Mammalian craniofacial embryology *in vitro*

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ABSTRACT   Our review demonstrates that the whole embryo culture system established by New and his colleagues, in combination with beneficial fluorescent dye cell-tracing techniques, has greatly contributed to many advancements in the field of mammalian craniofacial embryology, especially with regard to elucidating the developmental behavior of cephalic crest cells. In addition, based on recent results, further combining whole embryo culture with mandibular organ culture methods has allowed us to trace cranial crest cells for a much longer developmental period, i.e., presently up to the cap stage in odontogenesis.

KEY WORDS: *mammalian whole embryo culture, cranial neural crest cells, DiI, Small eye rat, Pax-6 gene, retinoic acid, mandibular teeth*

Introduction

Only after New and his colleagues had established a new type of *in vitro* mammalian whole embryo culture system early in the 1970's (New and Coppola, 1970; New, 1971; New *et al.*, 1973), was it possible to practically investigate normal development in mammalian embryos during organogenesis. Initially, these culture techniques were predominantly applied in teratological *versus* developmental studies, e.g., to examine embryotoxic effects of drugs without administrating them through the maternal placenta and to screen the teratogenicity of various agents. Their application in developmental studies went on to markedly increase due to the availability of suitable materials allowing cell tracing during various stages of development since the end of the 1980's, fluorescent vital dyes such as DiI and DiO have shown the greatest promise for practically tracing cells in cultured mouse and rat embryos, having significantly elucidated cell migration pathways and lineages, especially with regard to understanding developmental behavior of mammalian neural crest cells. Here, we review associated advances, particularly focusing on those derived by the employment of DiI techniques.

Improvements in whole embryo culture: advantages of the rotator system

Several techniques have been developed for performing mammalian whole embryo culture, of which the roller bottle system and rotator system are most commonly used (for review see New, 1971, 1978, 1990; Cockroft, 1990; Eto and Osumi-Yamashita, 1995). Based on its inherent advantages, we refined the original

rotator system designed by New and Cockroft (1979), with the equipment now being commercially available (Ikemoto Co., RKI 10-0310). The newly developed whole embryo culture system (Eto and Takakubo, 1985a,b; Ohbayashi and Eto, 1986; Takakubo *et al.*, 1986; Eto and Osumi-Yamashita, 1995) is highly advantageous due to the following features. First, oxygen is continuously supplied by gas mixture cylinders containing predetermined concentrations of oxygen that are based on meeting embryo requirements during developmental stages of culture (Table 1). This feature makes the system much more convenient in comparison with the roller bottle system in which the gas mixture in the bottle must be replaced once or twice a day. More importantly though, it allows the gas flow rate to be increased such that at advanced stages more oxygen can be supplied to culture whole embryos, which is not possible using the roller bottle system. In order to supply high oxygen it is required to open the yolk sac membrane at the certain developmental stage (Table 1; also see Cockroft, 1973; Eto *et al.*, 1981). Second, as embryos are housed in see-through glass bottles containing 2-3 ml of medium, each of which has a center-holed silicon plug connecting it to the hollow rotator drum, this makes it is easy to observe embryos without removal from the incubator. Moreover, up to 12 bottles can be connected to one rotator drum, and with one embryo per bottle, this provides ease in experimental manipulation of individual embryos.

Table 1 summarizes various protocols for culturing rat embryos using our system. Embryos at the primitive streak stage (9.5 days) and head fold stage (10.5 days) can be cultured for 3 days or more, with resultant development being nearly equivalent to that *in vivo* (Osumi-Yamashita *et al.*, 1994, 1996; Lee *et al.*, 1995; see also New *et al.*, 1973, 1976; Cockroft, 1976). Embryos explanted at later

TABLE 1

CULTURE PROTOCOLS FOR POSTIMPLANTATION RAT EMBRYOS[a]

Explantation (days)	Initial somites	Medium/embryo[b] (ml)	Time dependent proportion of O_2[c] (%)	Culture period (h)	Final somites
9.5 1	0	1	5% (0 h-) 20% (36 h-) 60% (48 h-) 95% (60 h-) OYS at 72 h	96 h	40-
10.5	-10	1.5-2	5% (0 h-) 20% (12 h-) 60% (24 h-) 95% (36 h-) OYS at 48 h	72 h	40-
11.5	22	2.5-3 MC at 48 h	95% OYS at 24 h	72 h	-50[d]
12.5 (OYS)	34	3[e] MC at 24-36 h	95%	60 h	-50[d]

[a]Based on our data using Sprague-Dawley rat embryos and whole embryo culture equipment (Ikemoto Co., RKI 10-0310). [b]Medium is 2 mg/ml glucose in 100% rat serum. At least 2 ml per bottle of medium is required. More than 3 ml of medium should not be poured to avoid its flowing into the rotating drum. [c]Gas mixture contains 5% CO_2 and the balance, if any, is N_2. Timing of changing gas mixture can be earlier or later up to 3 h, but high oxygen is toxic for embryos before neural tube closure. [d]Tail somites do not increase due to degeneration of the tail. [e]Substituted with serum diluted with Tyrode's solution at 2-3 times. OYS, opening of the yolk sac membrane. MC, medium change.

stages cannot be cultured for such a long period, as the placenta cannot develop in this culture and its functions are essential for normal development of embryos. It should be noted that if embryos are to be cultured beyond the 30-somite stage, then the timing of opening the yolk sac membrane becomes critical.

The protocol for rat embryos also applies to mouse embryos at the same developmental stage (Osumi-Yamashita *et al.*, 1992, 1994, 1996), though with lower oxygen requirements and less medium. The development of cultured mouse embryos is, however, somewhat poorer than that of rat embryos: a tendency that is possibly due to using a medium composed of heterospecific serum.

Cranial neural crest cell migration and facial development

The merits provided by the rotator system make it a powerful tool for investigating several important morphogenetic events occurring over various developmental stages, e.g., segmentation and patterning of the neural tube and migration of neural crest cells. In amphibian and avian embryos, transplantation of different species enables observation of migration patterns such that the differentiation fates of crest cells derived from certain regions can be elucidated. Regarding mammalian embryos, however, corresponding transplantation experiments are difficult; thus vital dye labeling is commonly applied for tracing crest cells, with fluorescent dye being frequently used (e.g., DiI and DiO). In addition, labeled crest cells obtained from donor embryos can be injected into host embryos, which allows observation of the behavior of such donor cells within the host environment. Towards gaining a better understanding of craniofacial morphogenesis, the present review briefly follows various results which have clarified the migratory behavior of cranial crest cells in normal and abnormal development.

Migration of cranial crest cells in normal development

Populations of neural crest cells are formed at the junction between the surface ectoderm and neural plate, and each migrates along particular routes toward selected regions within the embryonic body, subsequently differentiating into various types of cells. Populations of cranial neural crest cells derived from the rostral neural plate contribute to the formation of facial structures. Our tracing studies on mammals using whole embryo culture and those by others have elucidated the migration pathways of cranial crest cells forming the craniofacial structures (Tan and Morriss-Kay, 1986; Serbedzija *et al.*, 1992; Matsuo *et al.*, 1993; Osumi-Yamashita *et al.*, 1994, 1996; Trainer and Tam, 1995; Osumi-Yamashita, 1997).

In the frontonasal region, the mesenchyme is formed from crest cells emigrating from the forebrain and midbrain (Matsuo *et al.*, 1993; Osumi-Yamashita *et al.*, 1994). Specifically, midbrain crest cells migrate through the region between the presumptive lens placode and optic cup, reaching the frontonasal region (Fig. 2) and then forming the lateral nasal prominence (LNP). Forebrain crest cells also migrate into the frontonasal region (Fig. 2), contributing to the formation of the medial nasal prominence (MNP), which will be discussed later in relation to craniofacial defects observed in the mutant rat.

In the pharyngeal region, crest cell migration is segmentally organized, being especially apparent in the preotic area (Fig. 2) (Fukiishi and Morriss-Kay, 1992; Serbedzija *et al.*, 1992; Matsuo *et al.*, 1993; Osumi-Yamashita *et al.*, 1994, 1996; Trainor and Tam, 1995). The segmental distribution of crest cells is shown to be coordinated with mesodermal cells existing along pathways of crest cells (Trainor and Tam, 1995). Segmental pharyngeal arches in birds are known to be mainly formed by the migration of crest cells emigrating from even-numbered rhombomeres, while those from rhombomere 3 (r3) and r5 either join populations from adjacent even-numbered rhombomeres (Sechrist *et al.*, 1993; Birgbauer *et al.*, 1995; Shigetani *et al.*, 1995), or die soon after emigration (Lumsden *et al.*, 1991; Graham *et al.*, 1993). Thus, segmental migration of hindbrain crest cells in avian embryos is considerably based on alternating odd- and even-numbered rhombomeric organization. On the other hand, the manner in which hindbrain crest cells migrate into pharyngeal arches in mammals

is somewhat different at the initial stage. First, mammalian cranial crest cells start to migrate at the 4- to 5-somite stage before formation of rhombomeres or closure of the neural tube (Nichols, 1981, 1986; Tan and Morriss-Kay, 1985). Instead, hindbrain crest cells emigrate from *prorhombomeres*, structures which are unique to mammals and which subsequently develop into alternating odd and even rhombomeres (Bartelmez, 1923; Adelman, 1925; Bartelmez and Evans, 1925; Trainor and Tam, 1995; Osumi-Yamashita *et al.*, 1996). More specifically, crest cells from the midbrain and prorhombomere A (proRhA) (future r1 and r2) migrate to the first pharyngeal arch and those from proRhB (r3 and r4) migrate to the second (hyoid) arch, while cells from the caudal prorhombomeres, proRhC (r5 and r6) and proRhD (r7), migrate to the third and more posterior arches. Second, zones free of crest cells exist at the boundaries of proRhA/B (the preotic sulcus) and proRhB/C in mammalian embryos (Fig. 2) (Tan and Morriss-Kay, 1985). These crest-free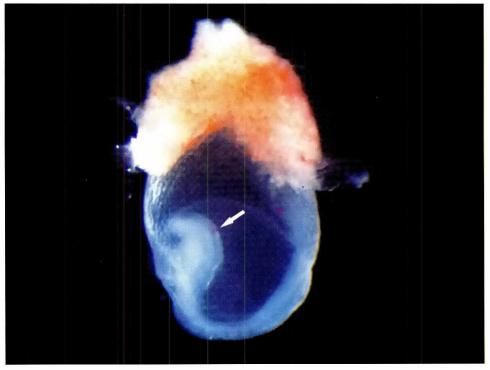 zones in the hindbrain correspond to the areas where expression of a neural crest-specific cell adhesion molecule cadherin 6 is absent at the onset of cranial crest cell migration (Inoue *et al.*, 1997). Contrastingly, in avian embryos, cadherin 7 is specifically expressed in the migrating crest cells, but its expression pattern is not metameric in the hindbrain neural plate (Nakagawa and Takeichi, 1995). Existence of such crest free zones is likely to play some key role in initial sorting of hindbrain crest cells emigrated from different prorhombomeres to form segmental pharyngeal arches in mammalian embryos. Later on, the situation in mammals becomes similar to that in avian since r3 and r5 do not produce crest cells and are obviously negative for cadherin 6 (Inoue *et al.*, 1997), while crest cells are extensively provided from even-numbered rhombomeres where roots of cranial ganglia are formed.

The expression of *Hox* genes appears interesting in respect of prorhombomeric organization of hindbrain crest cells. They show spatially restricted patterns of expression prior to the formation of rhombomeric morphology and later map to specific rhombomeric boundaries (for review see Krumlauf, 1993 and references therein). Most *Hox* paralogs have similar anterior boundaries of expression, being primarily expressed in a two-rhombomere periodicity and in pharyngeal arches as shown in Figure 3 (see also Fig. 9 in Osumi-Yamashita *et al.*, 1996). Derivatives of proRhB (r3 + r4 and second arch) express *Hoxb-2*, those from the anterior region of proRhC (r5 + r6 and third arch) express *Hoxa-2 + b-2* and *Hoxa-3 + b-3 + d-3*; and those from the proRhD (r7 and fourth arch) express *Hoxa-2 + b-2*, *Hoxa-3 + b-3 + d-3* and *Hoxa-4 + b-4 + d-4*. Apparently, expression of some other *Hox* genes, e.g., *Hoxa-1*, *Hoxb-1*, and *Hoxa-2*, deviate from this rule. *Hoxb-1* expression becomes to be restricted in a single rhombomere (r4) 6 h before rhombomeres are

Fig. 1. Lateral view of a rat embryo at the stage of cranial crest cell migration. *A fluorescent dye, DiI, is injected into the midbrain crest region (arrow) and labeled crest cells and their progeny can be traced after whole embryo culture.*

morphologically visible (Muphy and Hill, 1991). However, initial expression is observed along the neuroepithelium with the sharp anterior boundary at the preotic sulcus (proRhA/proRhB boundary), and this early expression pattern is identical to that of *Hoxa-1* (Murphy and Hill, 1991). Expression patterns of *Hoxa-2* is more complex. Its transcripts appear in the presumptive r3 domain at the 5-somite stage, and in r3 and r5 at the 10-somite stage, while strong expression is seen in r3, lower levels in r2 and r5, and in the neural crest cells derived from r4, yet importantly not in those from r2 at the 12-somite stage, and homogeneous low level of expression is seen in the neural tube posterior to the r1/r2 boundary, upon which higher levels in r3 and r5 are superimposed, in the 20-somite stage (Nonchev *et al.*, 1996). It should be noted that *Hox* genes are cloned as homologs of the *Drosophila* homeotic genes involved in specification of individual segments, which implies that a relation may exist between the expression pattern of the genes, or *Hox* code, and the pre-patterned information contained in hindbrain crest cells (Krumlauf, 1993; see also Noden, 1983 on pre-patterning of the cranial crest). Abnormal morphogenesis in mice due to transgenic disruption of these genes provides evidences that this is actually the case (Chisaka and Capecchi, 1991; Chisaka *et al.*, 1992; Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993). Accordingly, it is assumed that prorhombomeres serve as the basic metameric units in mammalian craniofacial development.

Although the first arch and frontonasal region are located just rostral to the domain regulated by the *Hox* code, other types of regulatory genes are thought to be involved in morphogenesis of the rostral head. For example, *Otx2*, a homeobox gene, is expressed in the first arch and frontonasal region (Ang *et al.*, 1994), and heterozygous disruption of the gene results in malformation of
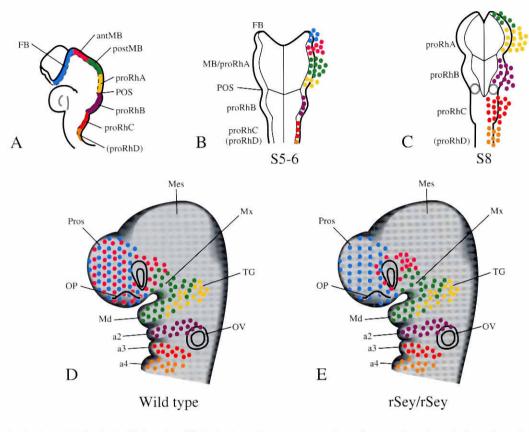
Fig. 2. Diagrams showing migration patterns of cranial crest cells in mouse and rat embryos. (A,B) *Lateral and dorsal views at the 5- to 6- somite stage.* **(C)** *Dorsal view at the 8-somite stage.* **(D,E)** *Lateral view of wild type and homozygous rSey embryos, respectively, at the pharyngula stage.* **(A)** *At the time of mammalian cranial crest cell emigration, four morphological units are present in the rostral neural plate: from anterior to posterior, (1) the forebrain (FB), (2) midbrain (MB) + presumptive prorhombomere A (proRhA, rostral hindbrain), (3)proRhB (preotic hindbrain), and (4) proRhC + presumptive proRhD (caudal hindbrain) (Bartelmez, 1923; Adelman, 1925; Bartelmez and Evans, 1925). Regions in which DiI labeling of crest cells was performed are shown by different colors.* **(B)** *Unlike crest cells in other animals and trunk crest cells, cranial crest cells in mammals emigrate from the neuroepithelium before its closure (Nichols, 1981, 1986; Tan and Morriss-Kay, 1985). At the 5- to 6-somite stage, 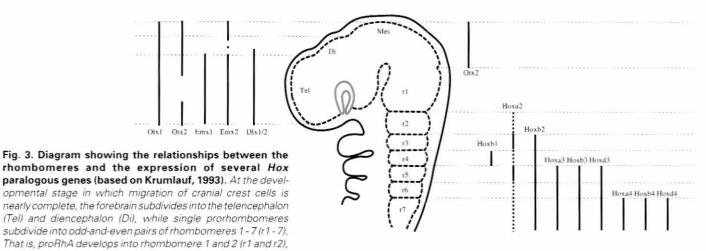crest cells begin emigrating from the forebrain and midbrain/proRhA region.* **(C)** *At the 8-somite stage, zones free of crest cells exist at the boundaries between proRhA/B (preotic sulcus, POS) and proRhB/C (Tan and Morriss-Kay, 1985); thereby making three streams in the hindbrain region. These crest free zones are corresponding to the areas where cadherin 6 gene expression is absent (Inoue et al., 1997). The forebrain cannot be seen in this view.* **(D)** *Normal embryo at the developmental stage in which migration of cranial crest cells is nearly complete. The most anteriorly situated facial primordium is the frontonasal prominence underlying the olfactory placode (OP) where crest cells emigrating from both the forebrain and midbrain migrate to. Caudally, the first pharyngeal (branchial) arch appears, later developing into the maxillary (Mx) and mandibular (Md) prominences which are respectively the primordia of upper and lower jaws. Situated further caudally are the second (hyoid), third, and fourth (and sixth, though not clearly identified) pharyngeal arches (a2, a3, and a4). Crest cells derived from the posterior midbrain and proRhA migrate to the first arch, those from the proRhB to the second arch, and those from proRhC and proRhD to the third and fourth arches, respectively.* **(E)** *In homozygous rSey embryos, migration of midbrain crest cells into the frontonasal region is specifically impaired, though crest cells from other regions migrate normally.* **(D)** *is based on DiI labeling results by Osumi-Yamashita et al. (1994, 1996).* **(B and C)** *are slightly modified from Figure 6 in Tan and Morriss-Kay (1985) who observed crest cell emigration in the rat by scanning electron microscopy.* **(E)** *is based on the results in Matsuo et al. (1993).*

the mandibular arch and the sphenoethmoidal region (Matsuo *et al.,* 1995); while disruption of *Dlx-2,* another homeobox gene expressed in craniofacial regions (Bulfone *et al.,* 1993a,b), results in malformation of the posterior portion of the sphenoid and larger wing (Qiu *et al.,* 1995). Clearly then, integrated analysis of gene expression and cell lineage tracing are needed to reveal the mechanisms involved in morphogenesis of the rostral head.

Abnormal behavior of midbrain crest cells in Small eye rat embryos

The *Pax-6* gene belongs to the *Pax* family of DNA binding transcription factors which are highly conserved in many species (Gruss and Walther, 1992), having been considered as a "master control gene" for eye development (Halder *et al.,* 1995). Mutations in mouse, rat, and fly *Pax-6* genes produce an eyeless phenotype in the homozygous condition, while those in the human gene have been found in patients with a variety of eye disorders including aniridia and Peter's anomaly (reviewed by Hanson and Van

Heynigen, 1995). As a means of investigating these interesting issues in morphogenesis, we have systematically analyzed developmental defects exhibited by the *Small eye* rat (*rSey*), a homozygous mutant lacking eyes and nose due to an impairment in which the frontonasal ectoderm fails to form lens and olfactory placodes (Fujiwara *et al.,* 1994). As will be described elsewhere, these homozygotes also show developmental disorders in the central nervous system (CNS). Newborn mutants exhibit a particularly interesting phenotype in the craniofacial region: the lateral wall of the nasal capsule is completely missing, and a cartilaginous rod-like structure is present in the midline (Osumi-Yamashita *et al.,* 1997). Regarding embryonic development, LNP is not formed, though other facial primordia appear unaffected (Matsuo *et al.,* 1993; Fujiwara *et al.,* 1994). Based on these malformations and the fact that the nasal region is populated by crest cells derived from the forebrain and midbrain as previously mentioned, we hypothesized that migration of midbrain crest cells is specifically impaired in homozygous *rSey* embryos. This was subsequently proven true

Fig. 3. Diagram showing the relationships between the rhombomeres and the expression of several *Hox* paralogous genes (based on Krumlauf, 1993). *At the developmental stage in which migration of cranial crest cells is nearly complete, the forebrain subdivides into the telencephalon (Tel) and diencephalon (Di), while single prorhombomeres subdivide into odd-and-even pairs of rhombomeres 1 - 7 (r1 - 7). That is, proRhA develops into rhombomere 1 and 2 (r1 and r2), proRhB into r3 and r4, proRhC into r5 and r6, and proRhD into r7 (also see Fig. 9 in Osumi-Yamashita et al., 1996). Most of 3' Hox genes exhibit two-rhombomeric periodicity in their expression patterns. It is of noteworthy that neuroepithelial and neural crest derivatives from a common prorhombomere have the same Hox code, a combinational expression of Hox genes (see Fig. 9 in Osumi-Yamashita et al., 1996). For example, derivatives of proRhB (r3 + r4 and second arch) express Hoxb-2, those from the anterior region of proRhC (r5 + r6 and third arch) express Hoxa-2 + b-2 and Hoxa-3 + b-3 + d-3; and those from the posterior region of proRhC (r7 and fourth arch) express Hoxa-2 + b-2, Hoxa-3 + b-3 + d-3, and Hoxa-4 + b-4 + d-4. Apparently, Hoxa-1, Hoxb-1, and Hoxa-2 deviate from this rule, though their expression patterns dramatically change with developmental stages (see text). Importantly, none of these genes are expressed in the first arch. Other classes of homeobox genes, e.g., Otx1, Otx2, Emx1, Emx2, Dlx-1, and Dlx-2, expressed in the more rostral segments in the developing CNS as well as craniofacial mesenchyme, are also shown due to their significance in craniofacial development.*

by performing DiI labeling *in vitro* experiments; i.e., as in normal embryos, homozygous crest cells emigrating from the midbrain were found to aggregate at the ophthalmic placode, the region adjacent to the anterior cardinal vein, whereas in contrast they did not subsequently enter the frontonasal region (Fig. 2E) (see also Matsuo *et al.*, 1993). Since *Pax-6* is strongly expressed in the frontonasal ectoderm at the stage when crest cell migration occurs, though no expression has been detected in the crest cells themselves (Li *et al.*, 1994; Osumi-Yamashita *et al.*, 1997), we surmised that such impaired migration is a cell non-autonomous event caused by loss of function of the *Pax-6* gene.

To clarify whether or not this is the case, we investigated migration of midbrain crest cells to determine if their impairment is a region-specific event dependent on the migratory pathway. Based on the knowledge that orthotopically injected crest cells will generally follow the migration pathway of neighboring host cells, normal midbrain crest cells were isolated from wild type embryos, labeled with DiI, and orthotopically injected into host *rSey* and wild type embryos. The cells injected into wild type embryos showed the same normal migratory behavior, while as expected, those injected into homozygous embryos still showed impaired migration (Osumi-Yamashita *et al.*, 1997), thereby demonstrating that migration of midbrain crest cells is regionally affected by the frontonasal ectoderm along which midbrain crest cells migrate.

Abnormal behavior of hindbrain crest cells induced by retinoic acid treatment

Treatment of embryos with retinoic acid (RA) induces various deformities in craniofacial structures depending on its administration procedure, dose, and embryonic stage at exposure (for review see Morriss-Kay, 1993; Osumi-Yamashita, 1996). Simeone *et al.* (1995) performed detailed time-course analyses to clarify the effect of maternal RA treatments on the development of the mouse

CNS, considering stages from the start of gastrulation throughout induction and patterning of the neural tube. At 6.5 days and later, RA induces dramatic changes in the anterior-posterior patterning of the craniofacial region, e.g., administration between 7.2 and 7.4 days produces atelencephalic microcephaly with the loss of anterior sense organs. Similar phenotypes have also been reported by Conlon and Rossant (1992) and Marshall *et al.* (1992). In particular, by observing patterns of motor neuron distribution in rhombomeres of mouse embryos treated with RA at 7.5 days, Marshall *et al.* (1992) found that the identity of the trigeminal nerve is transformed into that of the facial nerve, which suggests that RA induces "posterior transformation" on the identity of the hindbrain. We also observed similar phenotypes in Wistar rat embryos, i.e., after subjecting embryos at 9.0 days (corresponding to 7.5 days in the mouse) to a 6-hour treatment with all-*trans*-RA, posteriorization was induced in the CNS and pharyngeal arches (Fig. 4) (Lee *et al.*, 1995). In contrast, corresponding treatment at 9.5 days induced fusion of both the first and second arches and the trigeminal and acousticofacial ganglia (Fig. 4, see Lee *et al.*, 1995). Fused arches have also occurred in mouse embryos treated *in vitro* and *in vivo* with all-*trans* or 13-*cis* RA (Goulding and Pratt, 1986; Webster *et al.*, 1986; Brown *et al.*, 1992; Leonard *et al.*, 1995).

DiI labeling was also performed to examine the migratory behavior of hindbrain crest cells in such transformed and fused pharyngeal arches. Of particular interest, in embryos treated at 9.0 days, hindbrain crest cells derived from proRhA and proRhB showed ectopical migration into the second and first arches, respectively (Fig. 4), indicating that RA affects the identity of these cells. In embryos treated at 9.5 days, however, crest cells derived from these prorhombomeres migrated to the anterior and posterior region within the fused arch (corresponding to the first and second arch, respectively), indicating that RA does not affect the differential identities of these crest cell populations (Fig. 4). Therefore, the
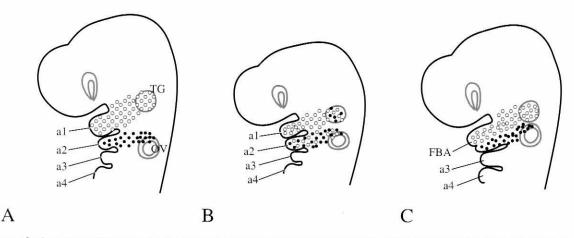
Fig. 4. Diagram of migration paths of hindbrain crest cells in (A) normal embryos and in embryos treated *in vitro* with RA at the (B) early and (C) late neural plate stage *(per from Lee et al., 1995). Neural crest cells from the midbrain/proRhA are shown as open circles and those from proRhB as solid circles. The migration of hindbrain crest cells appears to be stage-dependently influenced by RA in a manner leading to the formation of different phenotypes in craniofacial structures. (A) In control embryos, hindbrain crest cells show a segmental distribution pattern: i.e., cells from the midbrain/ proRhA migrate to the first branchial arch (a1) including the maxillary and mandibular prominences, and the trigeminal ganglion (TG); while those from proRhB migrate to the second branchial arch (a2) and acousticofacial ganglion. (B) In embryos treated at the early stage, the distribution patterns of hindbrain crest cells are different in that some of the midbrain/proRhA crest cells alter their migration path posteriorly to the second branchial arch and acousticofacial ganglion. (C) In late-stage RA-treated embryos, even though the fused branchial arch (FBA) is present and fusion of trigeminal ganglion and acousticofacial ganglion takes place, the segmental distribution pattern of crest cells remains the same as in control embryos; i.e., labeled cells from the midbrain/proRhA migrate to the anterior part of the FBA including the TG, while those from proRhB migrate to its posterior part including the acousticofacial ganglion. OV, otic vesicle.*

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Cranial neural crest cells and tooth development

It is generally known that teeth are formed by reciprocal interactions between the epithelium and mesenchyme in the first pharyngeal arch (for review see Thesleff *et al.*, 1995). Although mammalian midbrain and hindbrain crest cells both contribute to formation of the first pharyngeal arch (Tan and Morriss-Kay, 1986; Serbedzija *et al.*, 1992; Matsuo *et al.*, 1993; Osumi-Yamashita *et al.*, 1994, 1996; Trainer and Tam, 1995), no evidence exists that these cells are directly involved in forming the dental mesenchyme. Therefore, using Sprague-Dawley rat embryos, we carried out DiI labeling in order to determine the precise emigration sites and stages of crest cells migrating into the specific region in the mandibular prominence where tooth germs presumably form in the advanced developmental stage. Evaluation of the resultant distribution of labeled cells indicated that this "tooth-forming" region is predominantly populated by crest cells emigrating from the posterior midbrain up until the end of the 4-somite stage (Imai *et al.*, 1996).

Realizing that whole embryo culture cannot maintain embryos up to the stage in which tooth germs are formed, we established a new type of long-term culture system, i.e., a 60-hour whole embryo culture is followed by a mandibular organ culture (Imai *et al.*, 1996). This "combined" system is novel in that it allows labeled cells to be traced from the early somite stage up until the molars in explants reach the bud stage, which occurs after a 6-day organ culture. As expected, early-emigrating posterior midbrain crest cells were confirmed to contribute to the dental mesenchyme; the first-ever evidence that crest cells are involved in tooth formation. The improved utility gained by the use of our long-term culture system does not stop here, however, since other structures, e.g., Meckel's cartilage and the tongue, are also formed in the explanted mandibles, thereby indicating a high potential to become a valuable tool for elucidating morphogenetic mechanisms other than those related to teeth.

Concluding remarks

Although whole embryo culture techniques possess inherent limitations such that their application in mammalian developmental studies has been restricted to examining early- and mid-organogenesis, a vast number of developmental aspects nevertheless remain to be investigated. For example, in the near future, we intend to direct our efforts towards obtaining a fate map of the mammalian forebrain, the region forming the most highly developed cortex known to man. Moreover, new frontiers are fast appearing as a result of combining whole embryo culture techniques with recent molecular approaches involving application of antibodies, enzymes, antisense DNAs, and retroviruses (Augustine *et al.*, 1993, 1995; Sadler *et al.*, 1995; Båvik *et al.*, 1996; Solursh *et al.*, 1996). Initial findings indicate that a great potential exists for overcoming drawbacks associated with the use of knockout and transgenic mice.

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