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A novel approach to the growth analysis of hamster secondary palate by histone 3 mRNA *in situ hybridization*

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Summary. A study was undertaken to determine the cell proliferation kinetics during the development of hamster vertical palatal shelf *ad initium*. Hamster embryo heads, obtained at different times between days 10 and 12 of gestation (which is the period of vertical shelf development) were processed and sectioned to localize histone 3 mRNA, a cell cycle specific gene, by *in situ hybridization*. Sense and antisense ^{35}S-labelled histone 3 riboprobes were used as hybridization probes. Percent labelled cells were determined. The results showed that a high rate of random proliferation of both epithelial and mesenchymal cells was a major component of early vertical palatal growth. Subsequently, during the latter half of vertical shelf development, the proliferation rates of the epithelial and mesenchymal cells were different in a region specific manner. It was suggested that the spatio-temporal changes in the distribution of cycling mesenchymal and epithelial cells during vertical palate development may indicate their heterogeneity for subsequent segregation into appropriate phenotypes.

Key words: Palate development, Hamster, Cell Proliferation, Histone mRNA, *In situ hybridization*

Introduction

The differences in the formation of secondary palate among various vertebrates are obvious from the beginning of morphogenesis. For example, in fish and mammals, palatal primordia forms vertically *ad initium* (Greene and Pratt, 1976; Shah et al., 1990), whereas in birds and alligators they begin horizontally (Shah and Crawford, 1980; Ferguson, 1981). The primordial growth of palate in fish, birds and mammals is characterized by teratogen-sensitive, species-specific burst(s) of DNA synthesis, indicative of cell proliferation (Burdett et al., 1988; Shah et al., 1989a,b, 1991, 1994a,b). Subsequently, however, as the vertebrate palate morphogenesis advances, the rate of DNA synthesis decreases indicating that the palate formation does not proceed by an orderly series of cell doubling. It is a well recognized principle that the process of growth is an integral part of morphogenesis of an organ/structure and is accomplished through both the acquisition and organization of cells from the beginning (Waddington, 1959). It is, therefore, important to recognize the kinetics of cell proliferation (rate and distribution) in palate from the primordial stage of development.

Previous studies examined cell proliferation only during mammalian palatal shelf reorientation and fusion (Mott et al., 1969; Hudson and Shapiro, 1973; Jelinek and Dostal, 1974; Nanda and Romeo, 1975; Cleaton-Jones, 1976; Brinkley, 1984; Brinkley and Bookstein, 1986; Luke, 1989; Amwayi and Luke, 1990; Singh and Moxham, 1993). In the present study, we have focused on the hitherto unexplored issue of cell proliferation kinetics during the formation of the vertical palatal shelf. This aspect of vertical palate ontogeny is critical in that clinically, an overwhelming majority of human cleft palate patients exhibit severely hypoplastic palatal tissue. Also, from a biological standpoint, it seems that achieving a critical number of cells during early development may be important for completion of subsequent palate morphogenesis (Shah et al., 1989b). Furthermore, it is a common practice in the studies on experimentally induced cleft palate that teratogens are administered either prior to or at the time when the palatal shelves are still growing vertically. In hamster, the vertical growth of the palate is achieved between days 10 and 12 of gestation (Burdett and Shah, 1988). Hence, in the present study, the cell proliferation kinetics were determined during this period.

Histone proteins are active participants in gene regulation during development (Grunstein, 1992; Wolffe and Dimitrov, 1993). They are synthesized at significant rates and their accumulation increases 20-fold in eukaryotic cells during the S-phase of the cell cycle,

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which makes them excellent markers for cycling cells (Coffino et al., 1984; Stein et al., 1989; Heintz, 1991). In normal quiescent cells, replication-dependent histone genes are not expressed. Also, the activities of histones, in concert with other cell cycle related proteins, are important in various aspects of cell growth (Coffino et al., 1984; Sterner et al., 1989; Heintz, 1991). Hence, the correlation between the transcription of histone genes and DNA synthesis can be exploited to determine the kinetics of cell proliferation (Stein et al., 1989; Heintz, 1991). In the present study, we have used ^{35}S-labelled antisense single-stranded RNA probe to the mouse histone 3 cDNA to localize histone 3 (H3) mRNA transcripts by *in situ hybridization*, and detecting the dividing cells to analyze the rates and distribution of proliferating cells during hamster vertical palate development *ad initium*. In the developing tissues, such as palate, the approach also provides the further advantage of by-passing the maternal and placental metabolic effects on isotope incorportation in the cells, and yet allows *in vivo* observations. *In situ hybridization*, using an antisense single-stranded RNA probe to mouse histone 3 cDNA, have been shown to be an extremely sensitive method in detecting cycling cells during various disease processes as well as in developmental circumstances (Chou et al., 1990; Heintz, 1991; Erickson, 1993).

Materials and methods

The methods for environmental conditioning and mating for Golden Syrian hamsters were similar to that described earlier (Burdett and Shah, 1988).

Hamster embryos were obtained at various intervals between days 10:06 (ten days and six hours) and 12:00 of gestation (Fig. 4). The embryonic heads were dissected and fixed in 4% paraformaldehyde, dehydrated in ethanol and embedded in paraffin for 6 μm frontal sections as described by Zeller et al. (1987). Sections only from the middle third of the secondary palate were used for hybridization assays because, in hamster, the morphological steps of palate development begin in the middle third and then extend to the posterior and anterior thirds (Shah and Chaudhry, 1974). The sections were mounted onto gelatin-coated glass slides.

For *in situ hybridization*, the method of Zeller et al. (1987) as modified by Chang et al. (1989) was used. Prior to hybridization, each tissue section was pre-treated and pre-hybridized. Pre-treatment included tissue rehydration, mild acid hydrolysis with 0.2N HCl for 20 minutes at room temperature (RT), post-fixation of tissue with 4% paraformaldehyde, and finally, treatment with 0.25% (v/v) concentration of acetic anhydride in 0.1M triethanolamine (pH 8). Pre-hybridization of the pre-treated tissue sections involved a 2 hour incubation at 37 °C with sufficient quantities (~25 μl/section) of the hybridization solution (50% deionized formamide; 0.3M NaCl; 10mM Tris HCl pH 8.0; 1x Denhardt's solution; 500 μg/ml tRNA; 500 μg/ml poly[A]; 50mM dithiothreitol; 10% polyethylene glycol) without the probe. For hybridization, ^{35}S-labelled antisense single-stranded (ss) RNA probe to the mouse H3 cDNA was used (see below). The ss-RNA probes were added directly to the pre-hybridization solution on the glass slide ($2x10^6$ cpm per section), mixed well, and then incubated overnight at 50 °C under a glass cover slip, sealed with rubber cement. Post-hybridization washing included the following steps: 15 minutes twice in 50% formamide, 2 x SSC, 20mM 2-mercaptoethanol, at 50 °C; 15 minutes twice in 50% formamide, 2 x SSC, 20mM 2-mercaptoethanol, 0.5% Triton X-100, at 50 °C; 5minutes twice in 2 x SSc, 20mM 2-mercaptoethanol at RT; RNase digestion for 30 minutes at 37 °C (40 μg/ml of RNase A, 2 μg/ml RNase T1, 10mM Tris HCl pH 7.5, 5mM EDTA, 0.3M NaCl); 15 minutes in 2xSSC, 20 mM 2-mercaptoethanol at RT; four times each for 30 minutes in 2xSSC, 20 mM 2-mercaptoethanol at 50 °C with gentle shaking; once for 10 minutes in 2xSSC at RT. The slides were then dehydrated, dipped in Kodak NTB 2 emulsion and processed for autoradiography. Exposure time varied between 2-4 days at 4 °C. All slides were counter stained with Giemsa (Fisher #SG-28). In addition, adjacent sections were stained with H&E.

To determine the specificity of the labelling, sections were pre-treated for 30 minutes at 37 °C with 40 μg/ml of RNase A, 2 μg/ml RNase T1, 10 mM Tris HCl pH 7.5, 5mM EDTA, 0.3M NaCl. Specific labelling was not observed in these slides. Additional controls included: ^{35}S-labelled sense strand riboprobe control; and a lack of hybridizing signals in mitotic figure (Chou et al., 1990).

Molecular probes. The mouse histone 3 cDNA probe was provided by Dr. William F. Marzluff (Sittman et al., 1981). The fragment of DNA containing the mouse histone 3 cDNA was cloned into pGEM3 in an orientation such that transcription from the T7 promoter resulted in antisense RNA probe. Production of radio-labelled riboprobes was done by using a RNA transcription kit (Promega, Madison, WI), ^{35}S-UTP (Amersham, SJ. 40383, SP6/T7 & Grade, 850 Ci/mole, Arlington Heights, Illinois), and T7 RNA polymerase (Stratagene, La Jolla, CA). Typically, 80-90% incorporation was obtained. Each *in vitro* transcription reaction yielded ~230 ng of synthesized RNA with a specific activity of ~3 x 10^8 cpm/μg RNA. The final specific activity of the probe used for *in situ hybridization* was ~2 x 10^6 cpm per section.

The proliferation indices of E and M cells were determined in the vertically developing shelves at different times during development. Three to four slides, each carrying a set of 3-4 sections (one set for sense and the other for antisense riboprobe labelling), were prepared from each embryo. One section from each set was counted, simultaneously ensuring that the adjacent section was not used in the analysis. The individual embryos' average was determined by counting at least three sections per embryo. For each gestational age, 3-4 different embryos were counted to determine the percent

epithelial (E) and mesenchymal (M) cells undergoing proliferation. The proliferation index is defined as the number of labelled cells expressed as a percent of the total number of nuclei. A cell was considered labelled when the number of silver grains was five, or more, above the number of the grains in the background. The H3 riboprobe labels only those dividing cells which are in G_1, S, and G_2 phase. Cells in M phase are not labelled (Chou et al., 1990). The boundaries of the developing vertical shelf were identified by the points where the epithelium changed the direction (see Fig. 2 in Burdett et al., 1988).

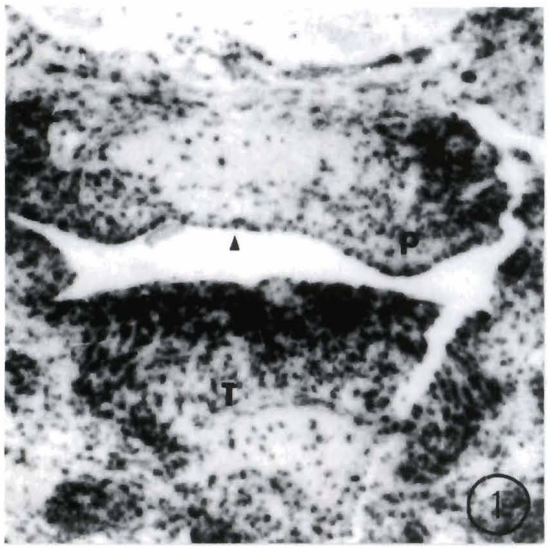
In order to further determine whether the cycling cells in vertically developing shelf were localized in a particular area, or were distributed randomly, the palate sections were divided into four segments by inserting a 10 x 10 marked glass grid into the eyepiece of the microscope. These segments were constructed from a line dividing: (1) a medial (ME) half (adjacent to the tongue) and a lateral (LA) half (away from the tongue), or (2) an upper (U) half (rostral, away from the growing tip of the shelf) and a lower (LO) half (caudal, towards the growing tip of the shelf). The proliferation index for E and M cells was determined in each of the four segments (U-ME, U-LA, LO-ME, LO-LA). The data were evaluated by Friedman two-way analysis of variance, nonparametric Wilcoxon test or Student's t-test (Zar, 1984).

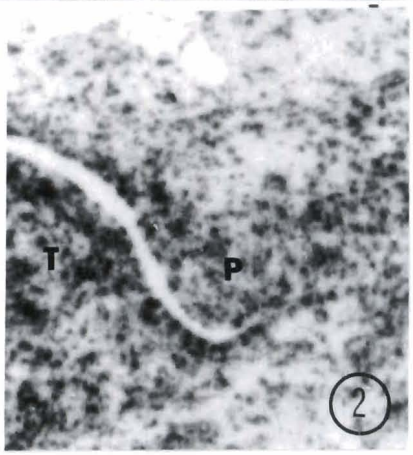
Results

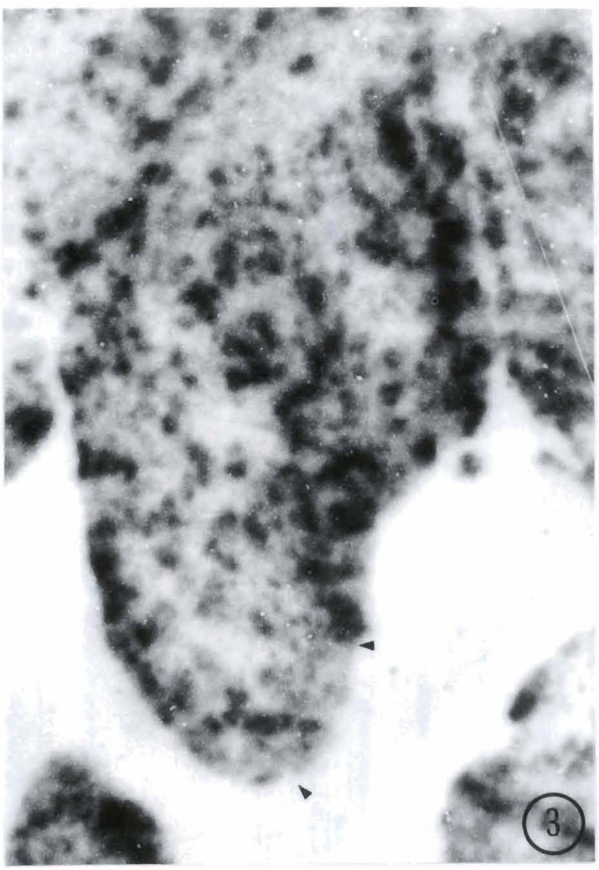
Figures 1-3 shows *in situ* localization of ^{35}S-labelled histone mRNA transcripts in the vertical palate at different times during development. It is clear that there are spatio-temporal changes in the distribution of labelled mesenchymal (M) and epithelial (E) during vertical palate development.

The data in Fig. 4 show that after an initial high on day 10:06, the percent of dividing cells in the mesenchyme of vertically developing palate peaked on day 10:18 of gestation ($P \leq 0.01$). In the ensuing 24 hours, the dividing M cells gradually declined, and on day 12:00 they were reduced by a third of that seen on day 10:18 of gestation.

The dividing E cells in the developing palate followed a trend similar to that seen for M cells during







Figs. 1-3. Frontal sections of vertically developing palate of hamster embryo showing *in situ hybridization* detection of histone 3 mRNA.
1. There are more heavily labelled cells in the palatal primordia (P) than in the roof of the mouth (arrowhead). Tongue (T). Day 10:06 of gestation. x 130.
2. Vertical shelf on day 10:18 of gestation. Tongue (T). x 190.
3. Vertical palatal shelf on day 12:00 of gestation. The epithelial cells in the lower medial half (between arrowheads) are not labelled. x 400

the initial 24 hours except that the peak percent of labelled cells was seen on day 11:00 of gestation (Fig. 4). During the next 12 hours, the percent of dividing E cells declined by 50% ($p \leq 0.001$) before showing an increase again.

Figure 5 shows that until day 10:12 of gestation, dividing M cells were equally distributed in all four segments of the developing palate. On day 10:18 of gestation, however, percent dividing M cells increased, albeit at different rates, in all segments ($P<0.01$). The increase was 4-fold in U-ME and approximately 2-fold in the other three segments. The rank order of the proliferation rate was U-ME, U-LA, and LO-LA = LO-ME. Subsequently, the rate of dividing M cells gradually declined in U-ME and both the lower segments ($P<0.01$), but remained unchanged in U-LA segment. On day 12:00 of gestation, although the dividing M cells were equally distributed between the two upper segments, and between the two lower segments, the rate of proliferation was twice as high in the upper half (U-MA and U-LA) than in the lower halves (LO-ME and LO-LA) ($P<0.01$).

On the otherhand, dividing E cells showed a different pattern of distribution (Fig. 6). Initially, until day 10:12 of gestation, the distribution of dividing E cells was higher in the two lateral than in the two medial segments ($P<0.05$). On day 10:12 of gestation, the dividing E cells were equally distributed in all four segments. Subsequently, with the exception of LO-ME segment (which is the region of the prospective medial edge epithelium of the horizontal shelf), the rate of E cell proliferation increased in the other three segments ($P<0.01$). The rank order of the proliferation rate was U-LA, LO-LA, U-ME and LO-ME. During the ensuing 24 hours, the rate of proliferating E cells remained 2-5 fold higher in the U-LA in comparison to the other three segments where the rate of E cell proliferation declined ($p<0.01$).

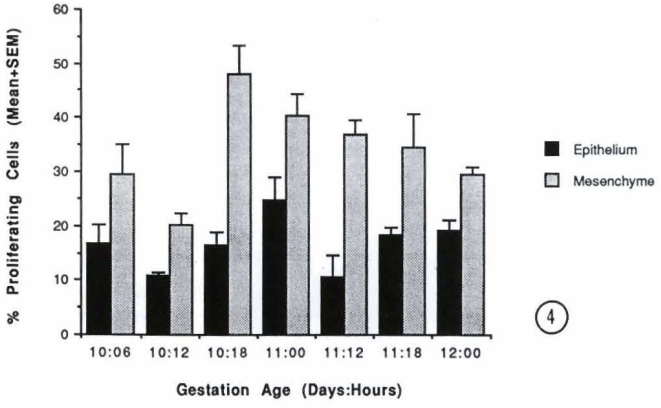
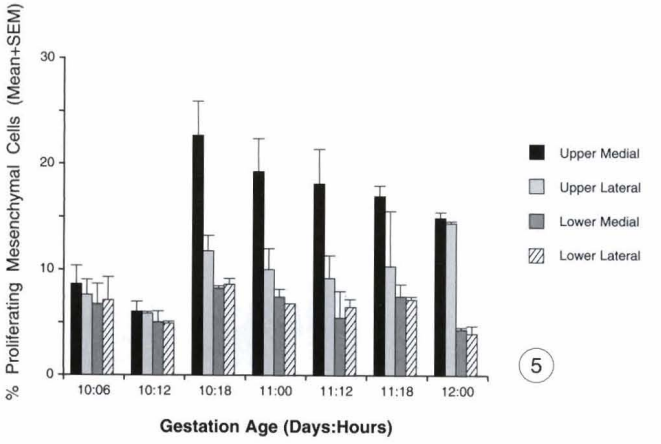


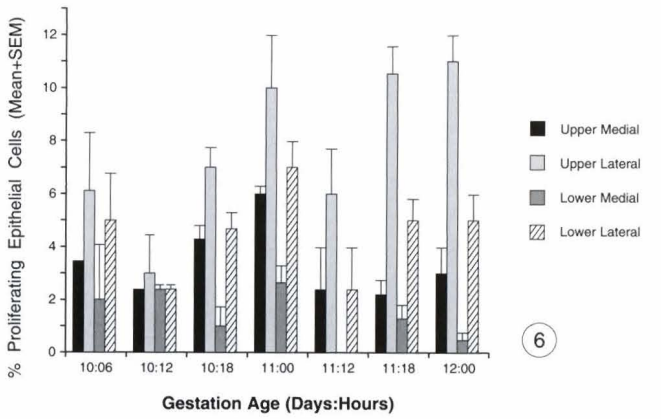
Fig. 4. Profile of the rate of cell proliferation in the epithelium and mesenchyme of the vertically developing palate at different times during gestation.

Discussion

A major component of early growth of palate is cell division. However, mitotic figures are not commonly seen in rapidly growing embryonic tissues, including palate (Walker and Fraser, 1956; Hughes et al., 1967; Shah and Chaudhry, 1974). Hence, proliferation indices are more reliable determinants of growth in a developing system (Miller et al., 1993). The present study sheds some light on the growth rate of cells during vertical palate development. It provides information not only on the proportion of cells entering cell cycle, and thus leading to the formation of heterogeneous pool of cells, but also on their location within the developing vertical shelf. Clearly, these baseline data should be useful in quantitatively assaying the roles of various morphogens, growth factors and hormones, and of teratological perturbations of the developing palate.

The results show that the overall trends in cell proliferation rates in the vertically developing palate parallel the biochemically measured rates of DNA synthesis described earlier in hamster (Burdett et al.,





Figs. 5-6. Profiles of the rate of cell proliferation in different segments of the vertically developing palate at different times during gestation. **5.** Mesenchymal cells. **6.** Epithelial cells.

1988; Shah et al., 1989a,b, 1991). Initially, the rate of proliferation was high; but it declined as the vertical palate development advanced. Similar temporal changes in the proliferation activity (DNA synthesis) during development have been observed in a variety of embryonic tissues including limb, eye, various facial primordia, pharyngeal plate, etc., in different animals (Searls and Janner, 1971; Summerbell, 1977; Minkoff and Kuntz, 1977, 1978; Truby, 1983; Bryant and O'Brochta, 1985; Bailey et al., 1988; Raynaud and Kan, 1992; Brewitt et al., 1992; Miller et al., 1993). A continuing proliferation of cells during the formation of vertical shelf would be intrinsically useful because it may allow greater flexibility in achieving necessary shape, volume (size), etc., during palate morphogenesis than a system in which the cellular decisions (for example, for position and lineages) were rigidly specified from the start by an invariant set of pre-localized determinants (Kimelman, 1993). In experimental studies on mutant mice, or following teratogenic treatment of rodents, or in the descriptive studies of human embryos with cleft palate, where vertical development of the palatal shelves were affected, both the volume and shape were affected (Vij and Kanagasutheram, 1971; Blocksema et al., 1973; Latham, 1973; Maher, 1977; Flint, 1980; Burdett and Shah, 1988; Shah et al., 1991).

The observations of the present study further show that there are temporal and spatial differences in the proliferative behaviour between M and E cells, which indicates that both their relationship and role during palate development may be very complex. Prior to day 10:12 of gestation, i.e., during the formation of the palatal primordia, the proliferation of both E and M cells was random as revealed by the segmental (upper and lower, medial and lateral) analysis. Based on the information derived from embryological and regeneration studies, a random cell division pattern was also described for the development of limb primordia in various species (Ede, 1971; Searls and Janner, 1971; Summerbell, 1977; Truby, 1983; Raynaud and Kan, 1992). Subsequently, however, M cells in the upper medial, and E cells in the upper lateral segments showed the most rapidly proliferating pools of cells. When the observations of the segmental analysis were pooled, it appeared that the proliferation gradient for M cells, in hamster palate after day 10:18 of gestation, was U-ME < U-LA < LO-ME = LO-LA; and for E cells, after day 11:00 of gestation, was U-LA < U-ME < LO-LA < LO-ME. This would suggest that during vertical palate development either: (1) there is a successive wave of cell proliferation from the upper toward the lower segment (an idea, which would be consistent with the general concept for morphogenesis of a structure, forwarded by Waddinton in 1956), or (2) the cells in the lower segment of vertical palate have a longer duration of cell cycle, i.e., G_1 phase, than the cells in the upper segment. Clearly, the information on the number and duration of cell cycles in the developing palate *ad initium* would be useful in further clarifying the biological aspects of vertical shelf formation. Furthermore, the data suggest that, at least on the basis of proliferative behaviour, heterogeneity, and thus perhaps phenotypics ommitment, of the palatal cells may be evident by day 11:00 of gestation in hamster, which is 24-30 hours prior to the reorientation and fusion of shelves (Shah and Chaudhry, 1974). This proposition is consistent with both the structural and *in vitro* observations of palate development in hamster as well as in other mammals on at least two grounds: (1) the E cells of LO-ME segment represent the prospective medial edge epithelium of the horizontal shelf where mitosis almost ceases 24-30 hours prior to the fusion (Hudson and Shapiro, 1963; Pratt and Martin, 1975; Shah et al., 1985); (2) when grown *in vitro*, hamster vertical shelves are capable of full differentiation only if explanted after day 11:00 of gestation (Shah et al., 1985; in other mammals also there are chronological restriction on palate differentiation *in vitro* - Pourtois, 1966; Vargas, 1968; Goss et al., 1970; Smiley and Koch, 1972; Vargas et al., 1972). Hence, it is plausible that the spatio-temporal changes in E and M cell proliferation rates, prior to day 11:00 of gestation in the vertically developing palate of hamster (or at a comparable time during palate development in other mammals), may be essential in gradually segregating cells into impeding myogenic, osteogenic, fibroblastic, odontogenic or glandular lineages, as appropriate, for subsequent phases of palate development. This, however, does not clarify whether the early palatal primordia possesses self organizing capacity, i.e., autonomy.

The foregoing discussion suggests that localized differences in mitotic rates in both the E and M cells may be critical in the vertical shelf development. It is, however, unclear whether the changes in cell proliferation pattern is a part of a temporal program of the vertical shelf development or is determined by the «positional information» of the cells (Wolpert, 1969), or by some other chemical (i.e., growth factors, hormones) or physical (i.e., length/volume of the vertical palate) factors.

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