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Adenovirus-mediated ectopic expression of Msx2 in even-numbered rhombomeres induces apoptotic elimination of cranial neural crest cells in ovo

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SUMMARY

Distinct cranial neural crest-derived cell types (a number of neuronal as well as non-neuronal cell lineages) are generated at characteristic times and positions in the rhombomeres of the hindbrain in developing vertebrate embryos. To examine this developmental process, we developed a novel strategy designed to test the efficacy of gain-of-function Msx2 expression within rhombomeres in ovo prior to the emigration of cranial neural crest cells (CNCC). Previous studies indicate that CNCC from odd-numbered rhombomeres (r3 and r5) undergo apoptosis in response to exogenous BMP4. We provide evidence that targeted infection in ovo using adenovirus containing Msx2 and a reporter molecule indicative of translation can induce apoptosis in either even- or odd-numbered rhombomeres. Furthermore, infected lacZ-control explants indicated that CNCC emigrated, and that 20% of these cells were double positive for crest cell markers HNK-1 and β-gal. In contrast, there were no HNK-1 and Msx2 double positive cells emigrating from Msx2 infected explants. These results support the hypothesis that apoptotic elimination of CNCC can be induced by 'gain-of-function' Msx2 expression in even-numbered rhombomeres. These inductive interactions involve qualitative, quantitative, positional and temporal differences in TGF-β-related signals, Msx2 expression and other transcriptional control.

Key words: Cranial neural crest, Cell migration, Chick, Microinjection, Fusion protein, Whole mount immunostaining, Explant culture, Pattern formation, Cell determination, Craniofacial development

INTRODUCTION

Congenital malformations in vertebrates involving the craniofacial region are often attributable to defects in the proliferation, migration, patterning, differentiation, or apoptosis of cranial neural crest cells (CNCC) that give rise to many of the tissue types that form this region (Webster et al., 1986; Sulik et al., 1988; Gorlin and Slavkin, 1997). The neural tube of the developing hindbrain is composed of seven paired compartments called rhombomeres. Three distinct populations of CNCC arise from the hindbrain and exit lateral to rhombomere (r) 2, 4 and 6 (Lumsden et al., 1991; Sechrist et al., 1993). The first population (composed of CNCC from midbrain, r1 and r2) emigrate into the first branchial arch and the trigeminal (V) ganglion. The second CNCC originate from r4 and emigrate into the second branchial arch and the facial (VII) and vestibulo-acoustic (VIII) ganglia. The third comprises CNCC from r6 and r7, entering the third branchial arch and superior ganglion of the glossopharyngeal (IX) nerve (Lumsden et al., 1991). The mandibular arch skeleton and muscle have a composite origin in which the proximal elements are r1+r2 derived, whereas more distal elements are exclusively midbrain derived (Köntgesis and Lumsden, 1996). The most proximal region of the mandible such as the retroarticular process or M. depressor mandibule is derived from the second arch (Köntgesis and Lumsden, 1996). Both the mandibular skeleton and tongue muscle display an organization which precisely reflect the rostrocaudal order of segmental crest deployment from the embryonic hindbrain in chick development (Köntgesis and Lumsden, 1996).

Using fluorescence videomicroscopy, a certain proportion of CNCC from r3 and r5 were observed to emigrate and join major populations from adjacent even-numbered rhombomeres (Birgbauer et al., 1995). However, CNCC from r3 and r5 are restricted to limited contributions within the branchial arches (Köntgesis and Lumsden, 1996). Most CNCC from odd-numbered rhombomeres (r3, r5) are eliminated by apoptosis, and this process is regulated by bone morphogenetic protein 4 (BMP4) (Graham et al., 1993, 1994). Concomitantly, the transcription factor muscle segment homeobox homologue 2 (Msx2) is expressed in r3 and r5. In vitro studies of rhombomere explants demonstrated that exogenous BMP4 treatment can up-regulate Msx2 expression (Graham et al., 1994). Localization

and expression data in various developing systems suggests that Msx2 is a downstream effector in the BMP signaling pathway. For example, where Msx2 expression in the chicken limb bud is spatially and temporally associated with apoptosis, expression of a dominant negative type I BMP receptor reduces interdigital apoptosis and Msx2 expression (Zou and Niswander, 1996). During epithelial-mesenchymal interactions of tooth and mammary gland development, during skeletal development of facial primordia and vertebrae and during dorsal differentiation of spinal cord BMP expression correlates with and induces Msx2 expression (Vanio et al., 1993; Monsoro-Burq et al., 1996; Phippard et al., 1996; Barlow, 1997; Liem et al., 1995). These developmental examples, along with many other studies, indicates that BMP signaling activity mediates many different physiological processes such as cell death and epithelial-mesenchymal interactions during craniofacial and skeletal development. In addition, there is extensive correlation between the expression of Msx2 and apoptosis in the neuroepithelium such as in the anterior neuropore, dorsal spinal cord and tail bud (Maden et al., 1997). Even though Msx2 expression is closely associated with these processes both temporally and spatially, a direct functional relationship between Msx2 and these developmental processes has not yet been established.

Recently, an in vitro study demonstrated that constitutive ectopic Msx2 expression in the P19 cell line results in an increase in apoptosis upon cell aggregation (Marazzi et al., 1997), but its significance in a physiological context is yet to be determined. The present studies were designed to test the hypothesis that the homeodomain transcription factor Msx2, expressed in r3 and r5, responds to BMP4 signaling to induce apoptotic elimination of CNCC during normal craniofacial development. We now report the development of an adenoviral gene delivery system into CNCC and we demonstrate that apoptotic elimination of CNCC can be induced by 'gain-of-function' Msx2 expression in even-numbered rhombomeres during chick embryogenesis.

MATERIALS AND METHODS

Recombinant adenovirus construction

Mouse Msx2 cDNA was a gift from Dr Yi-Hsin Liu (University of Southern California, Los Angeles, CA). Msx2-HA was generated by inserting the HA epitope tag at the C-terminal of the Msx2 construct after disruption of the termination codon by polymerase chain reaction (PCR). The Msx2-HA construct was inserted into the adenovirus shuttle vector pACEF under the control of the EF promoter (Mizushima and Nagata 1990). The E1 region was deleted in this shuttle vector and, therefore, the resulting recombinant viruses were replication defective and free from expression of the viral proteins in neuronal cells. Control adenovirus carrying a *lacZ* insert (AdV-lacZ) was a gift from Dr Silvio Gutkind (National Institute of Dental Research, NIH, Bethesda, MD).

Low passage HEK 293 cells (Micobix Biosystems Inc., Toronto, Canada) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Recombinant viruses were obtained in low passage HEK 293 cells by homologous recombination between the pACEF shuttle vector and pJM17 vector (Micobix Biosystems Inc., Toronto, Canada) containing the viral genome. The recombinant Msx2-HA adenovirus (Adv-Msx2-HA) was characterized by restriction mapping of the viral genome. The viral titer was determined by the simplified end point cytopathic effect assay (Miyake et al., 1996). The virus was amplified in HEK 293 cells and either the supernatant from infected HEK 293 cells or CsCl-purified virus were used as a source for infections in subsequent experiments.

Experimental manipulations of chicken embryos

Fertilized chicken eggs (Truslow Farms Inc., Chestertown, MD) were incubated at 37°C in a humidified atmosphere and the embryos were staged according to Hamburger and Hamilton (1992; HH stage). Using a 10 μm diameter glass micropipette, 10 nl of recombinant adenovirus solution (titer of 1×10^{10} pfu/ml) accompanied by 1% tetramethylrhodamine dextran (Molecular Probes, Eugene, OR), which was used as a visible marker, was microinjected between the cranial neural folds at HH stage 7-8, or into the medial regions of the maxillary processes or forelimb buds at HH stage 21-22 using a Transjector 5246 (Eppendorf, Hamburg, Germany) under a fluorescence stereomicroscope (Leica, Deerfield, IL). The embryos were returned to incubation and allowed to continue developing in ovo.

Rhombomere explant culture

Chick embryos infected at HH stage 7-8 with AdV-lacZ, AdV-Msx2-HA or uninjected were incubated for 12 hours in ovo to reach HH stage 10-11. Subsequently, the rhombencephalon was microdissected and then treated with 1 mg/ml dispase (Boehringer Mannheim, Indianapolis, IN) in L-15 medium (Gibco-BRL, Gaithersburg, MD) for 15-20 minutes to separate the rhombencephalic tissue from the surrounding mesenchymal cells. Individual rhombomeres were identified by anatomical landmarks and separated by microdissection. The isolated rhombomere explants were plated in 96-well tissue-culture plates which had been coated with fibronectin by overnight incubation at 4°C with a 50 μg/ml solution of fibronectin in F-12 medium (Gibco-BRL, Gaithersburg, MD). Explants were then cultured for 24 hours in F-12 medium supplemented with 10% fetal bovine serum.

Immunoprecipitation

Immunoprecipitation was performed as previously described (Harlow and Lane, 1988). Briefly, HEK293 cells were metabolically-labeled with 150 μCi/ml [^{35}S]cysteine, methionine pro-mix (Amersham, Arlington Heights, IL) for 12 hours. Cell lysate was incubated with 1 μl of anti-HA polyclonal antibody (Babco, Berkeley, CA) for 1 hour at 4°C and the immunoprecipitate was recovered by Protein A sepharose (Pharmacia Biotech, Uppusala, Sweden). The immunoprecipitates were separated on 8% SDS-PAGE. The gels were dried and exposed for autoradiography for 18 hours.

Whole-mount immunohistochemistry and immunocytochemistry

Whole chick embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 12 hours, washed briefly in PBS and dehydrated through a graded series of ethanol in PBS. The specimens were blocked in TBST (0.14 M NaCl, 10 mM KCl, 25 mM Tris-HCl pH7.0, 0.1% Tween-20) containing 10% normal sheep serum, followed by incubation for 12 hours at 4°C with anti-HA polyclonal antibody at a 1:2000 dilution or anti-HNK-1 antibody (Becton Dickinson, USA) at a 1:100 dilution in TBST. Following extensive washing in TBST, immunoreactions were detected by incubation for 12 hours in alkaline phosphatase-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibody (Jackson Immuno Research, West Grove, PE) at a 1:2000 dilution in TBST. The antibodies were pre-adsorbed with chicken embryo extract (Gibco-BRL, Gaithersburg, MD) to eliminate cross-reactivity. Embryos were then washed extensively in TBST, followed by further washings in NTMT (0.1 M NaCl, 0.1 M Tris-HCl pH9.5, 50 mM $MgCl_2$, 0.1% Tween-20) and finally NTMT with 2 mM levamisole. The bound alkaline phosphatase was visualized after incubation in NBT/BCIP solution (Boehringer Mannheim, Indianapolis, IN) in NTMT.

Specimens for frozen sectioning were first fixed in 4% paraformaldehyde for 12 hours, then infiltrated with a graded series of sucrose solution and embedded in OCT compound. A series of 5 μm cross-sections of each rhombomere were collected for immunohistochemistry. CNCC from rhombomere explant cultures or HEK293 cells infected with AdV-Msx2-HA were fixed for 10 minutes

and washed in PBS. Frozen sections as well as fixed cells were permeabilized in 0.1% Triton-X in PBS and blocked for 1 hour in antibody buffer (5% bovine serum albumin and 0.025% Triton X-100 in PBS) containing 10% normal goat serum. Incubations with antibody were performed at 4°C for 12 hours using anti-HA polyclonal antibody at a 1:1000 dilution in TBS or anti-HNK-1 monoclonal antibody at a 1:100 dilution. Following washing in PBS, frozen sections and HEK293 cells were incubated for 2 hours in rhodamine-conjugated anti-rabbit IgG secondary antibody at a 1:100 dilution, and CNCC were incubated in rhodamine-conjugated anti-mouse IgG secondary antibody or FITC-conjugated anti-rabbit IgG secondary antibody (Jackson Immuno Research, West Grove, PE) at a 1:100 dilution in antibody buffer for direct immunofluorescence.

X-gal staining

Whole embryos and cells were fixed in 4% paraformaldehyde for 10 minutes. Specimens were washed in PBS and incubated for 12 hours at 37°C in 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-galactopyranoside) solution, with 35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate and 0.02% Nonidet P-40.

Quantitative detection of β-gal

Chick embryos infected at HH stage 7-8 with AdV-lacZ or uninjected were incubated for 12 hours in ovo to reach HH stage 10-11. Subsequently, individual rhombomeres were identified by anatomical landmarks and separated by microdissection. Ten isolated explants in each rhombomere were collected and homogenized in lysis buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol, 2.5 mM EDTA and 0.125% NP-40). The amount of total protein in each lysate was determined by BCA protein assay (Pierce, Rockford, IL). The level of β-gal expression was quantitated by a colorimetric assay in which 200 μl of each rhombomere lysate containing 5 μg of protein and 8 mM CPRG (chlorophenol red-β-D-galactopyranoside) (Boehringer Mannheim, Indianapolis, IN) were incubated at 37°C for 20 minutes. The absorbance at 570 nm was measured with a MRX Microplate Reader (Dynatech Laboratories Inc., Chantilly, VA).

Detection of apoptosis

Apoptosis was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method using the ApopTag Plus In Situ Apoptosis Detection Kit-Fluorescein (Oncor, Rockville, MD) according to specifications from the manufacturer. For whole-mount TUNEL staining, embryos were fixed in 1% paraformaldehyde in PBS for 20 minutes. Specimens were washed briefly, dehydrated through a graded series of ethanol in PBS and subjected to labeling with the ApopTag Plus In Situ Apoptosis Detection Kit. One modification was introduced to optimize for whole-mount staining: embryos were blocked in TBST (0.14 M NaCl, 10 mM KCl, 25 mM Tris-HCl pH7.0, 0.1% Tween-20) containing 10% normal sheep serum, followed by 12 hours of incubation in alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim, Indianapolis, IN) at a 1:2000 dilution in TBST. Visualization of the label was performed as described for whole-mount immunohistochemistry.

Statistical analysis

Data were analyzed by one-way analysis of variance and Student's *t*-test, and statistical significance was determined at a confident level of $P<0.01$. All experiments were performed at least in triplicate.

RESULTS

Characterization of the recombinant adenovirus

Msx2 and β-gal were expressed under the control of the EF-1 promoter of the recombinant adenovirus to achieve high expression levels. An HA epitope tag was inserted at the carboxy terminus of Msx2 in order to identify and distinguish ectopic expression from endogenous Msx2 (Fig. 1A).

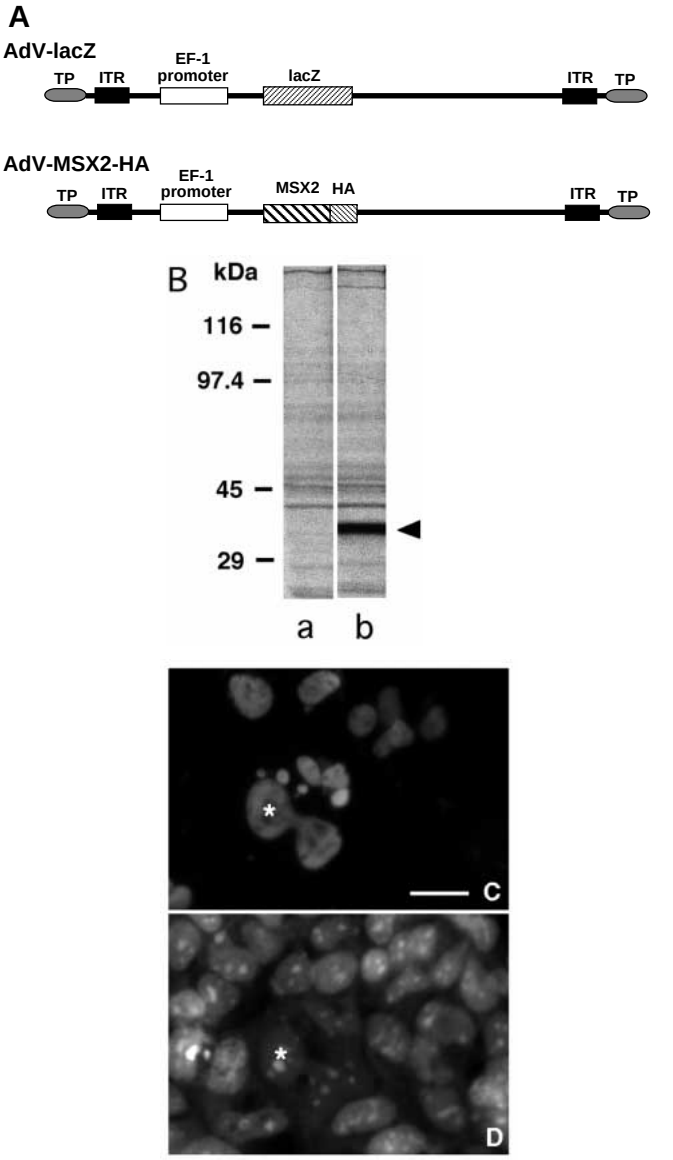

Fig. 1. Characterization of recombinant adenovirus. (A) Schematic representation of the structure of the recombinant adenovirus illustrating the relative positions of the inverted terminal repeat (ITR) and terminal protein (TP) on both ends of the adenoviral genome, the EF-1 promoter, the lacZ cDNA and the mouse Msx2 cDNA, and hemagglutinin (HA) tag fusion gene. AdV-lacZ; recombinant adenovirus expressing lacZ, AdV-Msx2-HA; recombinant adenovirus expressing HA-tagged Msx2. (B) Metabolically labeled whole cell lysate of uninfected (lane a) as well as infected HEK 293 cells by AdV-Msx2-HA (lane b) immunoprecipitated using an anti-HA antibody to detect the expression of Msx2-HA. Arrowhead indicates the 35 kDa-specific band of HA-tagged Msx2 present in the infected HEK 293 cells. Molecular weight sizes in kilodaltons (kDa) are indicated on the left. (C) Msx2-HA was localized to the nucleus of HEK 293 cells 24 hours after infection with AdV-Msx2-HA using an anti-HA antibody. (D) Nuclear counterstaining of HEK293 cells infected with AdV-Msx2-HA using propyoniodide. Asterisk indicates the same nucleus in C and D. Scale bar, 20 μm.

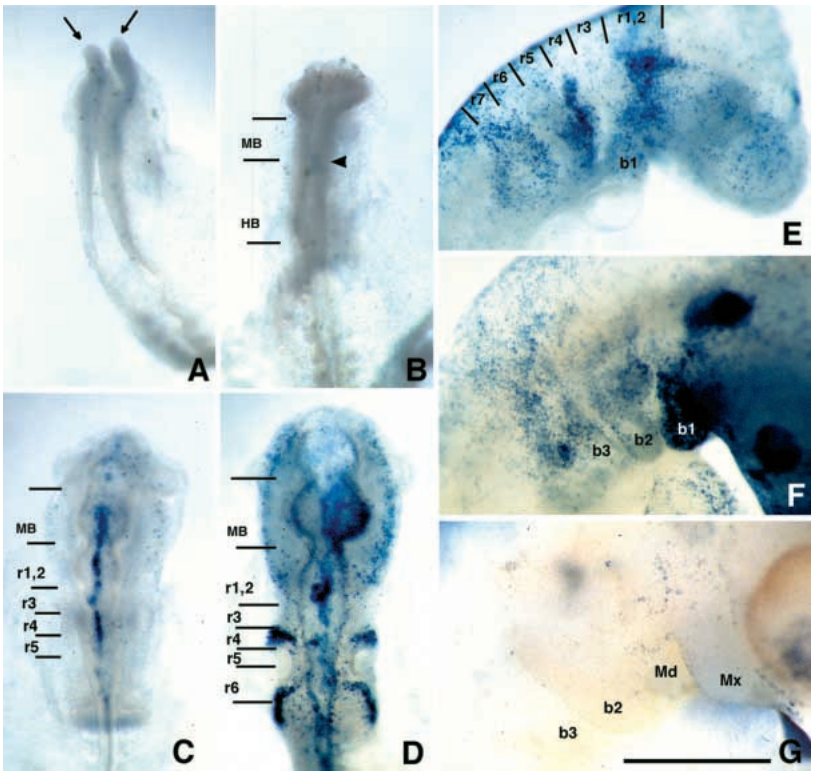

Fig. 2. Normal emigration pattern of AdV-lacZ-infected cranial neural crest cells (CNCC) during embryonic chick development. After infection at HH stage 7-8 the embryos were allowed to develop in ovo for 4 hours (A), 8 hours (B), 12 hours (C), 24 hours (D), 36 hours (E), 48 hours (F) and 72 hours (G) after which whole-mount β-gal staining was performed. (A-D) Dorsal views of chick embryos, and (E-G) lateral views of embryos. Three major populations of CNCC were identified as AdV-lacZ-infected cells at 36 and 48 hours (D and E). MB, midbrain; HB, hindbrain; r, rhombomere; Mx, maxillary process of first branchial arch; Md, mandibular process of first branchial arch; b1, b2, b3, first, second and third branchial arches respectively; arrows indicated open neural folds; and arrowhead showed earliest detectable β-gal-positive signal in the dorsal region of neural tube. Scale bar, 600 μm.

Immunoprecipitation and immunolocalization of the virally expressed Msx2-HA were performed after infection of HEK 293 cells to characterize the protein. Immunoprecipitation of Msx2-HA using an anti-HA antibody revealed a single prominent translation product of approximately 35 kDa, consistent with the predicted size from the DNA sequence, and was not present in control (Fig. 1B). Using the same antibody, HA-taged Msx2 was localized to the nucleus of HEK 293 cells (Fig. 1C,D), consistent with the native molecule and being a transcription factor. In fact, AdV-Msx2-HA as well as AdV-lacZ infected several types of chicken and murine cells in culture and expressed these proteins with high efficiency (data not shown). These characteristics allow the adenovirus-mediated gene transfer system to serve in cell lineage tracing and ectopic gene expression experiments.

Efficient adenovirus-directed gene delivery to cranial neural crest cells in ovo

In the developing chick embryo, mesencephalic and rhombencephalic neural crest cells first emigrate at HH stage 8-9 (6 somite stage; Tosney, 1982) to populate the branchial arches; the process is complete by HH stage 12 (16 somite stage; Lumsden et al., 1991), an 18 hours time span in ovo. In order to address the temporal-spatial pattern of recombinant adenovirus-derived gene expression in developing chick embryos in ovo, AdV-lacZ was microinjected between the neural folds at HH stage 7-8; prior to CNCC emigration. Histochemistry for β-gal expression, performed at progressive time points to monitor the pattern of cell migration, revealed no detectable staining at 4 hours of incubation (Fig. 2A).

The earliest detectable, though weak, expression of AdV-lacZ was at 8 hours after infection, in the dorsal region of the neural tube (Fig. 2B, arrowhead). The timing of onset of expression was consistent with cell culture experiments in which the first detectable expression was observed in HEK 293 cells at 7 hours after infection (data not shown). By 12 hours, the number and intensity of β-gal-positive cells increased, essentially confined to the dorsal neural tube with a few migrating CNCC (Fig. 2C). At 24 hours, β-gal-positive cells formed three major populations on the dorsal and lateral side of the rhombencephalic neural tube; cells originating from r 1, 2, 4 and 6 were directed towards each of the first three presumptive branchial arches (Fig. 2D). Since there is a rostral-caudal temporal sequence of CNCC emigration (Lumsden et al., 1991), the first streaming population originating

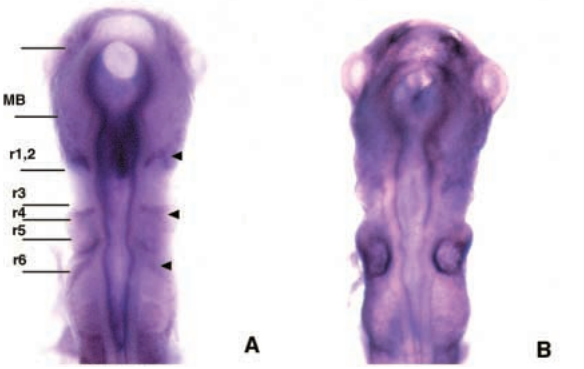

Fig. 3. Normal emigration patterns of HNK-1-positive CNCC during chick embryonic development. (A) Dorsal view of HH stage 13 embryo stained with anti-HNK-1 antibody demonstrating three major populations of migratory CNCC (arrowheads) originating from rhombomeres (r) 1 and 2, 4 and 6. (B) HH stage 13 embryo used as a primary antibody negative control for whole mount immunohistochemistry. Scale bar, 300 μm.

from r1 and r2 has already assumed a more lateral position than the population from r4 or r6, resulting in fewer migrating cells to be observed from the dorsal aspect. β-gal-positive cells along the dorsal region of the neural tube decreased by 36-48 hours, but intense labeling was observed in the developing branchial arches, reflecting the continued emigration of CNCC (Fig. 2E,F).

Minimal expression was detected at 72 hours when the branchial arches were well-defined (Fig. 2G). This emigration pattern was also observed by immunostaining for HNK-1 epitope on CNCC in stage 13 embryos (Fig. 3), and was consistent with a previous report (Kuratani and Eichele, 1993). These observations demonstrate that AdV-lacZ expression during CNCC emigration did not appear to have an adverse effect on normal patterning, and further provided evidence that this experimental approach could be used effectively employing molecular markers in cell lineage determination.

Ectopic Msx2 expression induced apoptosis

In order to test a direct relationship between Msx2 expression and CNCC apoptosis, AdV-Msx2-HA was microinjected between the neural folds of HH stage 7-8 chick embryos which were incubated in ovo for 12 hours. Similar to AdV-lacZ infection (Fig. 4A), Msx2-HA expression, as detected by whole-mount immunohistochemistry, was primarily localized along the dorsal region of the mesencephalic and rhombencephalic neural tube (Fig. 4B). Furthermore, to confirm the reproducibility of the infection in the anterior-posterior direction within rhombomeres, we assayed for the expression level of β-gal in each rhombomere using a quantitative colorimetric detection method. The result (an average of ten specimens from each rhombomere) showed that there were similar expression levels among all rhombomeres (Fig. 4C).

Apoptosis was assessed by whole-mount TUNEL detection after microinjection of AdV-Msx2-HA or AdV-lacZ. While both AdV-Msx2-HA- and AdV-lacZ-infected embryos exhibited normally occurring focal apoptosis in r3 and 5, AdV-Msx2-HA-infected embryos showed increased apoptosis including that in r1, 2 and 4 when compared with controls (Fig. 4D,E). Similar results were obtained when apoptosis was assayed using Nile Blue staining (data not shown). In addition, using high magnification and anatomical landmarks to delineate the rhombomeres, we counted the number of TUNEL-positive cells in r1 to 4 of AdV-Msx2-HA-infected embryos and showed that there were three to four times more TUNEL-positive cells than in control infected embryos in r1, 2 and 4. This difference was statistically significant (P<0.005) (Fig. 4F).

Analyses were performed to determine whether apoptosis was associated with Msx2 expression and whether it occurred in the cells expressing Msx2-HA. Cross sections through r2 and 4 of AdV-Msx2-HA- or AdV-lacZ-infected embryos were double-stained for HA and apoptosis using the TUNEL method, or apoptosis and β-gal. Nuclei that were double-positive for HA and TUNEL were detected along the dorsal surfaces of r2 (Fig. 5) and r4 (data not shown). Taken together, these results document that Msx2 expression directly up-regulated apoptosis in CNCC from r1, 2 and 4.

Msx2 expression eliminated precursor CNCC prior to migration

Our in ovo experiments showed increased apoptosis in the

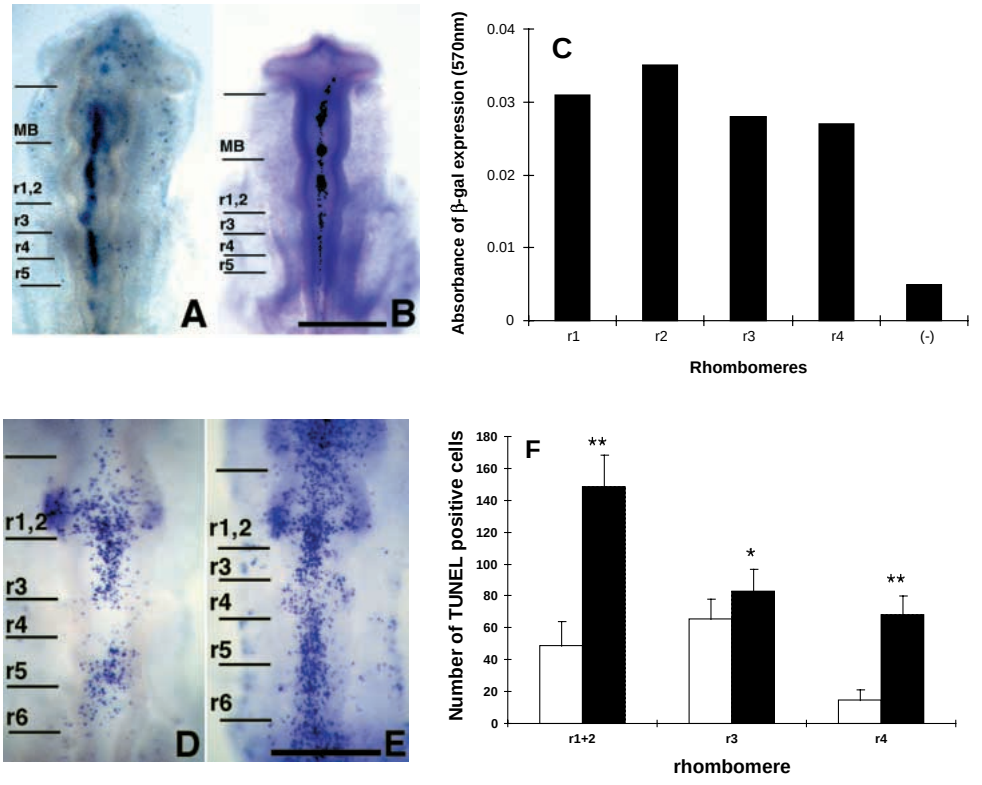

Fig. 4. Ectopic Msx2 expression induces CNCC apoptosis in r 1-2 and 4. HH stage 7-8 chick embryos were infected with AdV-lacZ (A, D) or AdV-Msx2-HA (B, E). Embryos were then incubated and further developed for an additional 12 hours in ovo. To monitor for adenovirus-mediated gene expression, embryos were subjected to either β-gal staining (A) or whole-mount immunohistochemistry to identify Msx2-HA (B). Scale bar, 300 μm. (C) Quantitative detection of β-gal expression level in the dorsal region of rhombomeres infected with AdV-lacZ. (-), uninfected control rhombomere. (D,E) Whole-mount TUNEL assay for apoptosis along the dorsal rhombencephalic neural tube. Dorsal views of embryos are shown. The apparent difference in size of the rhombomeres in D compared with E is due to the degree of curvature of the embryos and not to magnification. r, rhombomere. Scale bar, 750 μm. (F) Comparison of TUNEL-positive cells in the dorsal region of even- and odd-numbered rhombomeres infected with AdV-lacZ (white bar) (n=11) or AdV-Msx2-HA (black bar) (n=9). Standard error bars are placed on top of each respective data bar (*P<0.01; **P<0.005).

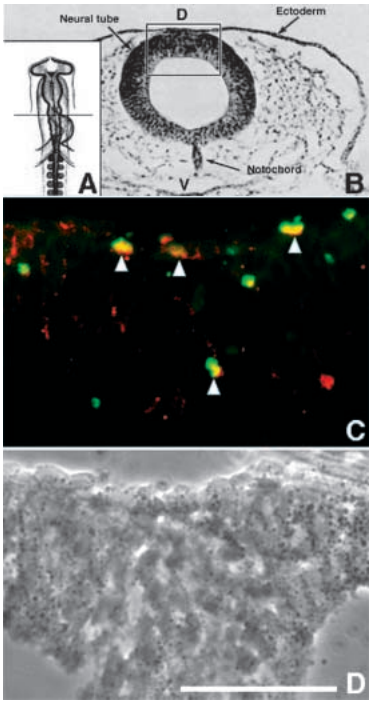

Fig. 5. Apoptosis was detected in cells expressing Msx2-HA at the level of r2. (A) Dorsal view of HH stage 10 chick embryo. The line indicates the level of r2. (B) Cross section of the r2 level at HH stage 10. V, ventral; D, dorsal. The rectangle indicates the area shown in C and D. (C) Dorsal region of rhombomere 2 infected with AdV-Msx2-HA at HH stage 7-8 and incubated for an additional 12 hours in ovo. Msx2-HA expression was visualized with rhodamine (red)-conjugated anti-HA antibody and apoptosis was monitored by FITC (green)-conjugated secondary antibody using the TUNEL method. Arrowheads indicate double positive nuclei (yellow). (D) Phase contrast image of the 5 μm cross section in C. Scale bar, 50 μm.

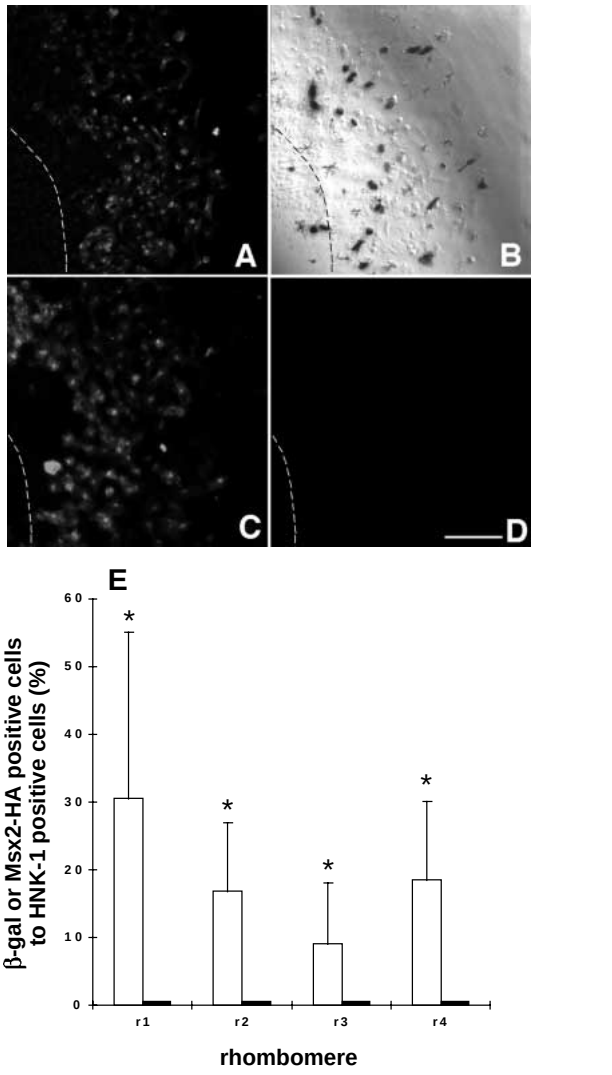

Fig. 6. Apoptotic elimination of precursor CNCC by ectopic Msx2 expression in even- and odd-numbered rhombomere explants. HH stage 7-8 embryos infected with AdV-lacZ or AdV-Msx2-HA were incubated in ovo for 12 hours. Subsequently, each of r 1-4 were microdissected and individually explanted in culture for an additional 24 hours. R2 explants were double-stained for neural crest specific marker HNK-1 (A) and β-gal (B) in AdV-lacZ control infected explants, or HNK-1 (C) and HA (D) in AdV-Msx2-HA infected explants. Scale bar, 100 μm. Dashed lines indicate the margins of rhombomere explants. Similar double stainings were performed for all other rhombomeres from r1-4 and comparison made between Msx2-expressing versus control β-gal-expressing cells. White and black bars indicate percentage of β-gal- and HA-positive cells, respectively, compared to total HNK-1-positive cells that had emigrated from the specific rhombomere explants (r1-4). Standard error bars are placed on top of each respective data bar. (*P<0.005).

dorsal region of the rhombencephalic neural tube associated with ectopic expression of Msx2. However, these studies did not address whether such induced apoptosis directly resulted in the elimination of precursor CNCC. Therefore, to determine whether Msx2 directs the apoptotic elimination of precursor CNCC in rhombomeres, chick embryos were microinjected with recombinant adenovirus and incubated in ovo for 12 hours until HH stage 10-11. Subsequently, individual rhombomeres were microdissected and placed in explant cultures for an additional 24 hours; a sufficient incubation to allow for CNCC emigration from the individual rhombomere explants. Cultures were stained for the CNCC marker HNK-1 and HA, or HNK-1 and β-gal. Infected Adv-lacZ control rhombomere explants showed that 20% of the emigrated cells were double positive for HNK-1 and β-gal indicating normal migration (Fig. 6A,B). In contrast, none of the emigrated cells were double positive for both HNK-1 and HA in rhombomere explants infected with AdV-Msx2-HA (Fig. 6C,D). In addition, we counted the number of emigrated HNK-1-positive CNCC that also expressed β-gal or Msx2-HA from individually isolated r1-4 explants. Similarly, a significant number of CNCC that also expressed β-gal was observed emigrating from each of the control AdV-lacZ-infected rhombomeres, whereas no HNK-1-positive cells that also expressed Msx2-HA were detected from rhombomeres infected with AdV-Msx2-HA (Fig. 6E). These results indicate that Msx2 expression directed apoptotic elimination of CNCC within even- and odd-numbered rhombomere explants.

Msx2 induces apoptosis within the neuroepithelium

To address whether different tissues are equally susceptible to

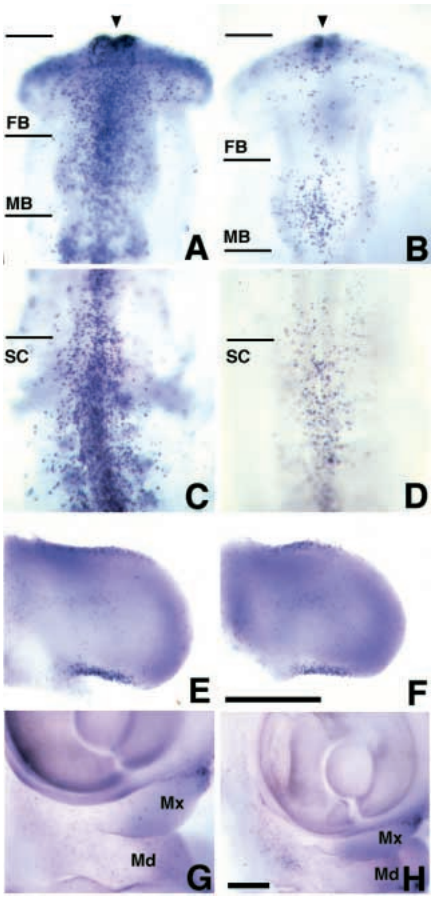

Fig. 7. Ectopic Msx2 expression induces apoptosis in the neuroepithelium. Forebrain and midbrain regions (A,B) and spinal cord region (C,D) of neural tube at HH stage 7-8 chick embryos, and distal region of the forelimb bud (E,F) and maxillary process (G,H) at HH stage 21-22 embryos were infected with AdV-Msx2-HA (A,C,E,G) or AdV-lacZ (B,D,F,H). Embryos were then incubated and further developed for an additional 12 hours in ovo. Whole-mount TUNEL assay for apoptosis was subsequently performed. Dorsal views (A-D) and lateral views (E-H) of embryos are shown. FB, forebrain; SC, spinal cord; arrowhead, anterior neuropore. Scale bar, 750 μm, same magnification from A-F, and from G-H.

apoptosis induced by ectopic expression of Msx2, apoptosis was assessed by whole-mount TUNEL detection after infection with AdV-Msx2-HA or AdV-lacZ in various other levels of the neuroepithelium such as forebrain, midbrain and spinal cord, and non-neuronal tissues such as the forelimb bud and the maxillary process. These sites were selected because they represent areas where endogenous BMP4 and Msx2 are present. While both AdV-Msx2-HA- and AdV-lacZ-infected embryos exhibited normally occurring focal apoptosis in the neuroepithelium, AdV-Msx2-HA-infected embryos showed extended apoptosis along the neural tube including the dorsal side of the forebrain, midbrain and spinal cord, when compared with controls (Fig. 7A-D). However, there was no increased apoptosis observed in the forelimb bud and maxillary process of embryos infected by both AdV-Msx2-HA compared with AdV-lacZ infected embryos (Fig. 7E-H). These results demonstrated that different tissues vary in their competence to respond to apoptosis induced by ectopic expression of Msx2 during chicken development.

DISCUSSION

Using an adenovirus-mediated gene delivery system, we have ectopically expressed Msx2 in the developing hindbrain of chicken embryos and demonstrated that Msx2 directly mediates apoptosis in the even-numbered r2 and 4 in addition to increased apoptosis also in r3 and 5 (Figs 4, 5). Furthermore, using rhombomere explant cultures we obtained experimental evidence that the apoptotic elimination of CNCC takes place prior to cell emigration (Fig. 6). Taken together, these data suggest that the normal patterning of CNCC differentiation involving apoptosis in the odd-numbered rhombomeres is directly attributable to the expression and function of Msx2. In addition, we also demonstrated that the neural tube is more competent than other tissues to respond to apoptosis induced by ectopic expression of Msx2 during chicken development. Even though Msx2 appears to be concomitantly up-regulated along with BMP signaling in diverse developmental events, this is the first in ovo evidence that Msx2 is an effector of BMP downstream events.

Functions of Msx2-directed apoptotic elimination of precursor CNCC in rhombomeres

Cellular apoptosis serves multiple functions during animal development (for recent reviews, see Jacobson et al., 1997; Clarke and Clarke, 1996; Sanders and Wride, 1995). The formation of digits in vertebrates involving interdigital cell death is a widely studied developmental phenomenon. Apoptosis of CNCC cells in rhombomeres may have a similar function as that during limb formation; patterning. The discontinuous production of CNCC from successive rhombomeres, controlled by apoptotic elimination of CNCC in r3 and 5, sculpts the CNCC into non-mixing streams of migrating cells destined for corresponding branchial arches, and consequently ensures the fidelity of patterning (Graham et al., 1996; Figs 2, 3). However, at least some proportion of CNCC from r3 and r5 emigrates but joins major streaming cell populations from adjacent even-numbered rhombomeres (Birgbauer et al., 1995), suggesting that other mechanisms may also contribute to the segregation of CNCC into distinct streams. Cells that survive the early apoptotic elimination of premigratory CNCC in r3 and r5 are restricted to tiny contributions within the second arch skeleton and muscle (Köntgesis and Lumsden, 1996). Several members of the ephrin and ephrin receptor family are expressed in premigratory and migratory CNCC, and the surface ectoderm of the branchial arches (Gale et al., 1996; Flenniken et al., 1996). Migrating CNCC may be guided by these molecules as was observed for trunk neural crest cells (Wang and Anderson, 1997).

In addition to pattern formation, apoptosis in CNCC may serve to eliminate a reserve population of cells that would be mobilized as a compensatory mechanism under circumstances when the normal population of cells would fail to develop. This is supported by the observation that ablation of the dorsal hindbrain in the chicken embryo before HH stage 9 resulted in the remaining hindbrain cells up-regulating to reform CNCC

(Hunt et al., 1995; Buxton et al., 1997), which subsequently re-routed and re-populated the rhombomeres (Saldivar et al., 1997). In addition, when individual rhombomeres were explanted into cultures, large numbers of CNCC from r3 and r5 survived and emigrated out of the explant similar to those from r2 and r4 (Graham et al., 1993, 1994; Fig. 6). Similarly, interdigital cells which would normally undergo apoptosis survived when released from digital constraints in a recombinant limb model (Ros et al., 1997). These results demonstrate the potency of r3 and r5 CNCC to contribute to normal craniofacial development.

Msx2-directed CNCC apoptosis may also be involved in mechanisms leading to craniofacial malformations. In transgenic mice overexpressing wild type or a point mutant form of Msx2, premature closure of cranial sutures mimicking Boston-type craniosynostosis was observed (Liu et al., 1995). In another Msx2 transgenic mouse line, multiple craniofacial defects, including mandibular and maxillary deficiencies, were characteristic of the phenotype (Winograd et al., 1997). These defects could be attributed to Msx2-induced excessive apoptosis in the differentiating CNCC destined to form the mesenchyme of the sutural structure (Couly et al., 1993) or the first branchial arch. This is further supported by observations that embryonic exposure to retinoic acid or ethanol resulted in deregulation of Msx2 expression and consequent craniofacial dysmorphogenesis (Brown et al., 1997; Rifas et al., 1997). Antisense deoxyoligonucleotide inhibition of Msx2 from whole embryos in culture also produced severe craniofacial defects although association with CNCC apoptosis was not explored (Foerst-Potts and Sadler, 1997).

Apoptotic signaling pathway of Msx2 and BMP4

BMP4 and Msx2 are normally expressed in the chicken hindbrain in r3 and r5. Their expression is closely associated with focal apoptosis of premigratory CNCC in those rhombomeres (Graham et al., 1993, 1994). Exogenous recombinant BMP4 added to rhombomere explant cultures upregulated Msx2 expression and induced CNCC apoptosis (Graham et al., 1994). BMP4 as well as BMP2 and 7 in the limb bud (Ganan et al., 1996; Macias et al., 1997) and BMP2 and 4 in the mandibular and maxillary processes (Barlow and Francis-West, 1997) can induce apoptosis, suggesting that the actions of BMP4 may be cooperatively mediated by heterodimers of BMP2 and 4, or BMP4 and BMP7. Expression of the dominant negative type IB BMP receptor in chicken embryonic hindlimbs greatly reduce interdigital apoptosis and Msx2 expression (Zou and Niswander, 1996), whereas expression of the constitutive active type IB BMP receptor results in increased apoptosis (Zou et al., 1997). In the mandibular and maxillary processes, ectopic application of BMP2 and BMP4 can induce Msx2 expression and apoptosis (Barlow and Francis-West, 1997). Taken together, these data suggest that BMP induced apoptosis by up-regulating Msx2 expression. Recent in vitro study using P19 cells demonstrated that the combination of retinoic acid and BMP2 or BMP4 synergistically induces apoptosis (Glozak and Rogers, 1996), and constitutive ectopic Msx2 expression results in an increase in apoptosis induced only upon cell aggregation, but has no effect when cells are allowed to interact with the substrate (Marazzi et al., 1997). Our in ovo data in this report is not only consistent with previous findings, but also demonstrates a physiological consequence of Msx2-mediated BMP-induced apoptosis.

It is likely that the initial steps of BMP4 signaling are transduced by transmembrane serine/threonine kinase receptors, BMPR-I and BMP-RII (for reviews, see Massague, 1996; Reddi, 1997), and cytoplasmic effectors of the Smad or TAK1 pathways (Massague, 1996; Baker and Harland, 1997; Fanger et al., 1997; Yamaguchi et al., 1995; Shibuya et al., 1996). It is unknown whether Msx2 is further downstream of Smads, TAK1 or as yet unidentified signaling cascades. Using the yeast two-hybrid system and in vitro binding assays, two Msx2 signaling partners have been identified: Miz1, a zinc finger transcription factor (Wu et al., 1997), and Dlx2 or 5 which are homeoproteins (Zhang et al., 1997). However, functional significance of these interactions is yet to be determined.

In summary, we have investigated the role of Msx2 within rhombomeres during chick embryogenesis. Previously, using even- as well as odd-numbered rhombomere explants in vitro, BMP4 was shown to increase Msx2 expression in the odd-numbered rhombomeres resulting in apoptosis (Graham et al., 1994). A temporal and positional change in the response of rhombomere neuroectodermal cells to BMPs might contribute to the diversification of neuronal differentiation, transdifferentiation of neuroectoderm into CNCC or apoptosis. Our results from ‘gain-of-function’ Msx2 expression in either odd- or even-numbered rhombomeres in ovo, prior to crest cell emigration, resulted in increased apoptosis of the three major populations of CNCC (Figs 4, 5) supports the assertion that Msx2 can serve as a gatekeeper for neuroectodermal cell diversification. The mechanisms by which BMPs and Msx2 control specific cell lineages and patterns may also be crucial through many subsequent stages of development.

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