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Genomic analysis of neural crest induction

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SUMMARY

The vertebrate neural crest is a migratory stem cell population that arises within the central nervous system. Here, we combine embryological techniques with array technology to describe 83 genes that provide the first gene expression profile of a newly induced neural crest cell. This profile contains numerous novel markers of neural crest precursors and reveals previously unrecognized similarities between neural crest cells and endothelial cells, another migratory cell population. We have performed a secondary screen using in situ hybridization that allows us to extract temporal information and reconstruct the progression of neural crest gene expression as these cells become different from their neighbors and migrate. Our results reveal a sequential 'migration activation' process that reflects stages in the transition to a migratory neural crest cell and suggests that migratory potential is established in a pool of cells from which a subset are activated to migrate.

Key words: Neural crest, Induction, Migration, Macroarray, Subtraction, Chick

INTRODUCTION

Neural crest cells are a uniquely vertebrate cell type that arises in the 'neural folds' at the border between neural and non-neural ectoderm in early embryos. Neural crest induction occurs via signaling between these tissues (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995), and appears to be mediated by the Wnt (Garcia-Castro et al., 2002) and BMP (Liem et al., 1995) signaling pathways. Although initially contained within the central nervous system, neural crest cells depart from their site of origin, migrate extensively throughout the embryo, and form many diverse derivatives including most of the peripheral nervous system, facial skeleton, and melanocytes of the skin (reviewed by LeDouarin and Kalcheim, 1999). However, the molecular events that take place following neural crest induction and prior to migration are poorly understood.

Because there is no way to physically or molecularly define a neural crest cell until it becomes migratory, it has not been possible to characterize in detail the events that give rise to migratory neural crest cells. Neural crest precursors do not exist as a segregated population in the ectoderm. When individual cells in the neural folds are labeled with a lineage tracer, progeny of those cells contribute not only to neural crest, but also to central nervous system and epidermal derivatives (Bronner-Fraser and Fraser, 1988; Selleck and Bronner-Fraser, 1995). Furthermore, there are no known genes that specifically mark only those cells in the neural folds that will migrate. A small number of molecules fortuitously found to be expressed in the neural folds have been shown to be required for neural crest formation, notably *slug* (Nieto et al., 1994; LaBonne and Bronner-Fraser, 2000), *sox9* (Spokony et al., 2002), *FoxD3* (Kos et al., 2001; Sasai et al., 2001), and the *rho* family of small GTPases (Liu and Jessell, 1998). Of these, *slug* is the best available marker of neural crest precursors; however, not all *slug*-expressing cells become migratory neural crest cells (Linker et al., 2000).

We have combined chick embryological techniques with genomics to obtain a detailed characterization of the early events in neural crest formation. To utilize the accessibility and thorough descriptive embryology of the avian neural crest (LeDouarin and Kalcheim, 1999), an arrayed, early chick cDNA library (a 'macroarray') was screened with a subtracted probe (Rast et al., 2000). This approach provided a functional genomics resource in an organism where such technology was previously lacking. To generate our subtracted probe, we took advantage of the ability to recapitulate de novo chick neural crest induction in vitro by co-culturing pieces of non-neural ectoderm and intermediate neural plate (Fig. 1A, B) (Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995). This afforded us a powerful tool for producing neural crest precursors under defined conditions. Finally, by characterizing the in situ hybridization patterns of the genes we identified, we were able to extract temporal information about the expression of each clone over the course of neural crest development. This combination of in vitro-induced neural crest with macroarray screening (Rast et al., 2000) and in situ hybridization allowed us to achieve a gene expression profile of a premigratory neural crest cell, a cell type that cannot be identified or isolated.

Here, we present a resulting collection of 83 genes that are expressed as a consequence of neural crest induction. This analysis identifies many novel markers of the early neural crest as well as candidate regulatory molecules for their development. Importantly, our results prompt comparisons with other migratory cell types and suggest a model in which migratory potential is sequentially acquired in neural crest

precursors, with this potential being activated in only a subset of cells by a signal to migrate.

MATERIAL AND METHODS

Embryos and explants

Fertile chicken eggs (White Leghorn) were incubated for 30-42 hours to obtain 4- to 16-somite embryos. Embryos were fixed overnight at 4°C in 4% paraformaldehyde for in situ hybridization. For explants, tissues were dissected in Ca^{2+}/Mg^{2+}-free Tyrodes saline plus 0.05% trypsin. Conjugates were assembled by wrapping freshly dissected ectoderm around intermediate neural plate in embryo extract plus 10% horse serum. Explants were allowed to recover for 1 to 4 hours on ice before embedding in collagen gels and culturing for 24 hours as previously described (Dickinson et al., 1995).

RNA preparation and RT-PCR

RNA was prepared from embryos or tissue in collagen gels using Stratagene's RNA isolation kit. Collagen gels were transferred to an Eppendorf tube with forceps, as much liquid as possible was removed, lysis solution was added, and gel and tissue were dissolved by trituration. Relative quantitative reverse transcriptase-polymerase chain reaction was performed as described previously (Gammill and Sive, 1997). Poly(A) RNA was prepared using the PolyA spin mRNA isolation kit (NEB).

Library construction and macroarray production

Oligo-dT primed cDNA with an average size of 1.7 kb was prepared from 4- to 12-somite chicken embryos using the Superscript cDNA Synthesis and Plasmid Cloning kit (Gibco BRL). The cDNA was directionally cloned in the vector CS107 (Baker et al., 1999) to create a library containing 1.5×10^8 clones/μg. 147,456 clones of the primary library were arrayed into 384-well plates and spotted as bacterial colonies on eight 20-cm square nylon membranes (Amersham) using the Q-bot (Genetix) in the Genomics Technology Facility at Caltech as previously described (Rast et al., 2000).

Neural crest subtracted probe and hybridization

cDNA from 72 non-neural ectoderm/neural plate conjugates were subtracted with cDNA pooled from 66 pieces of non-neural ectoderm and 68 pieces of neural plate using the hydroxylapatite chromatography method of Rast et al. (Rast et al., 2000). Macroarray filters were hybridized and analyzed as described (Rast et al., 2000). Real-time quantitative PCR was performed using a GeneAmp 5700 Sequence Detection System and SYBR Green Dye Mix (ABI) according to the manufacturers instructions. Primer sequences were as follows: slug forward 5′-CCGTCTCCTCTACCCAATGA-3′, reverse 5′-ATGGCATGAGGGTCTGAAAG-3′; S17, (Liu and Jessell, 1998); GAPDH forward 5′-GGACACTTCAAGGGCACTGT-3′, reverse 5′-TCTCCATGGTGGTGAAGACA-3′; S15 forward 5′-ACAACGGCAAGACCTTCAAC-3′, reverse 5′-CCCAAAGCTCCC-GTTTATTT-3′; EF1α forward 5′-TGTGCGTGACATGAGACAGA-3′, reverse 5′-CCGTTCTTCCACCACTGATT-3′; α-tubulin forward 5′-ACGAGGCCATCTACGACATC-3′, reverse 5′-CACCAGGTTGG-TCTGGAACT-3′.

In situ hybridization

Since signal intensity on the array is not a direct measure of absolute expression levels in the embryo because of the subtraction step, clones were assessed by in situ hybridization. This serves as an independent confirmation of macroarray results and enrichment in neural crest. Antisense, digoxigenin-labeled RNA probes were prepared from one clone in each contig. The probes were hydrolyzed and in situ hybridization performed as described previously (Wilkinson, 1992) using pools of embryos ranging from the 4- to 16-somite stage. Embryos were scored visually by examining the embryos at a variety of angles to reduce optical effects, and by cutting with a scalpel to examine internal localization. Selected embryos were sectioned by cryostat (Bright) and photographed with a Zeiss Axioskop2 Plus to confirm localization scores. Of 97 clones examined, 14 with ubiquitous expression and no obvious differences in expression levels between tissues were discarded.

Gene Ontology Annotator

Unbiased analysis of raw sequence data was performed using the Gene Ontology Annotator interface (http://udgenome.ags.udel.edu/gofigure).

RESULTS

Neural crest cells arise within the embryonic ectoderm at the border of future epidermis (non-neural ectoderm) and future central nervous system (neural ectoderm). During the process of neurulation, neural tissue thickens and rolls, transforming from a flat, rectangular 'neural plate' into a cylindrical 'neural tube'. During this process, cells at the borders of the neural plate bend and fold. These 'neural folds', which contain neural crest precursors, ultimately close and fuse to form a tube, with premigratory neural crest residing in the dorsal neural tube. Shortly thereafter, neural crest cells emigrate from the neural tube and migrate extensively along defined pathways. In avian embryos, the process of neurulation begins first in the presumptive head region and proceeds gradually tail-ward. As a result, multiple stages of neural crest development are evident in a single embryo; for example, a 10-somite embryo contains migrating neural crest cells in the head, premigratory neural crest in the closed neural tube of the trunk, and newly induced neural crest in the neural folds of the open neural plate.

Production of neural crest-enriched cDNA

A pool of premigratory neural crest cells was created by dissecting and juxtaposing pieces of non-neural ectoderm and intermediate neural plate from the open neural plate region of 8- to 12-somite embryos (Fig. 1A). Tissue co-cultured as 'conjugates' was compared with 'isolates' of neural plate or ectoderm alone (Fig. 1B). After a 24 hour period, conjugates exhibit abundant expression of the neural crest marker *slug*, whereas pieces of either tissue cultured in isolation do not express *slug* (Fig. 1C) (Dickinson et al., 1995). *slug*-positive cells represent newly induced, premigratory neural crest; conjugates produce definitive migratory neural crest cells (*slug* negative) only after 48 hours in culture (Dickinson et al., 1995).

Minimally amplified, neural crest-enriched cDNA was generated by subtracting conjugate cDNA with cDNA from isolates. The advantage of this comparison is that all tissue and culture conditions are constant except for the inductive interaction, allowing for a very specific subtraction. The subtractive step removes common (non-neural ectoderm and intermediate neural plate) and 'housekeeping' genes, enriching for sequences differentially expressed as a consequence of neural crest induction. Use of a subtracted probe has been shown to increase the sensitivity of macroarray hybridization so that differentially expressed genes can be detected at expression levels as low as 5 copies per cell, whereas before subtraction, a 40 copy per cell detection limit is observed (Rast et al., 2000). Quantitative PCR indicated that the neural crest

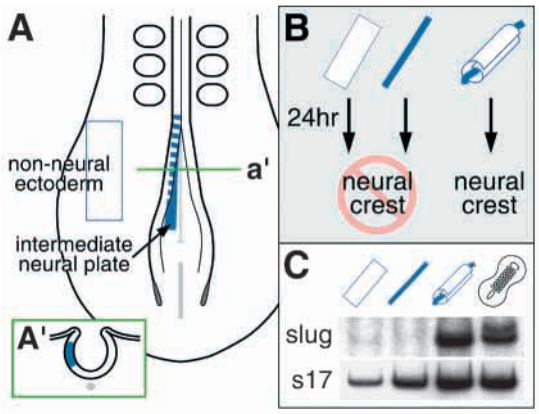

Fig. 1. Neural crest induction in vitro. (A) Non-neural ectoderm (open blue rectangle) and intermediate neural plate (solid blue line) were dissected from 8- to 12-somite chicken embryos (cross section of dissection shown in A′). (B) After 24 hours in culture, neural crest induction occurs only when neural plate and non-neural ectoderm are cultured in contact with one another (Dickinson et al., 1995; Selleck and Bronner-Fraser, 1995). (C) RT-PCR for the neural crest marker *slug*.

marker *slug* was enriched 10- to 26-fold relative to ubiquitously expressed genes such as *α-tubulin*, *EF1α*, *GAPDH*, and the ribosomal protein *s15*, confirming removal of common genes (data not shown).

Identification of genes upregulated following neural crest induction

An arrayed cDNA library containing 150,000 clones was prepared from a pool of 4- to 12-somite chick embryos, which includes the earliest events in neural crest development, from initial induction to the onset of migration. To retain the diversity of genes expressed and minimize non-specific loss of rare clones, we arrayed the unamplified, non-normalized library. EST studies in other organisms have demonstrated that gene expression during early development is complex, with many different genes expressed as opposed to the few, highly abundant transcripts observed in differentiated cells (Lee et al., 1999; Ko et al., 2000). We therefore expected complexity of gene expression to offset clone redundancy on the array, an assumption validated by the results.

To identify genes expressed as a consequence of neural crest induction, unsubtracted and subtracted conjugate cDNA were sequentially hybridized to the array. Spot intensities before and after subtraction were compared and 162 clones with the greatest fold increase in signal after subtraction were picked. 5′ sequence was obtained, assembled into contigs, and compared to the GenBank non-redundant database, identifying 97 different genes.

We then performed a secondary in situ hybridization screen to independently confirm the results of the macroarray screen. In situ hybridization patterns for all 97 genes were obtained using pools of embryos ranging from the 4- to 16-somite stage. Of these, 83 clones displayed strong expression in neural folds and/or migrating neural crest relative to neighboring tissues and were retained as differentially expressed clones (Table 1; see Fig. 2 for examples). In addition to serving as a screening tool, expression patterns in premigratory and migratory neural crest provided dynamic information about each clone over the course of neural crest development (Table 1).

Database sequence homology was used to assign a putative identity to each clone. With this information, we performed a literature-based functional scan to determine the known functions of each gene. As a secondary inspection of our data, we used the Gene Ontology Annotator as an unbiased bioinformatic analysis that provides a rapid means to obtain the potential functions of a gene from raw sequence data. This interface performs a BLAST search and links with public databases to obtain terms that describe molecular function, biological process and cellular components of a gene product. This combined analysis allowed the gene collection to be grouped into obvious functional categories (Table 1). Because many of these genes have well-defined functions in other systems, we can formulate testable hypothesis regarding their potential roles in the neural crest. This analysis revealed that many of our genes have previously been implicated in proliferation and migration events, consistent with the migratory, proliferative activity of neural crest cells. In addition, categories experimentally associated with neural crest development were also identified; for example, the rho pathway (Liu and Jessell, 1998) and extracellular matrix (Perris and Perissinotto, 2000). Taken together, the gene categories and temporal expression data create a picture of the changes taking place in premigratory neural crest cells as they prepare to leave the neural tube.

Neural crest cytoskeletal gene expression occurs in phases

Cytoskeletal components formed one of the largest gene categories we identified. Notably, these genes were expressed in phases. Intermediate filament genes (*cytokeratin 18* and *paranemin*, Fig. 2U), the proteins of which provide the structural integrity of cells, are characteristic of early premigratory neural crest, when cells are newly induced and stationary. In contrast, genes encoding elements of the actin cytoskeleton (for example *α-adducin* and *palladin*, Fig. 2B), which generate motile force during migration, are expressed in migrating neural crest. *palladin* is particularly interesting, as it preferentially localizes to growth cones and is required for neurite outgrowth (Boukhelifa et al., 2001), which is effectively a type of cell migration. While cytoskeletal alterations have been implicated in diverse cell migrations, including those of neural crest cells (reviewed by Duband et al., 1995), our results show for the first time that there is a sequential change in the type of cytoskeletal elements expressed following neural crest induction and leading up to migration.

The rho pathway in the neural crest

Components of the rho pathway formed another prominent gene category from our screen. This group included the rho effector protein MSE55 (Fig. 2I,W), as well as TIP-1, a PDZ domain-containing protein that interacts with the rho effector protein rhotekin to activate the serum response element (Reynaud et al., 2000). This finding is consistent with a previous demonstration that activation of the rho family of GTPases is required for delamination of neural crest cells from the neural tube (Liu and Jessell, 1998).

Table 1. Summary of functional categories and genes identified

Clone	Expression pattern pNC	mNC	SP		Notes
A. Chromatin					
Brahma-related group 1 (Brg1)	+	–	–		Central ATPase of human SWI/SNF, remodels nucelosomes
Adenosylhomocysteinase 1 or 2	+	+	–		Breaks down feedback inhibitor of transmethylation reactions
DNA (cytosine-5)-methyltransferase 3 beta	+	+	+/–		DNMT3b; repressive heterochromatin stabilization; ICF syndrome
Nuclear autoantigenic sperm protein (NASP)	–	+	–		Histone binding/transport protein, cell cycle regulated
Structure-specific recognition protein (SSRP1)	–	+	–		Unravels H2A/H2B histone dimers from nucleosome cores
B. Cytoskeleton					
Cytokeratin 18	+	–	+		Intermediate filament
Paranemin	+	–	+		Intermediate filament, forms co-polymers w/ vimentin or desmin
α-adducin	–	+	–	M	Spectrin /actin skeleton, rho phosphorylation promotes cell motility
Chaperonin TriC complex subunits 6a & 7	–	+	–		Molecular chaperone; required to fold actin and tubulin
Homogenin	–	+	–		Similar to gelsolin, which regulates actin polymerization
Palladin	–	+	+/–	M	Organizes actin cytoskeleton, critical for neurite outgrowth
C. ECM					
Collagen XVIII alpha I	–	+	–	EMP	Basement membrane collagen; C-terminal fragment is endostatin
Laminin α5	+	–	+/–	EM	Components of basement membranes of epithelia and endothelia;
Laminin γ1 precursor	–	+	–	EM	Laminin-10 (α5β1γ1) promotes endothelial cell migration
Procollagen alpha 1	–	+	–		Fibrillar collagen; extracellular matrix
D. Mitochondria					
Hexokinase 1, isoform HKI brain form	+	+	–		Glycolysis; glucose deficiency linked to birth defects
Aspartate aminotransferase E.C.2.6.1.1	–	+	–		Mitochondrial enzyme
ATP synthase, F1 complex, α & O subunit	–	+	–		Mitochondrial enzyme complex
Ubiquinol-cytochrome C reductase	–	+	–		Complex core protein 1; mitochondrial respiratory complex
E. Mitosis/cell cycle					
Condensation-related SMC-associated protein 1	+	+	+/–		CNAP1; subunit of condensin (chromosome condensation)
P57KIP2	+	+	–		CDK inhibitor, regulates proliferation and cell cycle exit
KIF4a/chromokinesin	+	+/–	+/–		Mitotic chromosome/non-synaptic membrane organelle transport
Proliferating cell nuclear antigen	–	+	–	P	DNA replication, repair, recombination
Pescadillo	–	+	+/–	P	Fish mutants have branchial arch defects; cell cycle progression
F. Nucleocytoplasmic export					
DEAD/H box protein 19 (Dbp5 homologue)	–	+	–		RNA helicase, nuclear pore protein, mRNA export
RanGTPase activating protein 1	–	+	–		Translocation of proteins into nucleus; spindle formation
Nopp140	–	+	+/–		Transport between nucleolus and Cajal bodies
G. Protein production/degradation					
Cathepsin D precursor	+	–	–		Lysosomal aspartic proteinase; abundant in brain
Alanyl-tRNA synthetase	+	+	–		Attaches alanine to tRNAs
150KD Oxygen-regulated precursor (ORP150)	–	+	–	E	ER chaperone, promotes VEGF processing/production
Chaperonin TriC complex subunits 6a & 7	–	+	–		Molecular chaperone; required to fold actin and tubulin
eIF3, subunit 8	–	+	–		Binds the 40s ribosome subunit; complexes with eIF4G
eIF4G	–	+	–		Multipurpose adaptor between mRNA and ribosomes
FTSJ-like methyltransferase	–	+	–		Heat-shock protein, 23s rRNA methylation, important for growth
Proteasome 26s regulatory subunit S2	–	+	–	P	Type 1 tumor necrosis factor receptor associated protein 2
similar to GCN1	–	+	+		GCN1/GCN20 complex in yeast, activate pathway for amino acid
GCN20/ATP-binding cassette transporter F2	–	+	+/–		Biosynthesis in amino acid starved cells
H. Receptors/downstream signaling					
KLG/protein tyrosine kinase-like 7 precursor	+	+	–		Receptor protein tyrosine kinase with unknown ligand
Neuropilin 2a1	+	+	+	EM	Receptor for semaphorin E, IV, IIIC, and F and VEGF145 and 165
BMP2-inducible kinase (BIKe)	–	+	–		Ser/thr kinase induced by BMP2 during osteoblast differentiation
Notch 1	–	+	+	EMP	Receptor for delta/serrate; lateral inhibition, neuronal vs. glial fate
PI3 Kinase Regulatory β Subunit	–	+	+/–	M	Binds activated protein-tyr kinases, PI3 catalytic subunit adaptor
I. Rho pathway					
MSE55	+	+	+		cdc42 effector protein, actin reorganization/pseudopodia formation
Rho GTPase activating protein (rhoGAP)	–	+	–	M	Stimulates the GTPase activity of rho, converting it to inactive state
Tax-interacting protein 1 (TIP-1)	–	+	–	M	PDZ domain interacts with rhotekin (binds activated rhoA)
PI3 Kinase Regulatory β Subunit	–	+	+/–	M	PI3K-independent pathway activates cdc42/cell migration
J. RNA binding proteins					
Dyskerin	+	+	+/–	P	Binds H/ACA sequence in snRNAs and telomere RNA
PAI RNA-binding protein	–	+	–	EM	Binds PAI-1 3′UTR, signals degredation/promotes ECM proteolysis
Pigpen/FUS	–	+	–	EMP	Upregulated in actively migrating and dividing endothelial cells
Pumilio 1	–	+	–	P	Binds nanos response elements, inhibits translation
K. Secreted signals/signal production					
Slit3	+	–	+/–	M	Secreted ligand for robo receptors, repellent, axon guidance
Cys-rich FGF receptor (CFR) associated protein	+	+	–		p70; ER and golgi localization; regulates at least FGF3 secretion
Thimet oligopeptidase (endopeptidase 24.15)	+	+	+/–		Extracellular peptide (neuropeptide) signal processing
Aldehyde dehydrogenase 9 A1	+	+/–	–		γ-aminobutyraldehyde oxidation, alternative for GABA synthesis
Collagen XVIII alpha I	–	+	–	EMP	Basement membrane collagen; C-terminal fragment is endostatin

Table 1. Continued

Clone	Expression pattern pNC	mNC	SP		Notes
L. Transcription factors					
SMART/HDAC1 assoc. repressor (SHARP)	+	+	–		Nuclear receptor corepressor, homology to Msx2 interacting protein
Slug	++	++	++	M	Zn-finger transcription factor
E12	–	+	–		Homo/heterodimerizes with other bHLH transcription factors
M. Transcription-coupled processes					
UV DNA damage-binding protein (DDB1)	+	+/–	+/–		Nucleotide excision repair; binds transcription factor E2F1, STAGA
Pre-mRNA splicing factor similar to PRP16	+	+	–		ATP-dependent RNA helicase; unwind RNA for intron recognition
Spliceosome-associated protein 130 (SAP130)	+	+	–		mRNA splicing and prespliceosome formation; binds STAGA
N. Transporters					
Monocarboxylate transporter 1	–	+	–		Uptake/removal of lactate, pyruvate; coupled to glutamate uptake
RIKEN 1810073N04/amino acid transporter	–	+	–		Sequence annotation
Cationic amino acid transporter 3, system y+	–	+	+/–		Transports cationic L-amino acids; L-arg needed for NO synthesis
O. Miscellaneous					
3-α-hydroxysteroid dehydrogenase-like protein	+	–	–		Homologous to members of aldo-keto reductase superfamily
Huntington-interacting protein 1	+	–	+/–		Clathrin-mediated endocytosis; actin binding; activates caspase-8
D52-like 2 tumor protein	–	+	–	P	Coiled-coil; cell proliferation, Ca2+-mediated signal transduction
SHIPPO 1	–	+	–		Sperm tail protein
P. Unknown/EST/no homology					
Glycine-rich protein/PAC clone RP5-1121A15	+	–	–		Sequence annotation
no significant homology (249C21)	+	–	+/–		
no significant homology (17N12)	+	–	+		Very specific for neural plate/early neural tube
no significant homology (312B4)	++	–	++		Fig. 3D
hypothetical protein	+	+	+/–		Human KIAA0147
Glutamine-rich protein	+	+	–		Sequence annotation
hypothetical proteins	+	+	–		Human CUA001, MGC10526
hypothetical proteins	–	+	–		Human FLJ12879, KIAA0174, KIAA1093, Drosophila CG3173
no significant homology	–	+	–		Screen clones 43L18, 30G1
hypothetical proteins	–	+	+/–		Human KIAA1036, XP_089437

Clones were organized into categories based upon functions assigned by sequence homology. Degree of homology is indicated by colored spots (●=BLAST bit score > 200; ●=bit score 80-200; ●=bit score 50-80; ●=bit score 40-50; ●=bit score < 40). Clones double-listed in two categories are italicized. In situ hybridization patterns in pools of 4- to 16-somite embryos were scored for expression in neural folds (premigratory neural crest; pNC) and cranial migratory neural crest (mNC). In vitro induced neural crest and macroarray results are assumed to correspond to an intermediate timepoint in neural crest development. General specificity of expression (SP) was also scored (–, expression in many tissues at varying levels; +/–, expression broad, but discrete patterns obvious; +, expression limited to only a few tissues; ++, expression highly specific). Clone homologies that have previously been linked to endothelial cell development ("E"), migration ("M"), or proliferation ("P") are indicated. In situ hybridization patterns and EST sequences for all clones will be available on the GEISHA (Gallus gallus EST and in situ hybridization) Database at http://geisha.biosci.arizona.edu/.

Neural crest expression of proliferation genes

Several genes identified in our screen were related to the cell cycle and mitosis (for example, *pescadillo* and *chromokinesin*, Fig. 2E), indicating that premigratory neural crest cells have proliferative capacity surprisingly early in their development. Neural crest cells divide many times as they leave the neural tube (Bronner-Fraser and Fraser, 1988). *pescadillo*, which was originally isolated as a fish mutant with branchial arch defects (a neural crest derivative) (Allende et al., 1996), is required for cell cycle progression and is abnormally expressed in transformed cells (Kinoshita et al., 2001). The mitosis/cell cycle function category is confirmed and expanded by the number of genes that were identified across categories that have previously been linked to proliferation and tumorogensis in other systems.

Intercellular signaling in the neural crest

We have identified genes encoding a number of receptors (such as *klg*, Fig. 2H), secreted factors (such as *slit3*), and proteins that create secreted signals (such as *thimet oligopeptidase*, Fig. 2K) that have not previously been linked to neural crest development. For example, the secreted factor slit3 is a chemorepellant that binds to robo receptors on axons during axon guidance (reviewed by Guthrie, 2001), although its expression and role in the formation of migratory neural crest cells was previously unrecognized. Meanwhile, BIKe is a serine/threonine kinase upregulated as a downstream response to BMP signaling (Kearns et al., 2001). Confirming the specificity of our screen, we isolated the receptor Notch1, which is expressed in migratory neural crest cells (Wakamatsu et al., 2000) and cell-autonomously required in premigratory neural crest for *slug* expression (Endo et al., 2002).

Transcripts for neuropilin 2a1, which is a receptor for chemorepellent semaphorins and vascular endothelial growth factor (VEGF) (Neufeld et al., 2002), was a particularly specific product from our screen. We found that *neuropilin 2a1* is expressed in premigratory and migratory neural crest, and in the somites (Fig. 3A-C‴). The elevating cranial neural folds prominently express *neuropilin 2a1* (Fig. 3A), and this expression appears intensified on the newly closed cranial neural tube just prior to neural crest migration (Fig. 3B,B′). A similar pattern is noted in the trunk neural folds (Fig. 3B,C). After emigration, *neuropilin 2a1* transcripts are maintained on migrating cranial neural crest cells (Fig. 3C,C′). *neuropilin 2a1* expression was also noted in the somites, with particularly high levels in the dorsomedial dermomyotome (Fig. 3C″).

Neural crest extracellular matrix gene expression

Our results indicate that changes in *laminin α5* (Fig. 2C,V) and *γ1* expression levels, never before described in neural crest cells, precede neural crest migration. Furthermore, the degradation of extracellular matrix (ECM) by plasminogen activator prior to neural crest migration (Valinsky and LeDouarin, 1985) appears to be regulated by expression of *plasminogen activator inhibitor RNA binding protein* in the neural crest. The expression of ECM molecules by premigratory and migratory neural crest is consistent with the proposal that interactions between neural crest cells and the ECM play important roles in initiation of neural crest migration and their guidance along stereotypic pathways (Perris and Perissinotto, 2000).

Neural crest genes involved in gene expression and metabolism

As cells recently induced to follow a new developmental pathway, presumptive neural crest cells appeared to be actively changing the genes they express (chromatin modification, transcription, RNA binding proteins, and mRNA export) and making new proteins. Interestingly, genes encoding transcriptional machinery components (chromatin modifiers such as *Brg1*, transcription factors such as *SHARP*, and transcription-coupled processes such as *DDB1*, Fig. 2M,S) appeared to be upregulated in newly formed neural crest (i.e. detected in neural folds), while those involved in protein production (such as *GCN1* and *GCN20*, Fig. 2G) were generally more abundant later (in migratory neural crest). This switch may be involved in regulating the migratory capacity of neural crest precursors. Neural crest cells also express transcripts for RNA-binding proteins that regulate RNA stability and translation as well as numerous genes associated with metabolic activity (mitochondrial gene expression, transporters) as they become migratory.

We identified surprisingly few transcription factors, with the exception of expected factors slug and E12 (Fig. 2L), which interacts with Id2, a factor that regulates neural crest cell identity (Martinsen and Bronner-Fraser, 1998), in a yeast two-hybrid assay (Y. Kee and M. B. F., unpublished observation). This is probably because we initially picked only a subset of enriched clones, and transcription factors that are expressed at

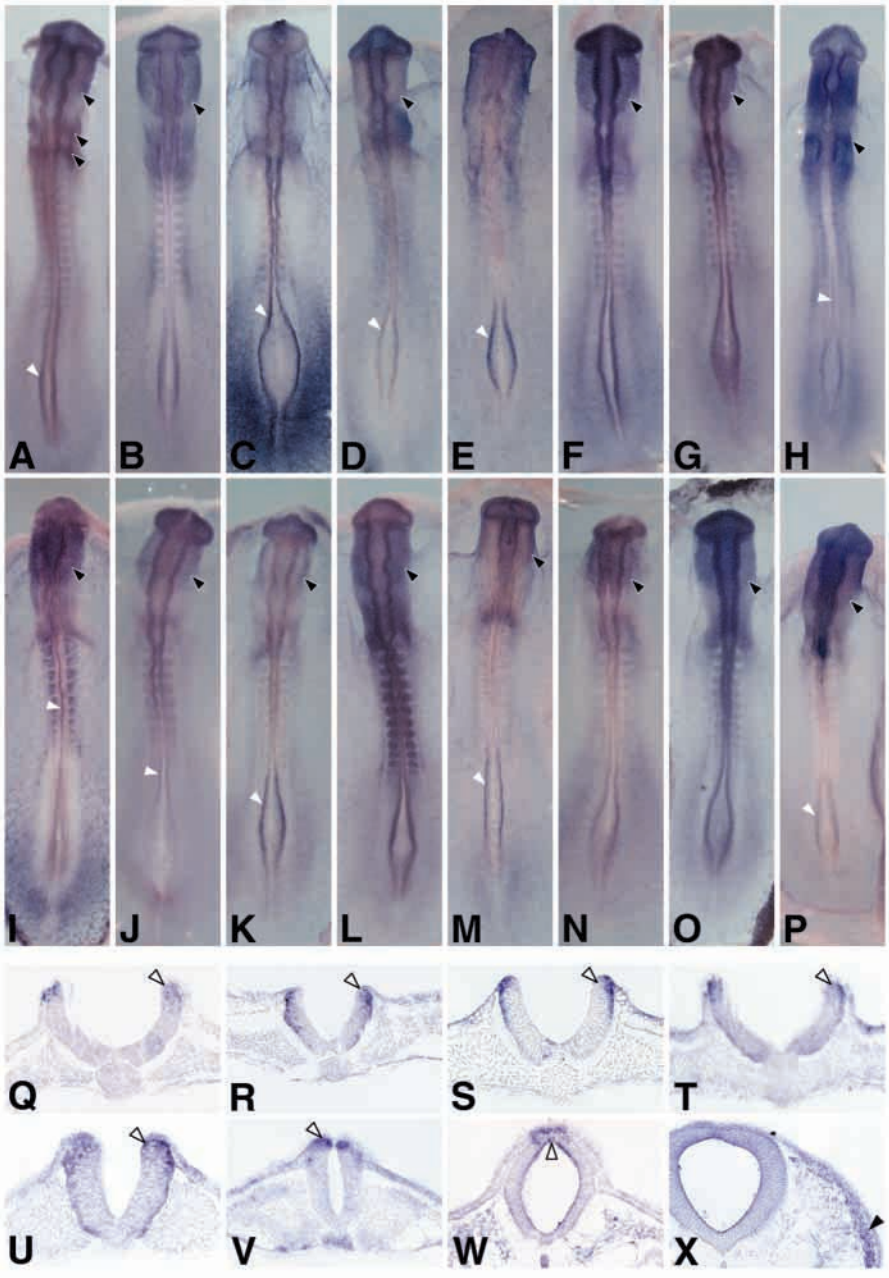

Fig. 2. Neural crest gene expression. In situ hybridization was performed to confirm localization in premigratory and migratory neural crest. Owing to the caudal to rostral gradient in avian development, these stage 10 embryos are illustrative of multiple events in neural crest development, including induction (open neural plate), premigratory (dorsal neural tube) and migratory (cranial) neural crest. Gene expression in the embryos shown is representative of that observed in the pool of 4- to 16-somite embryos scored for each clone. White arrowheads indicate gene expression in neural folds, black arrowheads mark gene expression in migrating neural crest. (A-P) Dorsal view, anterior up. Labels A-P correspond to categories in Table 1. Expression of: (A) *adenosylhomocysteinase 1 or 2*; (B) *palladin*; (C) *laminin α5*; (D) *hexokinase 1*, isoform HKI; (E) *KIF4a/chromokinesin*; (F) *Nopp140*; (G) *GCN20/ATP-binding cassette transporter F2*; (H) *tyrosine kinase receptor KLG*; (I) *MSE55*; (J) *dyskerin*; (K) *thimet oligopeptidase*; (L) *E12*; (M) *UV DNA damage binding protein 1*; (N) *cationic amino acid transporter 3*; (O) *D52-like 2*; (P) *human KIAA0147*. (Q-X) Transverse sections, dorsal up. (Q) *cathepsin D*; (R) *alanyl tRNA synthetase*; (S) *UV DNA damage binding protein*; (T) *CFR-associated protein*; (U) *paranemin*; (V) *laminin α5*; (W) *MSE55*; (X) *Nopp140*.

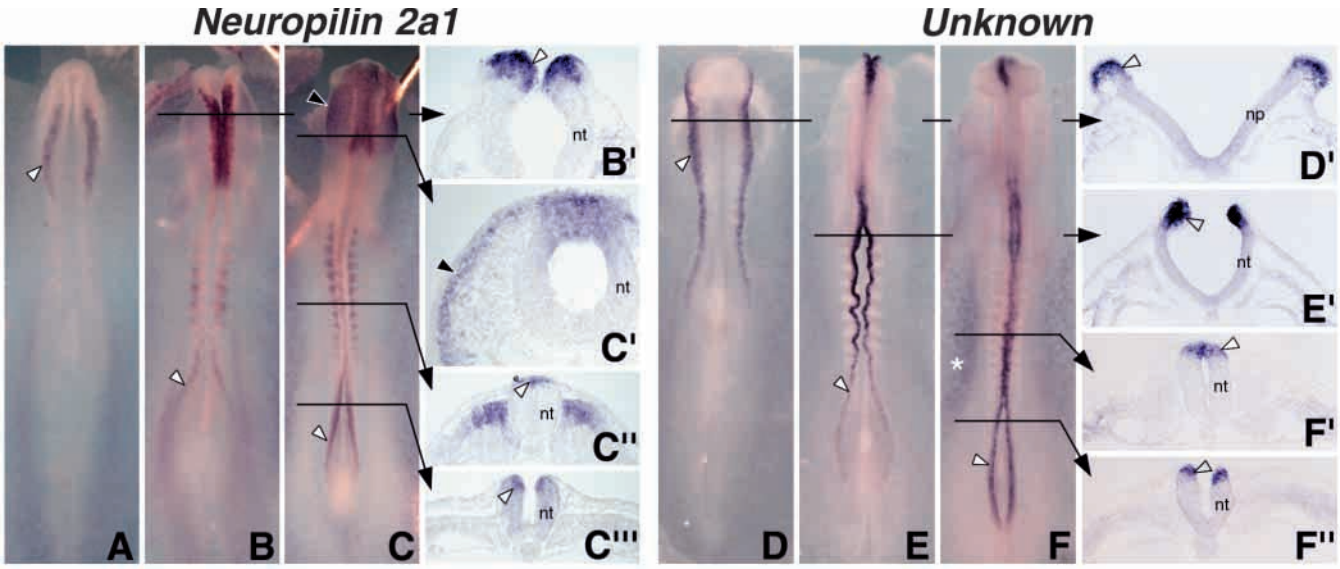

Fig. 3. Neural crest expression of *neuropilin 2a1* and an unknown gene. (A-C‴), *Neuropilin 2a1* expression is specific to cranial and trunk neural folds and dorsal neural tube (premigratory neural crest; white arrowheads), migratory cranial neural crest (black arrowheads) and somites. (D-F″) A gene with no homology to any sequences in the GenBank nr database is highly specific to neural folds from their earliest appearance and down-regulated at the time of neural crest migration. Expression is also apparent in splanchnic mesoderm (asterisk). (A,D) 4-somite embryo; (B,E) 7-somite embryo; (C,F) 12-somite embryo. Dorsal view, anterior up. (B′-C‴,D′-F′) Transverse sections (dorsal up) through embryos pictured in A-F at the levels indicated by lines. nt, neural tube; np, neural plate.

very low levels may be more difficult to detect. Alternatively, transcription factors could be temporally up- or downstream of genes expressed at the timepoint examined. For example, *FoxD3* is known to be involved in neural crest formation, but first expressed in avians only shortly before migration takes place (Kos et al., 2001).

Novel molecules expressed in neural crest

In total, 16 of 83 genes isolated are without an assigned function or are potentially novel based on lack of known homologues. For example, the gene with the most specific expression pattern obtained from this screen was one with no known homology in the database. This gene is exclusively expressed at the interface of non-neural ectoderm and neural plate as soon as these tissues are distinct (Fig. 3D-F″). Later, it is expressed in the dorsal neural tube (Fig. 3F′) and the splanchnic mesoderm (Fig. 3F), where endothelial cells arise. It resembles *slug* expression in its specificity; however, it is expressed earlier than *slug* and, unlike *slug*, is down-regulated when neural crest cells migrate (Fig. 3E,F). This presents the intriguing possibility that it plays a role in regulating delamination of neural crest cells from the neural folds. Importantly, it provides us with a novel, highly specific, and very early marker of cells with the potential to form neural crest.

DISCUSSION

In this study, we have taken advantage of our ability to recapitulate neural crest induction under defined conditions to screen a chick macroarrayed cDNA library. The resulting collection of 83 genes establishes new markers and identifies novel candidate regulators of neural crest development, prompting comparisons with other migratory cell types. A secondary in situ hybridization screen not only confirmed the specificity of our clones, but also provided temporal expression data about each clone over the course of neural crest development. This allows us to synthesize a model for the events that result in the migration of a subset of cells from the neural tube. The results permit us for the first time to analyze neural crest induction as a process rather than as a singular event.

A wealth of new markers for an ill-defined cell type

Despite its critical role in the formation of many different lineages, the molecular aspects of early neural crest development are poorly understood. This is due to a combination of factors, including the fact that the premigratory neural crest is not a segregated cell type that can be purified. The neural crest has been best studied in avians because of their ease of experimental manipulation; however, the avian system is relatively intractable to molecular analysis owing to the lack of genomic information and the current inability to consistently perform loss-of-function experiments. In addition, there has been a dearth of specific markers of neural crest precursors. By screening our own macroarrayed library, we have overcome some of these molecular roadblocks to identify a broad range of new markers for premigratory and migratory neural crest. In addition to known genes, nearly 20% of the genes identified in our screen have no known homologues in the database and likely represent novel gene products. Some of these are expressed earlier than *slug*, which prior to this study was the earliest known neural crest marker in birds. These markers will facilitate more comprehensive future analyses of neural crest development.

Similarities between neural crest and endothelial cell development

Eight of the genes we isolated from newly formed neural crest

cells have previously been implicated in endothelial cell development, including those of factors involved in VEGF production and signaling (such as *ORP150* or *neuropilin 2a1*) as well as proteins important for endothelial cell migration (such as *laminin α5* and *γ1*). This suggests that endothelial cells and neural crest cells may employ similar developmental programs.

Several points suggest that this correlation is significant and not due to random chance. First, ORP150 is an ER chaperone whose function is required for VEGF secretion (Ozawa et al., 2001), while neuropilin 2 is an isoform-specific VEGF receptor (Gluzman-Poltorak et al., 2000). Thus, we have identified both a factor that produces, and a receptor that responds, to VEGF. Second, although it has never been emphasized, VEGF is expressed in tissues that could affect neural crest development, including the headfolds, neural tube and cephalic mesenchyme of E8.5-9.0 mouse embryos (Miquerol et al., 1999). Furthermore, VEGF mutant embryos exhibit poorly developed and unsegmented branchial arches, a neural crest derivative (Ferrara et al., 1996). Third, only laminin-10 and laminin-11 contain both the laminin α5 and γ1 chains (Colognato and Yurchenco, 2000). Laminin-10/11 mediates adhesion and promotes migration of endothelial cells (Doi et al., 2002) and a broad spectrum of carcinoma cells (see below) (Kikkawa et al., 1998; Tani et al., 1999; Pouliot et al., 2001). Furthermore, these effects were shown to be mediated by α3β1 and α6β1 integrins, and neural crest expression of β1 integrin is required for their adherence to laminin (Lallier and Bronner-Fraser, 1991; Lallier and Bronner-Fraser, 1993). Fourth, the most specific product of our screen (Fig. 3D-F″) was expressed only in the neural folds and blood islands, which are where neural crest and endothelial cell precursors, respectively, arise. Finally, although not identified in this screen, endothelin signaling, which stimulates endothelial cell migration and proliferation (Wren et al., 1993; Morbidelli et al., 1995), is required for different aspects of neural crest development (reviewed by Yanagisawa et al., 1998).

Although a comparison of neural crest and endothelial cells is novel, it is logical when considered mechanistically, as both endothelial and neural crest cells migrate and invade tissues as they perform their embryonic functions. Similarities between vasculogenesis and axon outgrowth have been noted (Shima and Mailhos, 2000). Furthermore, Eph/ephrin signaling is involved in segmental organization of trunk neural crest migration, and in determination of arterial versus venous identity in the vasculature (reviewed by Shima and Mailhos, 2000). Our results indicate that early events during neural crest cell specification and initiation of migration share signaling pathways in common with endothelial cells, and that experimental comparisons between these two important cell types will be informative.

Similarities between neural crest cells and metastatic cancer cells

Phenotypically, neural crest cells and metastatic cancer cells are similarly motile and invasive and can follow the same migratory pathways (Erickson et al., 1980). The development of neural crest cells and malignant cells is believed to be related because of the sheer number of different cancers arising from neural crest lineages (reviewed by Hall and Hörstadius, 1988). Our results reveal that they are also comparable in the types of genes they express. When gene expression in metastatic melanomas is profiled, notable categories of gene expression include extracellular matrix molecules, proteins that regulate the actin cytoskeleton, and the rho GTPase rhoC (Clark et al., 2000). These same categories were also prominent products of our screen, indicating that the comparison between migratory neural crest cells and metastatic cancer cells is functional as well. In support of this idea, inhibition of rho GTPase activity can prevent both neural crest cell delamination (Liu and Jessell, 1998) and melanoma metastasis (Clark et al., 2000).

Post-transcriptional and post-translational regulation of neural crest cell properties

Our screen has revealed that the cytoskeletal components identified are expressed in phases, with intermediate filaments expressed in premigratory neural crest, and actin elements expressed in migratory neural crest. This phased cytoskeletal gene expression has not been realized in the past, and has interesting implications for the mechanism of neural crest migration. Enrichment of actin cytoskeletal elements in the premigratory, subtracted cDNA population indicates that presumptive neural crest cells in the neural folds alter their structural make-up in preparation for migration before migration actually takes place. However, not all cells in the neural folds will migrate (Bronner-Fraser and Fraser, 1988; Selleck and Bronner-Fraser, 1995), even if they express *slug* (Linker et al., 2000). Taken together, these apparently conflicting observations suggest a logical mechanism in which cells in the neural folds develop migratory potential by expressing the cytoskeletal machinery needed for migration, and that this potential is activated to initiate neural crest migration in a subset of cells through reorganization of existing structural elements.

By analogy with other migratory cell types (Ridley, 2001), the most likely mechanism for regulating such a cytoskeletal reorganization at the onset of neural crest migration is via post-translational modification by a rho pathway signal. Up-regulation of rho pathway genes following neural crest induction (Table 1) and the requirement for rho activity during neural crest emigration (Liu and Jessell, 1998), support this possibility. For example, α-adducin, which stimulates migration when phosphorylated by rho-associated kinase (Fukata et al., 1999), was enriched in premigratory neural crest. Cumulatively, these data suggest that activation of the rho pathway in a subset of cells in the neural folds causes post-translational cytoskeletal rearrangements that result in the migration of those cells from the neural tube. Post-translational regulation of migration is employed during other examples of cell migration events (reviewed by Ridley, 2001) and is also the simplest explanation for the collection of genes we have isolated from premigratory neural crest cells.

The identification of genes encoding RNA-binding proteins in our screen also presents the possibility that neural crest cell identity is regulated post-transcriptionally. Interestingly, 3 of the 4 RNA-binding proteins in our collection have been previously implicated in stem cell maintenance: pumilio controls germline stem cells in *C. elegans* and *Drosophila* (Crittenden et al., 2002), pigpen expression correlates with undifferentiated, proliferative endothelial cells (Alliegro and Alliegro, 1996; Alliegro, 2001), and dyskerin is a component of telomerase that appears to be essential for stem cell renewal

in a variety of tissues including the basal layer of the epidermis (reviewed by Marcinian et al., 2000). Since the neural crest is a multipotent stem cell population, these proteins are obvious candidates for post-transcriptional maintenance of the undifferentiated, stem cell state of neural crest cells as well.

The fourth gene encoding an RNA binding protein, *PAI RNA-binding protein*, also has interesting implications for post-transcriptional regulation of neural crest migration. Plasminogen activator (PA) is a serine protease that is expressed on migrating neural crest and degrades ECM for migration (Valinsky and LeDouarin, 1985). PA activity is regulated by plasminogen activator inhibitor (PAI), whose levels are in turn regulated post-transcriptionally by PAI RNA-binding protein (Heaton et al., 2001). The isolation of *PAI RNA-binding protein* suggests that PA activity is revealed shortly before migration through post-transcriptional regulation of PAI in the neural crest.

Selecting which cells in the neural folds will migrate

If neural crest cell properties are regulated post-transcriptionally and/or post-translationally, what provides the signal that selects certain cells in the neural folds for this regulation? Among the possible events, based on the genes we have identified in combination with data in the literature regarding neural crest development and other cell migration events, are: (1) that extracellular signals may stimulate migration of a subpopulation of cells; and/or (2) that asymmetric cell division may give rise to one daughter that leaves the neural tube and another that remains, perhaps as a stem cell. Of course, it is also entirely possible that cells with migratory potential within the neural folds are selected to migrate by a stochastic mechanism. Even if stochastic mechanisms are the primary determinant of whether a neuroepithelial cell delaminates to become a neural crest cell, intrinsic and extrinsic factors may provide bias that alters the probability of fate decisions in a particular direction.

We identified a number of genes encoding receptors and secreted molecules that are excellent candidates to send and receive signals instructing or maintaining premigratory neural crest cell specification, and/or stimulating migration. For example, *neuropilin 2a1* expression is highly specific to premigratory and migratory neural crest. Premigratory neural crest expression suggests that neuropilin 2a1 could be involved in establishing the premigratory neural crest pool, and is intriguing since neuropilins are typically associated with guidance and migration rather than cell-type specification. Expression in migrating neural crest cells suggests that the recentor neuropilin 2a1 could regulate neural crest migration or the response to migrational cues in the environment. Although no defects in neural crest development have been noted in *neuropilin 2* mutant mice (Chen et al., 2000; Giger et al., 2000), in *neuropilin 1* mutants, neural crest cells fail to reach their proper destinations and coalesce as ganglia even though early neural crest migration through the somites appears normal (Kawasaki et al., 2002). Like *neuropilin 2*, *neuropilin 1* is expressed on migrating neural crest cells from hindbrain and trunk levels (cranial neural crest was not examined) (Eickholt et al., 1999). Thus it is possible that neuropilin 1 and neuropilin 2 serve redundant roles in early neural crest formation and migration.

Other genes associated with signaling cascades were also noted in our screen. KLG is a receptor protein tyrosine kinase, although its function and ligand have not been determined. Inside the cell, BMP2-inducible kinase could modulate protein activity in presumptive neural crest cells in response to BMP signaling, which has been implicated in neural crest specification (Liem et al., 1995) and which is required for neural crest cell delamination from the neural tube (Sela-Donenfeld and Kalchiem, 1999). In support of the possibility that extracellular signals stimulate migration, there are active changes in the expression of adhesion molecules and alterations to the cytoskeleton associated with neural crest cell delamination (reviewed by Duband et al., 1995). Furthermore, epithelial-mesenchymal transitions are generally described as a process requiring external stimuli for their initiation (Boyer et al., 2000).

Asymmetric cell division is another attractive mechanism to select certain cells in the neural folds for migration. If cells in the dorsal neural tube divided asymmetrically, such that one cell lost attachment to the basement membrane, a migratory neural crest cell would result. Thus, a signal that stimulates proliferation could also be a signal to migrate. This mechanism has been postulated in the past (Erickson and Reedy, 1998), and is enticing given the number of proliferation-related molecules we have identified. The recent demonstration that neural crest cells delaminate from the neural tube specifically at the G_1/S transition of the cell cycle lends further support to a role for cell division in the selection of neural crest precursors for migration (Burstyn-Cohen and Kalcheim, 2002). Intriguingly, the snail family of proteins, which in vertebrates includes the neural crest marker slug, is required to regulate asymmetric cell division in *Drosophila* neuroblasts (Ashraf and Ip, 2001; Cai et al., 2001). Whether this is an evolutionarily conserved function of snail/slug remains to be determined.

A model for early neural crest development

Based upon the identities and temporal expression patterns of genes up-regulated 24 hours after neural crest induction, coupled with the knowledge that not all cells in the neural folds will become migratory (Bronner-Fraser and Fraser, 1988; Selleck and Bronner-Fraser, 1995), we hypothesize a sequential activation of migratory potential following neural crest induction (Fig. 4). First, after induction, precursor cells in the dorsal neural tube change their state by activating intermediate filaments, cell cycle components, and receptors/ligands and by increasing their transcriptional machinery. This begins the process of becoming a neural crest cell. Second, expression of genes associated with migration (e.g. changes in the actin cytoskeleton, rho targets, and extracellular matrix) may establish migratory potential. Finally, we postulate that a signal to migrate mobilizes migratory potential in a subset of cells through post-transcriptional or post-translational regulation by rho activation, downstream signaling events, and asymmetric cell division, ultimately leading to delamination of neural crest cells (Fig. 4). Other cells remain in the neural folds and become dorsal neural tube, never accessing this migratory potential.

This model best explains our collection of genes and their temporal expression patterns, and indicates a mechanism to regulate neural crest migration that is very different from those presumed in the past. Instead of postulating a gene(s) that specifically marks those cells that will migrate, our model is

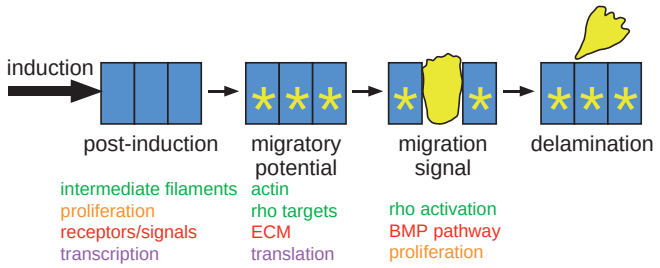

Fig. 4. Premigratory to migratory neural crest: sequential activation of migratory potential. The array of genes expressed 24 hours after neural crest induction, coupled with temporal analysis of expression patterns throughout neural crest development, make it possible to assemble a hypothesis for acquisition of migratory capacity. Following initial induction, cells in the neural folds begin the process of becoming neural crest cells (blue rectangles) by expressing intermediate filaments, proliferation factors, receptors and secreted signals, and increased transcriptional machinery (including known transcription factors such as slug). This is followed by the acquisition of migratory potential (yellow asterisks) with expression of components of the actin cytoskeleton, rho targets, extracellular matrix, and translational machinery. The signal to migrate mobilizes this migratory potential (yellow cell) through the activation of rho targets (Liu and Jessell, 1998) as a consequence of signaling through the BMP pathway (Sela-Donenfeld and Kalchiem, 1999) or asymmetric cell division (Erickson and Reedy, 1998). This results in the delamination of some cells from the neural folds. Cells that remain in the neural folds never access their migratory potential and become dorsal neural tube. Green: cytoskeleton; orange: proliferation; red: cell surface/signaling; purple: gene expression.

consistent with embryological observations of neural crest migration and with the events that take place in other migrating cell types. Our hypothesis is supported by the fact that no genes from this screen, or in the literature, exhibit a 'salt and pepper' distribution pattern within the neural folds, suggesting that there is no determined subpopulation within the neural tube with uniquely neural crest properties. Furthermore, there is ample evidence from cell lineage studies in avian and amphibian embryos that there is a shared neural tube/neural crest precursor and that the progeny of these precursors can mix even across the midline (Bronner-Fraser and Fraser, 1988; Collazo et al., 1993).

In summary, we provide a genomic analysis of the consequences of neural crest induction, creating a molecular profile of a cell type that cannot be purified or identified. Importantly, this analysis suggests previously unrecognized similarities with other migratory cells such as endothelial cells, and identifies new markers and new candidate regulatory molecules of neural crest development. Rather than examining the role of a single gene, we describe for the first time the consequences of an embryonic inductive event at a genomic level. This collection of genes, when incorporated with existing knowledge of neural crest development, suggests novel mechanisms for regulating the migration of a subset of cells from the neural folds, changing the way we view the process of neural crest migration. This approach has created testable hypotheses that open the door for future work on the developmental function of both known and novel molecules.

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