Regional variation of cell proliferation within
the facial processes of the chick embryo:
a study of the role of ‘merging’ during
development

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SUMMARY

Variation in rates of cell proliferation along the long axis of the maxillary process, within the lateral nasal process and in the zone of attachment between these structures was analyzed employing DNA labeling indices. Chick embryos were labeled with [3H]thymidine for 1 h and processed for histology and autoradiography. The percentage of labeled mesenchymal cells was determined in delineated areas.

Analysis of labeling indices indicated that rates of cell proliferation varied within each of the facial processes. Regions where rates of proliferation were maintained at elevated levels were the boundary areas of the facial processes (e.g. the anterior tip of the maxillary process) and the zones of attachment between the facial processes (e.g. between the maxillary process and the lateral nasal process). Despite the presence of elevated rates of proliferation in selected regions within the facial processes, however, the percentage of labeled cells in all areas declined with advancing developmental age. These findings support the hypothesis, proposed by Streeter and Patten, that the ‘merging’ of adjacent facial primordia, such as the maxillary and lateral nasal processes, is accomplished by elevated rates of cell proliferation within the zones of attachment compared to the rates of proliferation in adjacent regions.

INTRODUCTION

Descriptions of facial morphogenesis have often cited, and contrasted, the mechanisms of ‘fusion’ and ‘merging’ to describe the attachment and joining of the primordia that form the midface. Fusion – the attachment of the free ends of outpocketings with subsequent breakdown of the epithelial surfaces followed by mesenchymal penetration – has been most associated with joining of the palatal shelves of the maxillary process to form the secondary palate. Merging has been the mechanism associated with the joining of adjacent outpocketings such as the maxillary process with the lateral nasal process, the medial nasal processes with each other, and with the formation of the soft palate (Burdī &

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Faist, 1967; Patten, 1968; Ross & Johnston, 1972). This was described originally by Streeter (1948) as the means by which the contours of the developing face are molded. In Streeter's description of the coalesence of the facial processes, he stated: 'It is more precise to speak of these structures [the facial processes] as swellings or ridges which correspond to centers of growth in the underlying common mesenchyme. The furrows that lie between them on the surface are smoothed out as the proliferation and fusion of the growth centers fill in beneath.' Patten later used the term 'merging' to refer to the changes that Streeter described (1961).

Although these distinctions are presently recognized, the exact role of merging, mesenchymal penetration and epithelial fusion and breakdown in the attachment of certain facial primordia (e.g. the median nasal process with the maxillary process) remains unclear. Further, the basis for the mechanism of 'merging' has not been established. Streeter (and Patten) both believed that the merging of two adjacent primordia was accomplished by regional differences in rates of cell proliferation. In order to test this hypothesis, rates of cell proliferation were analyzed within the maxillary process, the lateral nasal process and in the zone of attachment between the two during the formation, enlargement and subsequent fusion of these structures in the chick embryo.

**MATERIALS AND METHODS**

**Labeling**

Chick embryos were incubated, windowed, staged according to Hamburger and Hamilton (1951), and labeled with \[^{3}H\]thymidine (specific activity 6-7 Ci/mmol, New England Nuclear Company) for 1 h by application of label on top of the embryo through the window in the egg shell. Doses ranged from 15 to 30 \(\mu\)Ci, depending on the age of the embryo; in all cases, the label was diluted to a total volume of 0.1 ml in a balanced salt solution. The developmental stages studied range from Stage 21 to Stage 30 and encompass the formation, enlargement and attachment of the maxillary process with the lateral nasal process.

**Histology and autoradiography**

For paraffin embedding, embryos were fixed in Bouin's solution, dissected, staged, washed in 50 % alcohol, dehydrated through graded alcohols, embedded, and serially sectioned in the long axis of the maxillary process (Fig. 1) at 4 \(\mu\)m (Humason, 1972). For plastic embedding, embryos were fixed in glutaraldehyde and paraformaldehyde (Graham & Karnovsky, 1966), washed in several changes of cacodylate buffer (0.1 M) for 24 h, dehydrated through graded alcohols to acetone, and infiltrated with Epon-acetone mixtures of increasing Epon concentration. They were then embedded in Epon, polymerized for 3 days at 60–70 °C (Luft, 1961), and sectioned parallel to the long axis of the maxillary process at 2 \(\mu\)m. Sections through the center of the maxillary process extending
Fig. 1. Scanning electron microscopic photographs (courtesy, K. K. Sulik) of 3-day (a) and 5-day (b) chick embryos in which the planes of sectioning through the maxillary process (MP), lateral nasal process (LNP) and the zone of attachment (ZA) are identified.
Fig. 2. Representative plastic sections through the maxillary and lateral nasal processes outlining the areas in which mesenchymal cells were counted to obtain labeling indices. At early stages (stages 21–23) (a) the reference point is the tip of the maxillary process from which counting areas were delineated along the maxillary process (numbered squares) and in the frontonasal region (lettered squares). At later stages (stages 25–30) (b) the reference point is the epithelial seam between the maxillary and lateral nasal process at their point of attachment. Counting areas were delineated from an area at this point (lettered x) along the maxillary process (numbered squares) and along the lateral nasal process (lettered squares).

through the zone of attachment into the lateral nasal process were analyzed (Fig. 1).

Slides were washed, coated with liquid autoradiographic emulsion (Eastman-Kodak NTB3), dried, and stored in light-proof boxes at 4 °C, for varying exposure times up to 4 months (Rogers, 1973). Slides were then developed and stained with either Harris’s haematoxylin and eosin (paraffin sections) or with Richardson’s stain (Epon sections).

**Cell counting**

Labeled and unlabeled mesenchymal cells were counted in areas delineated by an ocular grid for the determination of DNA labeling indices. The histological
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Table 1. Comparison of labeling indices in the tip, middle and base of the maxillary process

<table>
<thead>
<tr>
<th>Stage</th>
<th>Region</th>
<th>Tip</th>
<th>Middle</th>
<th>Base</th>
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<tbody>
<tr>
<td>24-5</td>
<td></td>
<td>35-9</td>
<td>35-0</td>
<td>35-9</td>
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<td></td>
<td></td>
<td>39-7</td>
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<tr>
<td>28-8</td>
<td></td>
<td>33-6</td>
<td>22-2</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td></td>
<td>45-6</td>
<td>39-0</td>
<td>41-4</td>
</tr>
<tr>
<td>34-0</td>
<td></td>
<td>24-6</td>
<td>30-4</td>
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</tr>
<tr>
<td>37-3</td>
<td></td>
<td>33-0</td>
<td>34-9</td>
<td></td>
</tr>
</tbody>
</table>

Embryos were labeled, embedded in paraffin, serially sectioned in the sagittal plane along the long axis of the maxillary process and processed for autoradiography as described in the text. Each horizontal line represents values obtained from one embryo.

sections examined for analysis were separated from each other by a sufficient amount of tissue to prevent recounting the same population of cells. Labeled cells were defined as those containing three or more grains above background over the nucleus and at least four sections from each embryo were examined and counted. Cell counting was conducted under oil-immersion at a magnification of 1250 x. Labeling indices for each anatomical area were obtained by dividing the number of labeled cells within a given area by the total number of cells contained within that area.

Paraffin sections through the center, along the long axis of the maxillary process, were analyzed. Counting areas were outlined in the tip, at the base (near the attachment of the maxillary with the mandibular process) and in the middle, at a point equidistant from the counting areas at the tip and base.

Counting areas on Epon sections were outlined along the length of the maxillary process, beneath the epithelium, into the zone of attachment between the maxillary and lateral nasal processes, and in the lateral nasal process (Fig. 2). The anterior limit of the maxillary process in younger embryos and the epithelial seam in the zone of attachment between the maxillary and lateral nasal processes in older embryos were used as reference landmarks. The remaining counting areas were then marked off from this location in both directions. Comparable counting areas on each slide were summed and averaged and a labeling index was obtained for each area with reference to the landmarks noted above.

RESULTS

A preliminary study, employing serially sectioned paraffin-embedded embryos, demonstrated regional variation in rates of cell proliferation in selected regions in the maxillary process. Labeling indices in older embryos (Stage 27) tended to be lower in the middle of the maxillary process than at the tip or at the base.
Fig. 3. Graphs of DNA labeling indices of mesenchymal cells along the long axis of the maxillary process and of mesenchymal cells in the fronto-nasal region anterior to the maxillary process in embryos at Stages 21–23. Embryos were labeled for 1 h and embedded in Epon. Labeling procedures, delineation and sequencing of counting areas, determination of labeling indices, etc., are described in the text and illustrated in Fig. 2. Numbers refer to counting areas in the maxillary process and letters refer to counting areas in the fronto-nasal region.

(i.e. the junction of the maxillary with the mandibular process). This difference was not observed in younger embryos (stage 24-5) (Table 1).

A detailed study employing plastic-embedded embryos was then performed in which labeling indices were obtained in sequence along the entire length of the maxillary process. This analysis was extended into regions anterior to the
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Table 2. Comparison of the means of labeling indices in the zone of attachment and the maxillary and lateral nasal processes

<table>
<thead>
<tr>
<th>Stage</th>
<th>Region</th>
<th>Mean</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>Nasal process</td>
<td>17.4</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>Zone of attachment</td>
<td>23.2</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Maxillary process</td>
<td>19.2</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Nasal process</td>
<td>28.1</td>
<td>11.9*</td>
</tr>
<tr>
<td></td>
<td>Zone of attachment</td>
<td>40.0</td>
<td>11.3*</td>
</tr>
<tr>
<td></td>
<td>Maxillary process</td>
<td>28.7</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Nasal process</td>
<td>15.4</td>
<td>24.5*</td>
</tr>
<tr>
<td></td>
<td>Zone of attachment</td>
<td>39.9</td>
<td>14.6*</td>
</tr>
<tr>
<td></td>
<td>Maxillary process</td>
<td>25.3</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Nasal process</td>
<td>14.2</td>
<td>19.9*</td>
</tr>
<tr>
<td></td>
<td>Zone of attachment</td>
<td>34.1</td>
<td>17.4*</td>
</tr>
<tr>
<td></td>
<td>Maxillary process</td>
<td>16.7</td>
<td></td>
</tr>
</tbody>
</table>

The means of labeling indices in the zone of attachment between the maxillary process and the lateral nasal process (counting areas a, x and 1 of Fig. 4) are compared to those in the maxillary process (counting areas 3, 4 and 5 of Fig. 4) and the lateral nasal process (counting areas c, d and e of Fig. 4). An asterisk denotes significant differences between regions.

maxillary process, including, at later stages, the contiguous lateral nasal process and the zone of attachment (Figs. 3, 4). At early stages (Fig. 3, Stage 21–23) labeling indices along the maxillary process tended, in general, to be higher than those anteriorly in the frontonasal region. Beginning at Stage 25 (Fig. 4), however, when the maxillary and lateral nasal processes approximate each other, a reproducible pattern of variation emerged. Areas of high and low labeling indices, which corresponded to specific anatomical regions, appeared. For example, labeling indices in the zone of attachment between the maxillary and lateral nasal processes and the regions immediately adjacent to the zone of attachment (sections x, a and 1; Fig. 4 and Table 2) remained high. On either side of this zone, where labeling indices declined (sections c, d, e and 3, 4, 5; Fig. 4 and Table 2), the regions coincided, approximately, with the middle of each process. A rise in cell proliferation was again found as the most proximal region of the maxillary process and the most superior region of the lateral nasal process was approached. These regions also correspond to areas of attachment.
Fig. 4. Graphs of DNA labeling indices of mesenchymal cells through the center of the maxillary process, beneath the epithelial seam in the zone of attachment between the maxillary and lateral nasal processes and along the long axis of the lateral nasal process as illustrated in Fig. 1, in embryos at Stages 25 and 26 (a) and at Stages 28 and 30 (b). Embryos were labeled for 1 h and embedded in Epon. Labeling procedures, delineation and sequencing of counting areas, determination of labeling indices, etc., are described in the text and illustrated in Fig. 2. Numbers refer to counting areas in the maxillary process. Letters refer to counting areas in the lateral nasal process. X refers to the counting area in the zone of attachment, beneath the epithelial seam.
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Fig. 4. For legend see opposite.

of one process with another (e.g. the maxillary process with the mandibular process, and the superior borders of the lateral nasal with the medial nasal process).

Data from these and additional embryos were used to calculate labeling indices within 100 μm of the tip of the maxillary process from Stages 21 through 30. As seen in Fig. 5, there is a progressive decline in the labeling indices of the mesenchyme at the tip of the maxillary process throughout this period, despite the fact that rates of cell proliferation near the tip of the maxillary process are as high, or higher, than any other region within it.

The differentiation of cartilage and bone in the regions of the chick embryo examined in this study occurs after 7 days (Murray, 1963). To confirm this, however, we prepared cleared whole-mount 7-day embryos and stained them with Alcian blue and Alizarin red S. No evidence of cartilage or bone was found in any of the regions analyzed in this study.

DISCUSSION

The data presented in this report on regional analysis of cell proliferation support the view that adjacent facial primordia attach by the mechanism
Fig. 5. DNA labeling indices of mesenchymal cells within 100 μm of the tip of the maxillary process from Stages 21–30. Chick embryos were labeled for 1 h with [3H]thymidine, embedded in Epon and processed for autoradiography as described in the text. Counting areas were delineated with an ocular grid; each point represents one embryo in which four or more sections were counted. A regression line was determined by the method of least-squares fit.

described as merging and, further, this mechanism is mediated by regional differences in rates of cell proliferation. Regions such as the boundary between the maxillary and lateral nasal processes contain mesenchyme which is proliferating more rapidly than mesenchyme in the center of the maxillary process or the adjacent region in the lateral nasal process. The data suggest that higher rates of cell proliferation correspond to other anatomical regions where primordia attach (e.g. the maxillary and mandibular processes, the medial and lateral nasal processes superiorly). Differential rates of decline in cell proliferation were observed as the primordia became joined. Preliminary data indicate that the decline in cell proliferation as well as the differences in rates of decline can be attributed to the exit of cells from rapidly proliferating pools and the appearance of subpopulations of quiescent cells. The size and distribution of these quiescent subpopulations may account for the differences in rates of cell proliferation that were found and this is currently being investigated.

The attachment of the facial processes has been extensively discussed in the literature (Streeter, 1948; Warbrick, 1960; Patten, 1961; Andersen & Matthiessen, 1967; Smith & Monie, 1969; Lejour, 1970; Stark, 1973; Waterman & Meller, 1973; Johnston, Hassell & Brown, 1975; Trasler & Fraser, 1977, and others) but the details of the sequence of events that occur during attachment remains unclear. While merging has been considered to be primarily responsible for the
joining of the lateral nasal process with the maxillary process, the mechanism for attachment of the medial nasal process with the lateral nasal and maxillary processes appears to be more complex. The formation of an epithelial 'nasal fin', formed by adjacent epithelial surfaces, followed by breakdown and mesenchymal consolidation is considered by many to be an important early step during attachment (e.g. Streeter, 1948; Warbrick, 1960; Lejour, 1970; Johnston et al. 1975). Others (Andersen & Matthiessen, 1967) consider the formation of a mesenchymal 'isthmus', produced by the invagination of the nasal placode, to be of primary importance. After these initial events, merging has been postulated to occur as a means of consolidating the attachment of the processes. The relative importance of epithelial seam fusion and breakdown, mesenchymal consolidation, and merging during the attachment of these structures, however, has not been determined and a spatial and temporal analysis of regional differences in rates of cell proliferation of mesenchyme during attachment of the medial nasal process with the lateral nasal and maxillary processes, correlated with the accompanying developmental changes in the epithelium, would be warranted.

The findings in this report of higher rates of cell proliferation in areas of attachment of the facial primordia indicate that developmental patterns have been established which regulate the cell cycle with reference to boundary regions. The maintenance of elevated rates of cell proliferation in the zone of attachment could be due to factors associated with the tip of the maxillary process prior to attachment or to changes which occur in this region (and the corresponding one in the lateral nasal process) when the primordia approximate each other.

Recently theoretical models have been proposed in which cells receive 'positional information' with respect to boundary or reference regions and then interpret this information by following a specific course of development (Wolpert & Lewis, 1975). Proximo-distal pattern specification in the chick limb bud has been attributed to the acquisition of positional information in a 'progress zone' within 300–400 μm of the tip under the influence of the apical ectodermal ridge (Summerbell, Lewis & Wolpert, 1973); cells establish their positional values by a timing mechanism with respect to an internal autonomous 'clock' in which the successive cell divisions within the 'progress zone' record time (Lewis, 1975).

The diffusion of a substance whose concentration could be detected by cells from a reference source of fixed concentration has also been proposed as a means of establishing a positional field (Crick, 1970). Development of the distal parts of the limb along the antero-posterior axis appears to be regulated by a small region at the posterior margin of the limb bud (Saunders & Gasseling, 1968) and recent experiments have implied that a diffusible graded signal emanates from this region to provide positional information along the antero-posterior axis of the limb (Tickle, Summerbell & Wolpert, 1975; MacCabe & Parker, 1976; Summerbell, 1979).

Whether these or alternative models can be applied to the development of the
facial primordia remains to be determined. Analogies can be found in the pattern of change in cell proliferation between the developing limb (Hornbruch & Wolpert, 1970; Searls & Janners, 1971; Janners & Searls, 1970) and recent work on cell proliferation in the primary palate (Minkoff & Kuntz, 1977, 1978; Flint & Ede, 1978). The findings in this report reinforce these comparisons and indicate that models which have been proposed to explain limb development may be of value in the analysis of facial morphogenesis.

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REFERENCES


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