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TECHNIQUES

Use of Fluorescent Dextran Conjugates as a Long-Term Marker of Osteogenic Neural Crest in Frogs

Joshua B. Gross* and James Hanken

The neural crest is a population of multipotent stem cells unique to vertebrates. In the head, cranial neural crest (CNC) cells make an assortment of differentiated cell types and tissues, including neurons, melanocytes, cartilage, and bone. The earliest understanding of the developmental potentiality of CNC cells came from classic studies using amphibian embryos. Fate maps generated from these studies have been largely validated in recent years. However, a fate map for the most late-developing structures in amphibians, and especially anurans (frogs), has never been produced. One such tissue type, skull bone, has been among the most difficult tissues to study due to the long time required for its development during anuran metamorphosis, which in some species may not occur until several months, or even years, after hatching. We report a relatively simple technique for studying this elusive population of neural crest-derived osteogenic (bone-forming) cells in *Xenopus laevis* by using fluorescently labeled dextran conjugates. *Developmental Dynamics 230:100–106, 2004.* © 2004 Wiley-Liss, Inc.

Key words: *Xenopus*; amphibian; cranial neural crest; fate map; lineage marker

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INTRODUCTION

Fate maps are critical to the science of embryology because they assess the capacity of embryonic cells to develop into specific tissues later in development (Clarke and Tickle, 1999). They generally are the first step toward defining the molecular and morphological underpinnings of a variety of developmental processes (Le Douarin and Kalcheim, 1999). In the context of neural crest biology, an amphibian fate map would enable a better understanding of the evolution of cellular contributions of cranial neural crest (CNC) to the vertebrate head. Yet, despite the fact that neural crest has long been attributed a direct role in cranial bone development in amphibians (de Beer, 1947; Andres, 1949; Wagner, 1959), a fate map depicting neural crest contributions to the bony skull in these tetrapods has never been produced. Larval development times that typically last several months or longer, combined with inadequate long-term labeling techniques (Table 1), have retarded efforts to map these and other late neural crest-derived tissues, which first form at or after metamorphosis.

We present a method for labeling early amphibian tissues with fluorescent dextran as a long-term marker for neural crest-derived cells. Dextran is a nontoxic, water-soluble polysaccharide produced by *Leuconostoc* bacteria, which is commercially available conjugated to a variety of fluorophores (Krotoski et al., 1988; Delarue et al., 1997). Fluorescent dextrans have been used over shorter developmental periods because they can be targeted to a particular cell or tissue very early in development (Cox and Hemmati-Brivanlou, 1995; Doniach and Musci, 1995). We find this technique superior to other methods of amphibian fate-mapping, because labeled cells can be tracked immediately in living (as well as fixed) tissues due to the fluorescent tag associated with the injected dextran (Selleck and Stern, 1991). Moreover, unlike many other fluorescent labels, these conjugates are stable over very long developmental periods (e.g., up to several months), and they can be

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Museum of Comparative Zoology and Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, Massachusetts
*Correspondence to: Joshua B. Gross, Museum of Comparative Zoology and Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138. E-mail: jgross@fas.harvard.edu

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TABLE 1. Summary of Methods Used in Fate Mapping the Cranial and Trunk Neural Crest in Amphibians[a]

Method of cell tracking	Cell types and tissues identified	Advantages	Disadvantages	References
Histological sections	Origin of visceral cartilages	Relatively simple technique	Nonmanipulative approach makes monitoring cell movements difficult	Landacre, 1921
Focal ablations of the cranial neural ridge	Pigment cells, muscle connective tissue, chondrocranium	Early method that greatly informed our understanding of the vast contribution of the neural crest to the vertebrate head	Results can be difficult to interpret since neighboring cell populations may replace (or rescue) ablated cells; damage from ablation can limit survival of embryos	Stone, 1926
Chromatic dyes, e.g., Nile blue sulphate	Cranial nerve ganglia, early migratory streams of the neural crest	Allows for live-cell tracking early in development using light microscopy; led to the first fate map of the early migratory streams of the neural crest	Dilution of label; dyes may unintentionally spread to surrounding cells	Hörstadius, 1950
Heterospecific transplantation	Sympathetic neuronal cells, early migratory streams of the neural crest	Intrinsic cellular marker allows for the identification of all cells derived from initial transplanted tissue	Species may differ in intrinsic growth rates, derivatives, etc.; improperly grafted neural crest cells may replace tissues normally derived from the mesoderm	Wagner, 1949; Sadaghiani and Thiébaud, 1987; Krotoski et al., 1988; Schneider, 1999
Tritiated thymidine labeling	Larval cranial skeleton in *Pleurodeles waltlii*	Reliable intrinsic marker that will not spread to surrounding cells or tissues	Radiological marker dilutes over time and can be toxic in high concentrations	Chibon, 1967
Carbocyanin dyes, e.g., DiI, DiO	CNC contributions to cranial musculature	Fluorescence allows for tracking live cells over only short time periods	Dilution of label; dyes may spread unintentionally to surrounding cells	Olsson and Hanken, 1996
GFP mRNA labeling	Ventral cranial cartilages in *Xenopus laevis*	Fluorescence allows for live-cell tracking.	Fluorophore fades after several weeks. Dilution of the original label with cell divisions	Borchers et al., 2000; Carl et al., 2000
Fluorophore-labeled dextran	Pluripotentiality of trunk neural crest cells; assessing the direct neural crest contributions to the anuran skull	Fluorescence allows for live-cell tracking; stability of dextran conjugates allows for cell labeling over long time periods (e.g., several months); can be combined with intraspecific transplantation or single-cell labeling; fluorophores behave as epitopes and can be used in secondary antibody amplification	Dilution of the original label with cell divisions	Collazo et al., 1993; this study

[a]References column highlights a minor list of reports utilizing each method. CNC, cranial neural crest; GFP, green fluorescent protein.

amplified in sectioned or whole-mount preparations by using the original fluorophore as an epitope for secondary antibody detection (Kaneko et al., 1996).

By combining the long-term stability of fluorescent dextran with a method for embryonic cell labeling, we are deriving a fate map that will define the neural crest contributions to the bony anuran skull. Such data will likely inform our understanding of several developmental processes that are unique to amphibians, including the dramatic remodeling and replacement of the cartilaginous larval skull during metamorphosis (Trueb and Hanken, 1992). By combining this technique with in situ hybridization, one may also assess the genetic machinery that underlies neural crest contributions to both endochondral and dermal bone. Finally, a fate map of CNC contribu-

tions to the anuran skull may greatly enhance our understanding of the dramatic changes in cranial form that accompanied the evolutionary transition from "lower" to "higher" vertebrates.

RESULTS AND DISCUSSION

Single-celled embryos were injected with lysine-fixable fluorescent dextran and allowed to develop to NF stage 14 (Nieuwkoop and Faber, 1994) for embryonic grafting of the cranial neural crest. Two types of fluorophore-conjugated dextran were tested, Cascade Blue and fluorescein. Survivorship to stage 14 was evaluated to assess the harmful effects of each dextran type (Table 2). Injections using Cascade Blue dextran appear more harmful than those using fluorescein dextran. Because the survivorship of fluorescein dextran-injected embryos does not differ from water-injected controls, we believe that the increased mortality noted in these embryos relative to uninjected controls is an artifact of the injection procedure rather than any potentially harmful effects of dextran on development. The majority of our experiments were carried out with fluorescein dextran.

TABLE 2. Survival of Injected Embryos Through NF Stage 14[a]

Injection compound	Number of single-cell, fertilized embryos injected	Number surviving to NF stage 14
Cascade Blue dextran	244	85 (35%)
Fluorescein dextran	564	326 (58%)
Water-injected controls	368	214 (58%)
Uninjected controls[b]	615	534 (87%)

[a]Data combined from six separate spawnings. NF, Nieuwkoop and Faber.
[b]Rearing conditions (i.e., temperature and media) were identical to those used for injected embryos.

TABLE 3. Success and Survival of CNC-Grafted Chimeras Through NF Stage 44[a]

Number of chimeric transplants performed	Number of successful chimeras[b] at NF stage 33/34	Number of successful chimeras surviving to NF stage 44[c]
133	95 (71%)	91 (96%)

[a]Data combined from five separate spawnings. CNC, cranial neural crest; NF, Nieuwkoop and Faber.
[b]In successful chimaeras, migratory streams of neural crest are specifically and clearly labeled at 24 hr postoperation (ca. NF stage 33/34).
[c]Approximately 5 days of rearing beyond NF stage 14 at room temperature.

Next, we evaluated the success of the CNC grafting procedure as well as the survival of CNC chimaeras into the larval period. After transplants were performed, chimeric embryos were reared for 24 hr (to NF stage 33/34) and analyzed for successful integration of the dextran-labeled CNC graft (Table 3). Roughly 71% of the chimeras showed successful integration of the graft, viz., streams of cranial neural crest were clearly labeled with fluorescent dextran and appeared to be following typical migratory pathways (Fig. 1D,E). We analyzed chimeras at this stage because CNC migration is nearly complete, and the presence of grafted cells within the mandibular, hyoid, and branchial arches is most prominent. Analyses at earlier stages would potentially miss some of the cells that are still migrating from the dorsal region of embryo. Also, because many cranial structures have begun to differentiate by stage 33/34 (Fig. 1F,G), we can effectively determine from cross-sections whether grafts accidentally included cells from adjacent embryonic tissues (see below).

The survivorship of CNC-grafted chimeras was assessed again at NF stage 44, which corresponds to a swimming and feeding larva. Because the survival of animals reared from early embryonic stages through metamorphosis can be affected by several factors unrelated to our experimental procedure, we arbitrarily chose this stage to determine whether the CNC grafting procedure had lethal effects on long-term development. The high survival rate obtained in our experiments shows that the grafting procedure did not impair normal development to later stages (Table 3).

To determine the specificity of neural crest grafts, several chimeric embryos at stage 33/34 were analyzed in cross section for various tissue markers. All results suggest that the CNC-labeling method is highly specific for neural crest and that it did not accidentally introduce labeled cells from the surrounding mesoderm, non-neural crest ectoderm, or endoderm. For example, the developing neural tube did not include grafted dextran-labeled cells (Fig. 2A,B). Occasionally, fluorescent label was apparent just beneath the epidermal ectoderm, but this labeling always was associated with pigment cells, a normal derivative of the neural crest (Fig. 2C,D), rather than epidermal cells.

We specifically tested whether grafts accidentally included cells from head mesoderm, because cranial bone tissue is derived in part from mesodermal cells in other vertebrates (Couly et al., 1993). By using anti-12/101 as a mesodermal marker, we never found dextran-labeled cells colocalized with 12/101-positive cells (Fig. 2E–H). This finding suggests that our grafts are specifically composed of CNC cells.

To determine the degree to which the grafts contained cells specific to the CNC, we assessed the presence of a migratory CNC marker. HNK-1 is a carbohydrate epitope that is highly specific for CNC cells during migration in several vertebrate species (Tucker et al., 1984). HNK-1 immunoreactivity has been used primarily as a neural marker in *Xenopus laevis* (Nordlander, 1993), but it is lo-

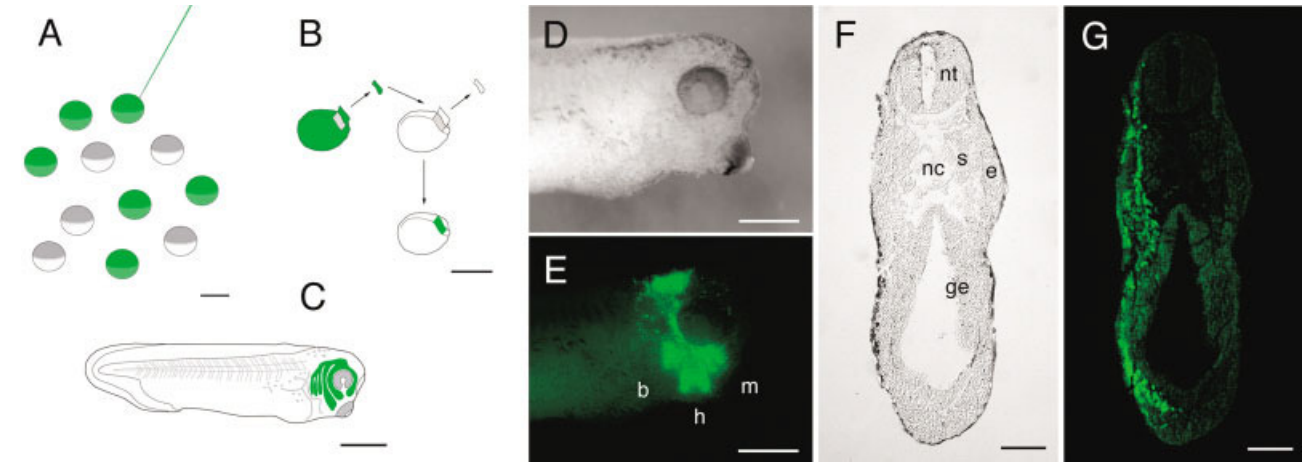

Fig. 1. Methodology. **A:** Single-cell *Xenopus* embryos are injected with 5–10 nl of fluorescent dextran (green), while uninjected embryos are set aside as hosts. **B:** At NF (Nieuwkoop and Faber) stage 14, ectoderm overlying the cranial neural ridge is carefully peeled away to expose the underlying cranial neural crest (CNC). The CNC is removed from unlabeled host embryos and replaced with homotopic explants from dextran-labeled donors. After grafting, the overlying ectoderm is carefully replaced over the explanted tissue. **C:** NF stage 33/34 embryos are examined in whole-mount for the presence of migratory, dextran-labeled CNC. **D,E:** Brightfield and fluorescent image of a representative NF stage 33/34 chimeric embryo. Fluorescently labeled cells have migrated from the grafting site around the eye and into CNC migratory streams of the developing craniofacial region (green, anti-fluorescein). **F,G:** Brightfield and fluorescent images of a cross-section through the hyoid region of a chimeric embryo. Note the differentiation of various head structures as well as migratory fluorescent CNC cells. m, mandibular stream; h, hyoid stream; b, branchial stream; nt, neural tube; nc, notochord; s, somite; e, eye; ge, gut epithelium. Scale bars = 1 mm in A–C, 500 μm in D,E, 200 μm in F,G.

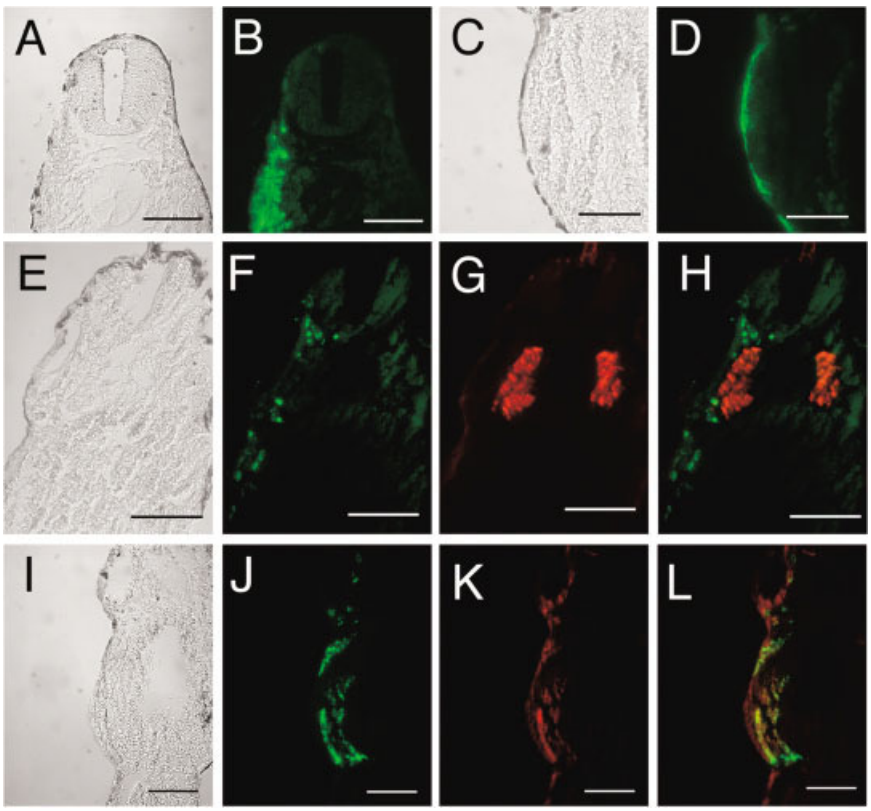

Fig. 2. Migrating donor cells are derived exclusively from the cranial neural crest (CNC). **A,B:** Brightfield and fluorescent images of the cranial region of a NF (Nieuwkoop and Faber) stage 33/34 chimaera. There are no dextran-labeled cells in the developing neural tube. **C,D:** Brightfield and fluorescent images of pigmented epithelium. CNC cells (green) appear to be associated with this layer. **E:** Brightfield image through the developing branchial stream of a chimeric embryo. **F–H:** Labeled CNC cells (F, green) and 12/101-positive cells (G, red) derived from the head mesoderm do not colocalize (H). **I:** Brightfield image through one of the migrating branchial streams. **J–L:** Labeled CNC cells (J, green) and HNK-1-positive cells (K, red) are colocalized (L, yellow). Scale bars = 200 μm.

calized to the CNC in other amphibians (Olsson et al., 2002). Anti–HNK-1 yielded positive immunoreactivity within the neural tube (data not shown) but also among migratory cells within the developing posterior branchial arches, which are populated by cells derived from the CNC. Indeed, there was specific colocalization of the HNK-1 antibody with cells derived from CNC grafts in chimeric embryos (Fig. 2I–L).

Finally, thorough analyses of cross-sections through the cranial region of chimeras never revealed labeled cells in any endodermal derivatives, e.g., the gut epithelium (Fig. 1F,G).

Each animal was grown in a light-protected box covered with aluminum foil. Larvae were reared through metamorphosis to NF stage 66, when many cranial bones have ossified. Following a simple protocol for immunohistological staining (see Experimental Procedures), the sectioned skull bones were analyzed for presence of fluorescent dextran.

Label is clearly evident in several bones. In the snout, for example, the anterior portion of the nasal bone is labeled with several punctate clusters of dextran (Fig. 3B). This area can be diagnosed as bone based on comparison with adjacent sections stained for

skeletal tissues (Fig. 3A; bone is stained red, cartilage is blue).

In another example, labeled cells have contributed to portions of the frontoparietal, a paired bone that contributes to the dermal skull roof (Fig. 3D). Based on the corresponding Tri-Chrome-stained section (Fig. 3C), the dextran-labeled area is located entirely within this bone. On the ventral portion of the skull, dextran is clearly present in the parasphenoid bone (Fig. 3E,F) as well as the squamosal bone (Fig. 3G,H). In all cases, the dextran-labeled bone is present only on the right side of the head, corresponding to the operated side of the chimeric embryo at stage 14.

Long-term labeling techniques have been used to examine the development of only a small number of model organisms. Hence, much of our understanding of the embryonic derivation of the vertebrate skull is based on data from relatively few species. Fate maps depicting the neural crest contribution to skull bones have been available for many years for fishes (Langille and Hall, 1986, 1988) and the domestic chicken (Le Douarin and Barq, 1969; Le Lievre, 1978; Noden, 1978; Couly et al., 1993), and, more recently, for the domestic mouse (Morriss-Kay, 2001; Jiang et al., 2002). Whereas many features of neural crest contribution are shared by these species, there also appear to be important differences among taxa regarding particular cranial regions or individual bones (Morriss-Kay, 2001; Santagati and Rijli, 2003).

These differences suggest that either the pattern of neural crest contribution to the vertebrate skull has changed significantly during vertebrate evolution or that widely accepted homologies of skull bones across major clades may be incorrect. Given the critical phylogenetic position of amphibians as an evolutionary "fulcrum" between fishes and amniotes, a comprehensive neural crest fate map for the anuran skull will clarify the developmental changes that occurred during vertebrate evolution and, thus, help to explain the differences reported among other taxa. With the application of fluorescent dextran conjugates and comparable labeling techniques, such a fate map is now within our reach.

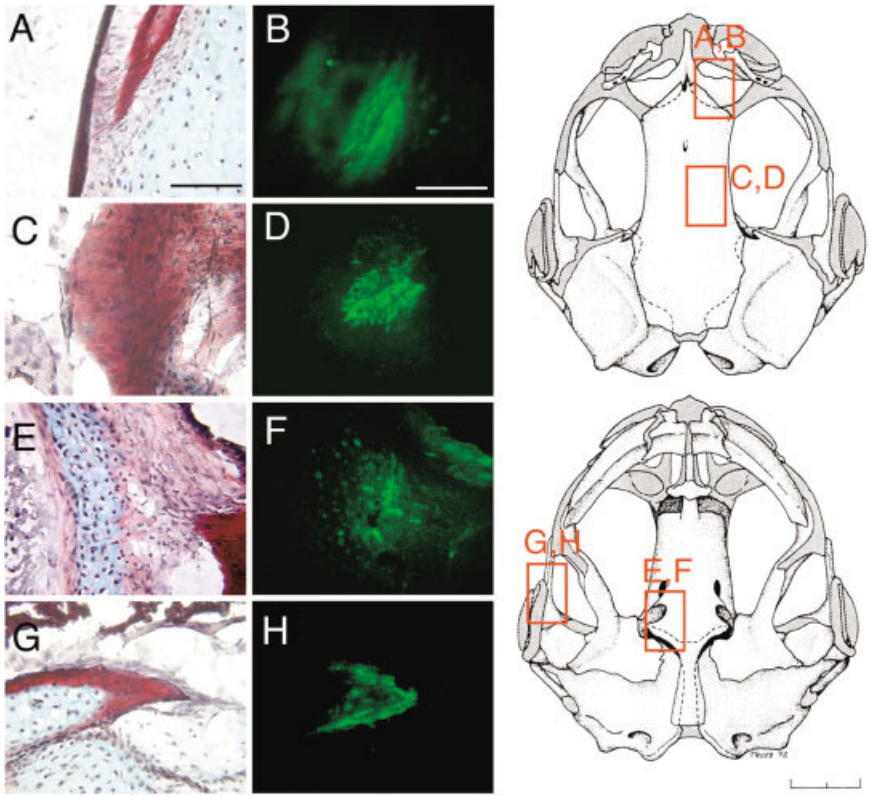

Fig. 3. Grafted cranial neural crest (CNC) cells divide and give rise to labeled progeny in the postmetamorphic skull. When chimeric larvae reached NF (Nieuwkoop and Faber) stage 66, the skull was sectioned and processed to reveal the original CNC-labeled fluorescent dextran. **A–D:** Two major bones were labeled dorsally, the nasal (A,B) and the frontoparietal (C,D). Both mature bones show several areas of punctate fluorescent staining (B,D). These regions correspond with the direct red-stained calcified portion of the nasal and frontoparietal bones in adjacent Tri-Chrome–stained sections (A,C). **E–H:** Two additional bones are labeled in the ventral skull, the parasphenoid (E,F) and the squamosal (G,H). Regions stained in A–H are depicted within the boxes noted on the skull diagrams to the right. Skull diagrams are modified from Trueb and Hanken (1992). Scale bars = 200 μm in A (applies to A,C,E,G), in B (applies to B,D,F,H), 2 mm in diagram 2 (applies to diagrams 1,2).

EXPERIMENTAL PROCEDURES

Embryo Preparation and Dextran-Fluorophore Injection

Eggs of *Xenopus laevis* were collected and fertilized in vitro according to standard protocols (Sive et al., 2000). Embryos were transferred to 0.3× MMR supplemented with 4% Ficoll (EM Science, Darmstadt, Germany) and stabilized in a collection dish. Several injection needles were prepared with a WPI PUL-1 Micropipette Puller (Delay = 4; Heat = 10). By using a sharp razor blade, the pipette tips were bevel-cut to a diameter of 10 μm. By using a PicoSpritzer II injection apparatus (Parker Instrumentation, Fairfield, NJ) connected to a vacuum pressure pump, 1 μl of either Cascade Blue- or fluorescein-labeled dextran (25 mg/ml, 10,000 molecular weight, lysine-fixable; Molecular Probes, Eugene, OR) was transferred into the needle.

Embryos were injected with 5–10 nl of dextran and allowed to develop at room temperature (ca. 22°C) overnight until they reached NF stage 14 (Nieuwkoop and Faber, 1994). Embryos were then assessed for brightness and survival before grafting. The healthiest, most brightly fluorescing embryos were selected for grafting experiments (Fig. 1A–C).

Embryonic CNC-Grafting Procedure

Stage 14 embryos were stabilized and oriented in modeling clay to best expose the neural ridge, which

overlies the developing neural crest. All grafting was carried out in 1× MBSH solution. Once both dextran-labeled donors and unlabeled hosts were stabilized side by side in clay, cranial neural crest explants were grafted from donor to host according to standard methods (Borchers et al., 2000; Carl et al., 2000).

This grafting technique requires that the overlying ectoderm of the neural ridge be temporarily lifted from the unlabeled embryo to allow removal of the region of the cranial neural crest destined to give rise to mandibular, hyoid, and branchial streams (Sadaghiani and Thiébaud, 1987). The same procedure is used with the fluorescent dextran-labeled donor, and the explant is then carefully placed into the unlabeled host. Finally, the overlying ectoderm is replaced on the chimeric embryo and stabilized with a piece of a glass cover-slip for several minutes. Special care was taken to replace the overlying ectoderm to avoid accidental labeling of neurogenic placodes, some of which are destined to develop from this region (Knouff, 1935).

Embryonic Immunohistology

Some embryos were fixed at stage 33/34 to analyze the specificity of neural crest grafts. Embryos in which all three CNC streams were fluorescing brightly were anesthetized in MS-222 and fixed in MEMFA (pH 7.6) at room temperature for 1 hr. They were preserved in 100% EtOH before being processed for immunohistology.

Several tissue-specific antibodies were used in concert with the dextran fluorophore label (fluorescein or Cascade Blue, Molecular Probes) as an epitope for secondary amplification. In all experiments, a rabbit polyclonal primary antibody (1:500 dilution in PBS + 0.1% Triton X-100 (PBST)) was used to amplify the dextran fluorophore marker. We used several antibodies to determine whether CNC grafts accidentally included cells from adjacent embryonic tissues. These include anti-12/101 (1:100 in PBST; DSHB, Iowa City, IA), as a marker of somitic mesoderm; and anti–HNK-1 (1:100 in PBST; gift of Marianne Bronner-Fraser; Cal Tech, Pasadena, CA), as a marker of migratory cranial neural crest.

All embryos were stained as whole-mounts, embedded in Paraplast tissue embedding medium (Oxford Labware, St. Louis, MO), and sectioned on a Leitz 1512 microtome (Leitz, Oberkochen, Germany). Serial sections (10–15 μm) were collected to a glass slide, cleared with Hemo-De (Fisher Scientific, Pittsburgh, PA), and mounted using Cytoseal 60 (Richard-Allen, Kalamazoo, MI) before fluorescent visualization.

Embryo and Tadpole Rearing and Analysis

Embryos were allowed to recover for 24 hr in a solution of 0.3× MMR + 4% Ficoll and then moved to fresh rearing medium (0.1× MMR). For the remainder of embryonic and larval development, rearing medium was changed every 2–3 days. Larvae were fed *Xenopus* tadpole food (Carolina Biological Supply, Burlington, NC). Because fluorescent dextrans are light sensitive, all individuals were reared in the dark after injection to maximally preserve integrity of the fluorophore. All individuals were reared through metamorphosis (NF stage 66), when cranial bone development is advanced, and then anesthetized by immersion in 1% MS-222 (Sigma, St. Louis, MO). They were immediately fixed overnight at 4°C in 3.7% paraformaldehyde in phosphate-buffered saline (PBS).

Skull Dissection and Decalcification

After fixation, specimens were rinsed thoroughly in PBS to remove paraformaldehyde. Under a dissecting microscope, all soft tissues were carefully removed from the head by using a pair of #5 forceps and microscissors (Fine Science Tools, Heidelberg, Germany), leaving only cartilage and bone. The skull was then soaked overnight in 0.5 M EDTA solution (pH 7.6) to dissolve mineralized calcium deposits before cryosectioning.

Enzyme Treatment and Bone Immunohistology

Skull bone tissue was sectioned (10–20 μm) by using a Leica CM 3050S cryostat (Leica, Nussloch, Germany). Cryosectioning was performed after decalcification with EDTA. Sections were then treated directly on the slide with a solution of Proteinase K (10 μg/mL) in PBS (pH 7.6) for 20 min at room temperature. Slides were rinsed in PBS for 5 min and then re-fixed in 3.7% paraformaldehyde for 30 min at room temperature. Specimens were rinsed extensively in PBS and then PBST before staining.

To determine whether the CNC marker specifically labeled skull bone tissue, all skulls were cryosectioned in three sequential series. One series was stained for the presence of the dextran label according to the above protocol. A second series of sections, in which the primary antibody incubation was omitted, was used as a negative control. A third series of sections was processed by using chromatic stains to identify cartilage and bone (see below).

Dextran-labeled tissues were incubated overnight in a cocktail of 5% normal goat serum (a blocking agent; Gemini Bio-Products, Woodland, CA) and rabbit polyclonal anti-Cascade Blue or anti-fluorescein (1:500 in PBST; Molecular Probes, Eugene, OR) depending on the type of fluorescent dextran used in the particular experiment. The next day, the tissue was rinsed all day in PBS and PBST for several hours to remove the primary incubation cocktail. Then, it was incubated overnight in goat anti-rabbit Alexa 488 (1:500 in PBST; Molecular Probes, Eugene, OR). The following day, the tissue was again rinsed all day in PBS and PBST. The same protocol and dilutions used above are applied to staining earlier embryos and tissues. To determine whether the dextran label was present in bone, the third series of cryosections was processed for routine histology with chromatic stains, viz., direct red (bone), Alcian blue (cartilage), celestine blue (muscle), and hematoxylin (nuclei) according to standard Tri-Chrome

staining protocols (Presnell and Schreibman, 1997).

Specimen Viewing and Image Acquisition

Slides were mounted by using Fluoromount G (Southern Biotech, Birmingham, AL) and viewed with a Leica fluoroscope (Model DMRE). High-resolution digital images were obtained with a 12-bit, black and white CCD camera (ORCA, Hamamatsu) using Openlab software (Improvision, UK). Images were saved as TIFF files and pseudocolored and contrast-adjusted in Adobe Photoshop 7.0 for Macintosh.

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