD 151, lactate dehydrogenase H, MIF2 suppressor, and Hairy1 (Figs. 7C–O) were examined in detail and compared with Pax2 and 3 at this stage. Whereas Pax3 expression was obvious in the condensing trigeminal ganglion at St. 15 (Fig. 7A), CD 151 was only present in a restricted region of ectoderm (Figs. 7C, H) from which trigeminal placode cells continue to be produced in a pattern that appears to presage the path that the ophthalmic lobe projections will follow. This staining may represent trigeminal placode cells that will continue to delaminate to form the ganglion. An interesting possibility is that cells expressing CD 151 may be making the transition from a placode cell to a migrating neuron whose cell body will line the axon track of the trigeminal nerve. At the level of the eye, CD 151 was seen in the dorsal surface ectoderm (Fig. 7G).

Lactate dehydrogenase H was maintained in the trigeminal and otic ectoderm through St. 15 (Figs. 7D, I, J). It was restricted to the area of the mandibular lobe of the trigeminal ganglion (Fig. 7I), as well as the surface ectoderm overlying the otic cup (Fig. 7J).

At St. 15, Hairy1 was expressed in the olfactory, lens, trigeminal, and otic placodes (Fig. 7F). Fig. 7L shows intense staining in the olfactory placode. In the lens, the expression of Hairy1 was restricted to the area adjacent to the surface ectoderm (Fig. 7M). A few cells at the ophthalmic level of the trigeminal ganglia (Fig. 7N), surface ectoderm, and otic cup also expressed Hairy1 (Fig. 7O).

Few markers have been found for epibranchial placodes that form in the ectoderm of branchial arches 2 and 3. Interestingly, MIF2 suppressor was observed in two of the epibranchial placodes, as well as the condensing mandibular trigeminal ganglia (Fig. 7E). These results show that at least some of the genes obtained in our screen of ~St. 10-induced placodal ectoderm were maintained through later stages. This is consistent with either long-term or sequential roles for these genes in multiple stages of placodal development.

Discussion

Here, we present the first array of candidate genes implicated in placode development. By combining tissue recombination to recapitulate placode induction in vitro with array technology, we have identified numerous new molecular markers for ectodermal placodes that are up-regulated as a result of induction. The results reveal previously unrecognized similarities in genes expressed by multiple placodes as well as shared properties between placodes and neural crest cells.

Genes up-regulated after placode induction indicate a change in cell state

Many of the genes identified in this screen are involved in processes associated with a cell type undergoing a transition. These categories include genes involved in cell division, cytoskeletal rearrangement, receptor/ligand interactions and their downstream signaling pathways, and
transcription factors. This is expected following induction because placode cells change from an epithelial to a migratory cell that will condense into a ganglion or form a sensory organ. In the trigeminal placode, most cells become postmitotic before they delaminate and migrate (Begbie et al., 2002; D’Amico-Martel and Noden, 1980); however, other placodal cells such as otic and epibranchial continue to divide while migrating (Begbie et al., 2002). In addition, placodal cells are thickened compared to neighboring ectodermal cells, both events requiring changes in the cytoskeleton. They respond to inducing signals (Baker et al., 1999; Stark et al., 1997) that in turn activate discrete signaling pathways. Finally, new transcription factors are likely to be activated in a cell adopting a placodal lineage. Many of the genes identified were expressed not only in placodes, but also in the neural crest and dorsal neural tube, consistent with their shared ectodermal lineage.

One of the genes with the most specific placodal expression pattern is CD 151 (PETA-3/SFA-1), a member of the tetraspanin superfamily of proteins with four membrane-spanning domains. These are involved in diverse cellular functions such as regulation of cell growth and differentiation, cell adhesion, intracellular signaling (rev. Hemler et al., 1996; Maeccker et al., 1997; Wright and Tomlinson, 1994), cell polarity (Yáñez-Mó et al., 2001), and epithelial cell–cell adhesions (Shigeta et al., 2003). CD 151 directly interacts with and is thought to modulate signaling of several integrins (Berditchevski and Odintsova, 1999; Fitter et al., 1999; Serru et al., 1999; Sincock et al., 1999; Stipp and Hemler, 2000; Yauch et al., 2000). Tetraspanin proteins are able to influence phosphoinositide-dependent signaling by recruiting PI 4-kinase to specific membrane locations (Yauch and Hemler, 2000), as well as interacting with PKCs (Shigeta et al., 2003; Zhang et al., 2001). Given these intriguing functions, it is tempting to speculate that CD 151 could play a role in changing the morphology of an ectodermal cell to a placode cell and perhaps transduce signals that prepare the placodal cell for migration.

Another gene product, Fzb1, is expressed in the trigeminal ectoderm but down-regulated as placodal cells ingress. Fzb1 is a soluble Wnt-binding protein that has been shown to antagonize Wnt signaling (rev. Moon et al., 1997). Fzb1 is involved in myogenesis (Borello et al., 1999; Tzahor et al., 2003), condensation and differentiation of cartilage (Wada et al., 1999), and digit formation (Chimal-Monroy et al., 2002) and is hypothesized to play a role in neural crest specification (Baranski et al., 2000). Its placodal distribution suggests a role for inhibition of Wnt signaling in trigeminal placode specification.

**Placodes have common gene expression during development**

All cranial placodes are thought to have an early common origin (rev. Baker and Bronner-Fraser, 2001), deriving from focal thickenings of the ectoderm that arise from cells adjacent to the neural plate and ectoderm border. Evidence in support of this hypothesis comes from the gene Six-4 which is expressed in a horseshoe shape corresponding to the placodal fate map (“preplacodal area”), and these cells continue to express the gene as they develop into distinguishable placodes (Esteve and Bovolenta, 1999). It has been argued that placodes have more differences than similarities, such as different tissue interactions for induction (Graham and Begbie, 2000), and therefore are not likely to be related. On balance, our data support commonality of gene expression in the placodes, with 43 out of the 52 genes...
being expressed in multiple placodes in St.-10 embryos. In addition, we found that the expression of some of the genes was maintained in the olfactory, trigeminal, and otic placodes, as well as in the newly forming epibranchial placodes.

Clearly, differences exist between individual placodes; most notably, this is reflected by their manner of induction and differentiation. The olfactory and otic placodes can be induced by mesoderm, endoderm, and neuroectoderm, whereas the trigeminal placode is induced by the dorsal neural tube, the epibranchial placodes by pharyngeal endoderm, and the lens placode by the neural ectoderm (rev. Baker and Bronner-Fraser, 2001; Francis-West et al., 2002; Graham and Begbie, 2000). Some of the signaling molecules involved in the induction of specific placodes have been identified, such as BMP7 for the epibranchial placodes.
placodes (Begbie et al., 1999) and several FGFs, such as FGF3 (Represa et al., 1991) for the otic placode. Our data indicate that the placodes may up-regulate common repertoires of genes in response to induction, regardless of the manner of induction.

It may seem contradictory that induction is different for each placode, yet they utilize many of the same genes for specification or delamination. However, induction is not likely to be a single step process, nor is it likely to occur at one distinct time point. For example, in the case of the trigeminal placode, cells are delaminating over time, and the response to induction may be similarly spread out. In addition, the same genes could be used in various combinations at different time periods to eventually result in different placodal derivatives. One intriguing possibility is that the inductive interactions may vary between placodes, but their subsequent responses may involve activation of some overlapping or intersecting signaling pathways.

Consistent with the possibility that different placodes may share common signaling pathways, cranial ectoderm appears to be able to respond appropriately to inducing cues from different placodal environments. For example, the midbrain level ectoderm, which would normally form the trigeminal placode, can be transplanted into the otic level and give rise to otic placode derivatives (Groves and Bronner-Fraser, 2000). In general, the competence of cranial ectoderm to give rise to different placodal derivatives is restricted over time, indicating that the placodes initially use the same genes but later diverge as placodes assume individual fates. Taken together, the location of the preplacodal area, the competence of placodal ectoderm to substitute for other placodal ectoderm early in development, and the overlapping gene expression after placode induction support the idea that placodes share some common genetic machinery.

Placodes and neural crest share similar gene expression

The peripheral nervous system arises from both neural crest and placodes, two cell populations that possess many similar characteristics: both arise at the neural plate/ectoderm border, contain migratory cells that undergo an epithelial to mesenchymal transition, and generate sensory neurons, glia, and other support cells, sometimes within the same ganglion. However, only neural crest cells from melanocytes and much of the craniofacial skeleton.

Because of these shared properties, it has been hypothesized that neural crest and placodal cells may be derived from a common ancestral cell type (Baker and Bronner-Fraser, 1997, 2001; Northcutt and Gans, 1983). Although our screen does not directly test a common origin between placodes and neural crest cells, 39 out of 48 genes were expressed by both the trigeminal level ectoderm and neural crest, suggesting remarkable cellular similarities. The large number of common genes expressed by these two cell populations now provides the tools to test whether neural crest and placode cells may use the same genetic machinery to accomplish similar tasks, such as delamination and migration.

Acknowledgments

We would like to thank Anitha Rao for technical support, Titus Brown for his help with bioinformatics programs, Dr. Jonathan Rast for answering questions regarding the library screen, and Drs. Vivian Lee and Lisa Tanehill Ziener for their critical reviews of the manuscript. This work was funded in part by NIH F32 DE14131-03 and NIH RO1 DE016459-05.

References


