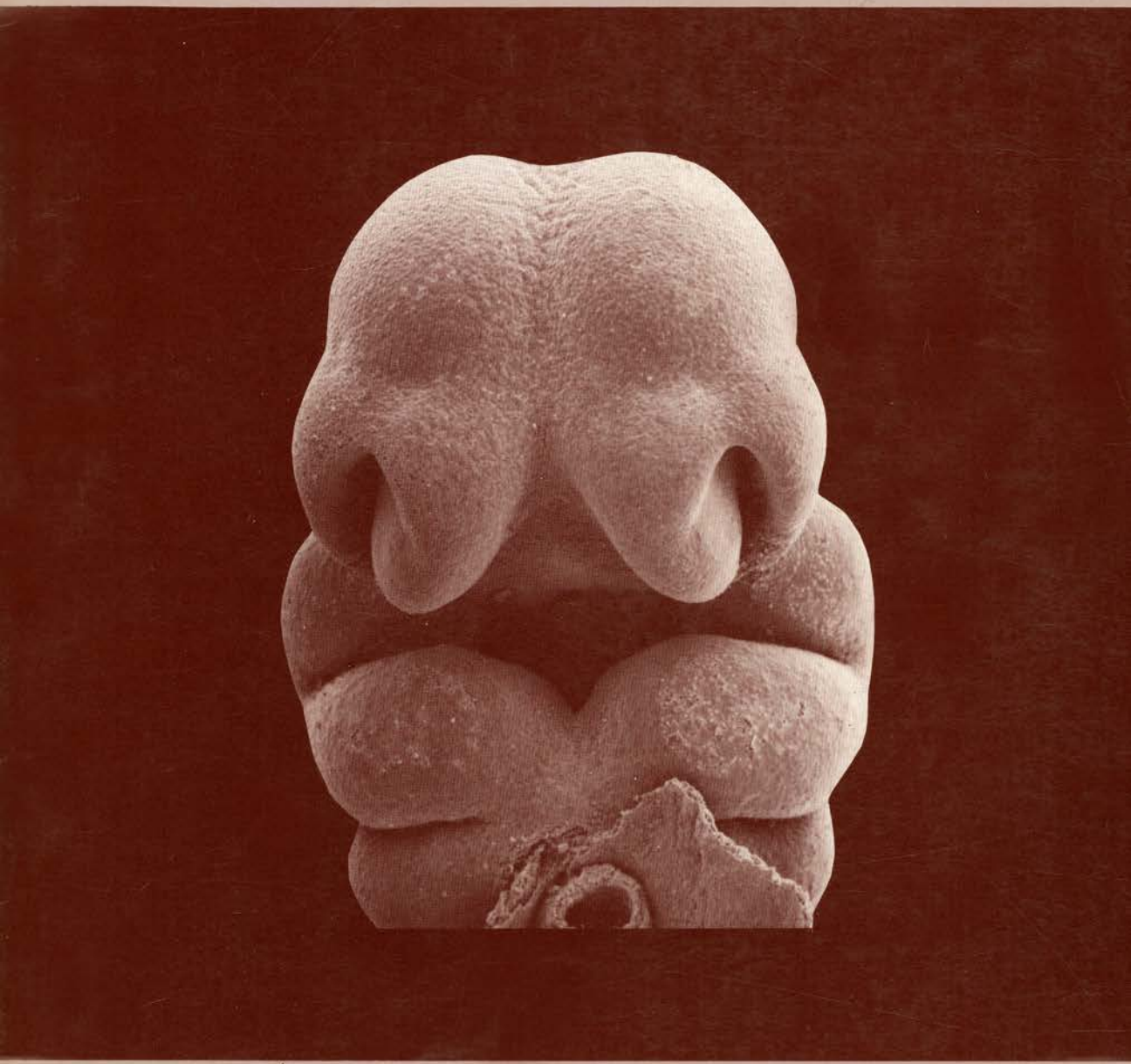
Journal of Craniofacial Genetics and Developmental Biology

Volume 9, Number 3, 1989



Alan R. Liss, Inc., New York

ISSN 0270-4145

Journal of Craniofacial Genetics
and Developmental Biology 9:271–283 (1989)

Reduced Epithelial Surface Activity Is Related to a Higher Incidence of Facial Clefting in A/WySn Mice

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The epithelial surfaces of the facial primordia were evaluated by scanning electron microscopy (SEM) during primary palatogenesis in two genetically related mouse strains, the A/J and the A/WySn strains. These two strains were selected because the reported frequency of spontaneous cleft lip with or without cleft palate [CL(P)] in the A/J strain approximates 0% whereas the spontaneous frequency of CL(P) in the A/WySn strain is 20–30% The embryos were examined prior to (two to six tail somites) during (seven to ten tail somites) and after (ten to 14 tail somites primary palate fusion. During fusion, epithelial surface activity characterized as cellular debris, dissociated cells, cellular projections, and epithelial bridging) was more pronounced in A/J embryos A/WySn embryos with spontaneous cleft lip exhibited a marked deficiency in epithelial activity when compared to their normal littermates. No discernible differences were detected in the facial morphology with the exception of the distal end of the medial nasal prominence, which appeared longer in the A/J strain. This study suggests that the degree of epithelial surface activity at the putative site of fusion and the relative length of the medial nasal prominence may account for the observed differences in facial clefting of the two strains. Face shape, related to prominence divergence, was similar in the two strains and could not explain the higher incidence of clefting observed in the A/WySn strain.

Key words: primary palatogenesis, face shape, fusion, facial, SEM

INTRODUCTION

Animal models of primary palate development have provided a great deal of information relative to both normal and abnormal development. The murine model has been particularly useful, because several strains have relatively high frequencies of spontaneous cleft lip with or without cleft palate [CL(P)] [Bornstein et al , 1970; Millicovsky et al , 1982, Kalter, 1979; Juriloff, 1982], and others have no spontaneous frequency of CL(P) [Trasler, 1968]

A relationship between strain frequencies of CL(P) in the mouse and the shape of the embryonic face at the time of primary palatogenesis was first established by Trasler [1968] The C57BL/6J strain, which has virtually no spontaneous incidence

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of CL(P), was compared to the A/J strain, which had, at that time, a spontaneous frequency of 10–12% for CL(P) Facial comparisons by Trasler [1968] indicated that the medial nasal prominences of the A/J embryos were more prominent, more medially placed, and less divergent than the medial nasal prominences of the C57B1/6J embryos This decreased divergence between the medial nasal prominences in the A/J strain was speculated to reduce the competency of the fusion between the facial prominences As a result, the A/J strain required greater cell proliferation by the facial prominences to establish contact. Thus greater demands were placed on the prominences of the A/J strain to fuse successfully These observed differences in spatial relationships of the facial prominences, which were shown to be strain-variable, were hypothesized to account for the differences in spontaneous CL(P) frequency

This face-shape hypothesis held that the genotype determined, to a large degree, embryonic face shape as well as the size of the facial prominences Genes that altered the facial shape and decreased the degree of contact of the prominences would increase the embryo's susceptibility to other factors (genetic or environmental) that interfered with lip formation [Fraser, 1971]

In 1982, the reported incidence of spontaneous clefting in the A/J strain was nearly zero, a dramatic drop from the previous incidence of 10–12% When compared to the Cl/Fr, which has a reported incidence of 36% of spontaneous clefting, the A/J and C57BL/6J strains displayed greater levels of epithelial activity during pri mary palatogenesis in the presumptive site of fusion [Millicovsky et al , 1982] Although the surface epithelium of the primary palate of some Cl/Fr embryos appeared competent, there was such a wide gap between the medial and lateral nasal prominences that the epithelial activity was thought ineffective Epithelial activity, which has been shown to be important in primary palate development, appears to be related to the incidence of spontaneous clefting in the various mice strains

The intent of our current scanning electron microscopy (SEM) investigation was to test the facial hypothesis and evaluate the amount of epithelial activity occurring in the primary palate of the A/WySn strain, which has a spontaneous CL(P) frequency of 20–30%, and the A/J strain, which has all but lost the ability for spontaneous clefting Facial prominence proportions and orientation, along with surface epithelial activity (cellular debris, blebs, globules, and signs of motility) were evaluated at intervals prior to and after fusion of the primary palate This information is important since the two strains have a similar genotype but very different frequencies of spontaneous facial clefting

MATERIALS AND METHODS

A/WySn and A/J strains of mice were housed separately in solid-bottom plastic cages, fed Purina Mouse Chow and tap water ad libitum, and maintained in alternate 12 hr periods of light and darkness Males were placed in cages with females of the same strain overnight.

Timed pregnancies were determined by the presence of vaginal plugs, checked for the following morning; if positive this was designated as day 1/4 of gestation Pregnant females were then separated and killed by cervical dislocation at various times from day 9 to day 12 of gestation The uterine horns were removed, and the embryos were rapidly dissected free of the uterus and placed in cold 0 2 M phosphate-

buffered glutaraldehyde (pH 7 3) for at least 24 hr Some of the embryos were fixed in their amniotic membranes, and others were dissected free of all extraembryonic tissue In all, 58 embryos were studied. 38 A/WySn and 20 A/J

Following fixation, the embryos were rinsed several times with 0 2 M phosphate buffer (pH 7 3) and then allowed to stand overnight. All residual embryonic membranes were then removed. The embryos were then staged and placed in a graded series of ethanol (ETOH) (50%, 70%, 90%, 95%, 100%, 100%) for 1 hr each and finally in absolute ETOH at least overnight. Specimens were then critical-point dried using an SPC 1500 critical-point drying apparatus (Bomar Co) from liquid CO_2, and the embryonic heads were dissected from the remainder of the embryo Dried heads were mounted with silver paint onto aluminum stubs, as were the tails to confirm the number of tail somites The mounted specimens were then sputter coated with gold for 6 min. Heads were examined in a Cambridge stereoscan 250 Mk 2 scanning electron microscope at accelerating voltages of 20 kV

The growth and development of the facial prominences of the A/WySn and A/J embryos were examined during the gestational periods before primary palate closure (two to six tail somites), during closure (seven to ten tail somites), and after fusion (ten to 14 tail somites) The ten to 14 tail somite stages were used to compare the gross facial morphology of normal embryos to those with spontaneous CL(P) All specimens were staged according to the following criteria. Number of tail somites distal to the genital tubercle [Sulik et al., 1979] and nasal placode stage of development [Trasler, 1968; Jurıloff and Trasler, 1976] Tail somite number was counted during specimen preparation and confirmed from tail specimens mounted on alumi num stubs and viewed by SEM The nasal placode state was judged and recorded during microscopic examination

RESULTS

SEM analysis of the A/WySn and A/J strains of mice indicates minor differences in facial form, which were primarily confined to the medial nasal prominence In particular, the distal end of the medial nasal prominence of the A/J strain appeared proportionately longer than that of the A/WySn strain The size and position of the maxillary and lateral nasal prominences appeared to be similar in the two strains This regional variation of size in the two strains may reflect a difference in proliferative activity of the underlying mesenchyme and surface epithelium. With regard to the degree of divergence of the medial nasal prominences, no differences were detected (Fig 1)

Epithelial activity (Fig 2) on the surface of the facial prominences was observed as different types. 1) cellular debris, globular masses, blebs, and spherical cells, which appeared to be either dead cells, migratory cells, or surface secretions, 2) dissociated cells from the surface epithelium, and 3) cellular projections and bridging across the fusion site Not all epithelial cells participated equally These activities appeared to aid in the union of the prominences In embryos that appeared to be developing normally, the A/J strain consistently showed far greater epithelial activity than the A/WySn embryos Most noticeable was the amount of surface cellular debris, surface blebs, globules, and rounded cells (Figs 3–5) The A/WySn embryos that showed potential facial clefting were strikingly deficient of these surface blebs (Fig 6)

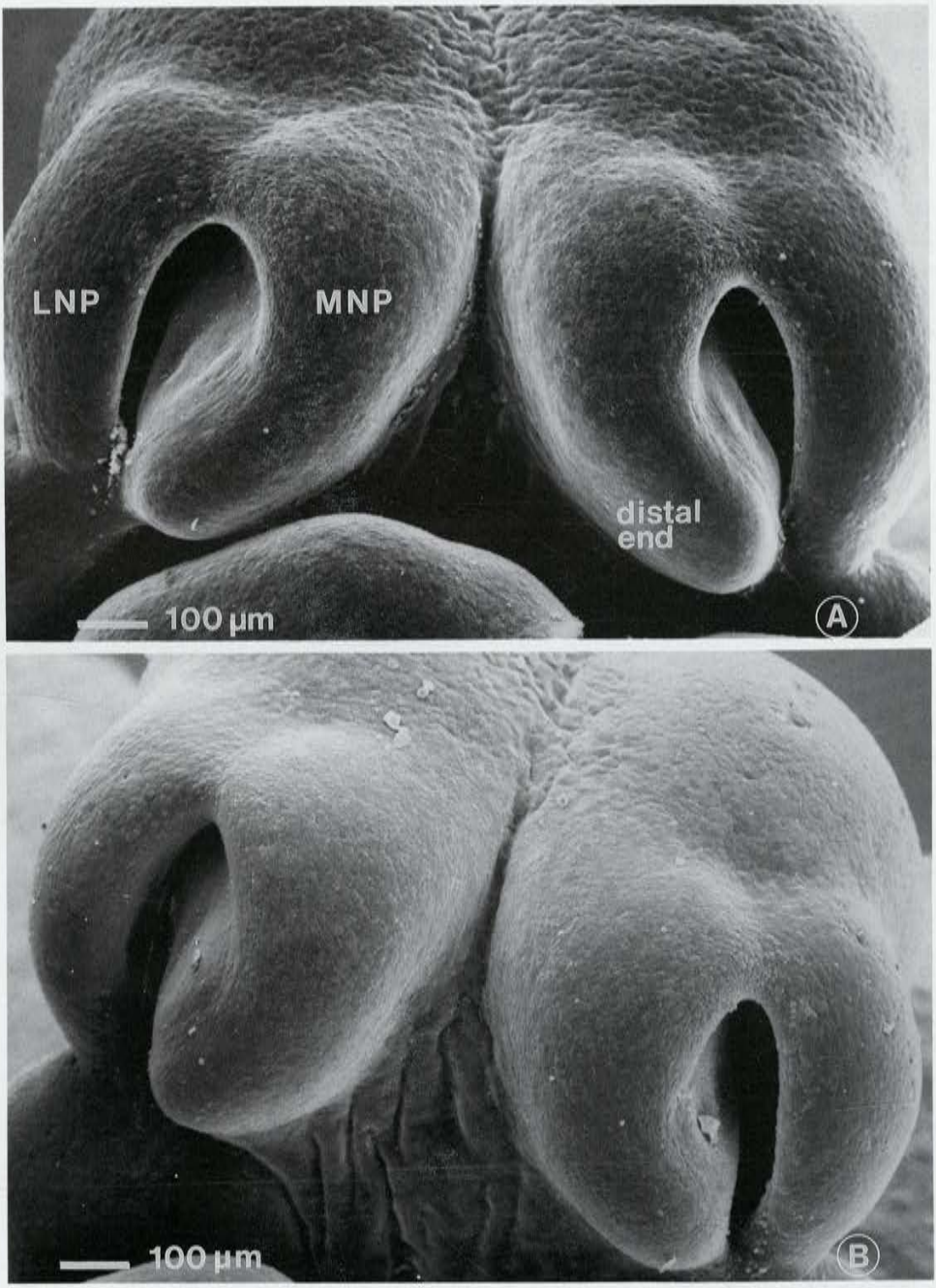


Fig. 1 Photomicrographs of the A/J **(A)** and A/WySN **(B)** embryos at the time of fusion of the medial and lateral nasal prominences, 11.5 days of gestation, nine tail somites. Both these mice appear to be developing normally There were no discernible differences in the degree of divergence of the medial nasal prominences. The major distinction regarding the facial prominences between the two strains was in the relative length of the medial nasal prominence: The length of the distal end of the medial nasal prominence of the A/WySn strain was relatively shorter than that of the A/J embryos. The size and positions of the other prominences were similar LNP, lateral nasal prominence; MNP medial nasal prominence.

DISCUSSION

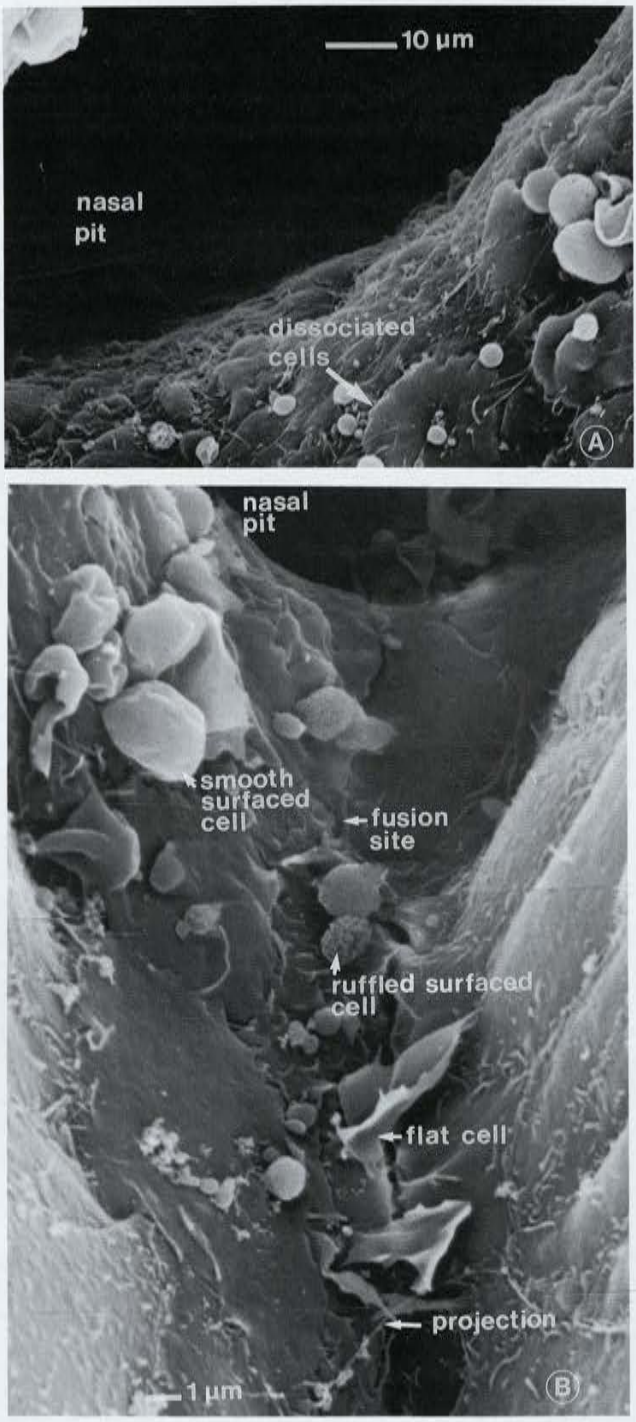
The frequency of spontaneous CL(P) in A/WySn mice is currently 25% and in A/J mice 0–2% (Johnston, 1988, personal communication) These differences in the spontaneous frequency of CL(P) are interesting in that the mice are inbred strains of the same family, the A strain. Although the A/J strain has experienced a reduction of the spontaneous cleft phenotype to the point of elimination, it still is very responsive to teratogens [Sulik et al , 1979; Melnick et al , 1981]

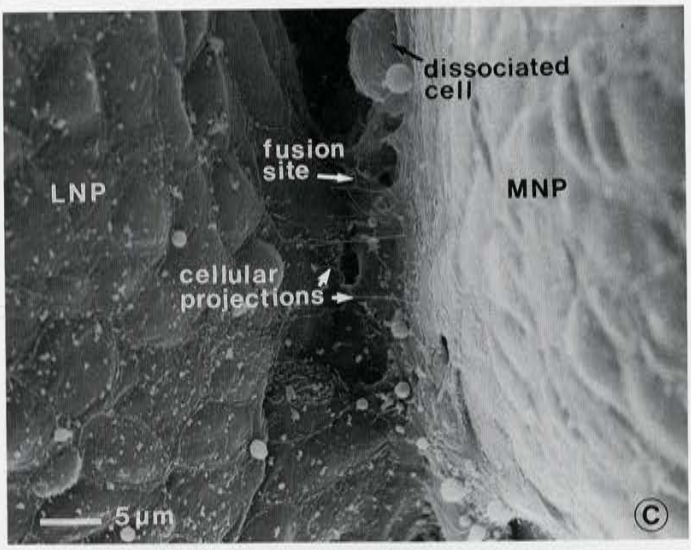
This study attempted to account for the difference of clefting frequency in A/J and A/WySn strains by comparing the facial shape and epithelial surface morphologies The major difference between the A/J and the A/WySn strains in facial shape was the proportionate length of the distal end of the medial nasal prominence, which was shorter in the A/WySn than in the A/J A short distal end is also present in the Cl/Fr, strain which also has a high frequency of spontaneous clefting When the Cl/Fr mice were treated with hypoxia, the distal end became even shorter, and higher frequencies of spontaneous facial clefting resulted [Bronsky et al , 1986] A shortened distal end either would reduce the frequency of contact among the facial prominences or, when contact did occur, would reduce the amount of overlap of the prominences and thus reduce the amount of contact surface area. As a result, fusion among the prominences would be more difficult to achieve This morphologic variation of size in the A/J and A/WySn strains may reflect a difference in proliferative activity of the underlying mesenchyme and surface epithelium.

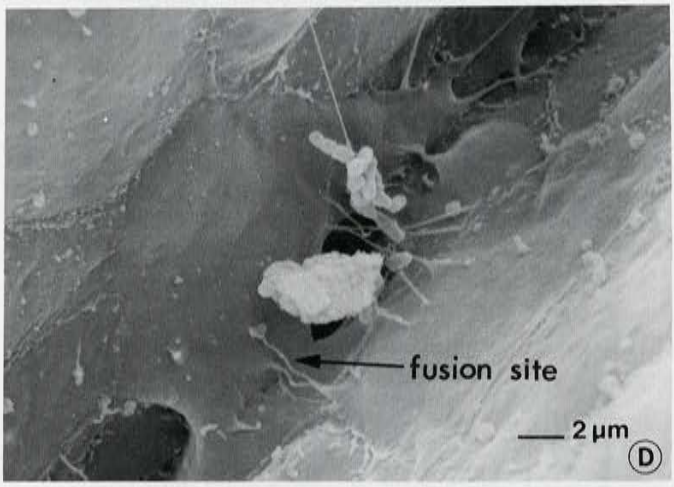
In concert with proliferative activity of the epithelium, the fusion process reveals a very active role for the epithelial cells located at the fusion site. Besides programmed cell death, increases in surface alterations, such as epithelial bridges, large and small globular cells, cytoplasmic projections, and cellular secretions have been reported. These structures may be responsible for promoting an epithelial bridge during the time of unification of the underlying connective tissue This epithelial bridging may be used to secure the tenuous initial attachment (Fig 2) and may also provide a necessary framework for epithelial fusion. Most likely these cell projections contain cytoskeletal elements, such as F-actin, and are suggestive of cell motility [Takeuchi, 1987, Greenburg and Hay, 1988] Kasaka and Eto [1986] reported the appearance of a superficial, migratory cell above the site of the isthmus, which aids in the fusion process Schupbach and Schroeder [1986] reported similar migratory activity of cells in the putative site fusion of the secondary palate

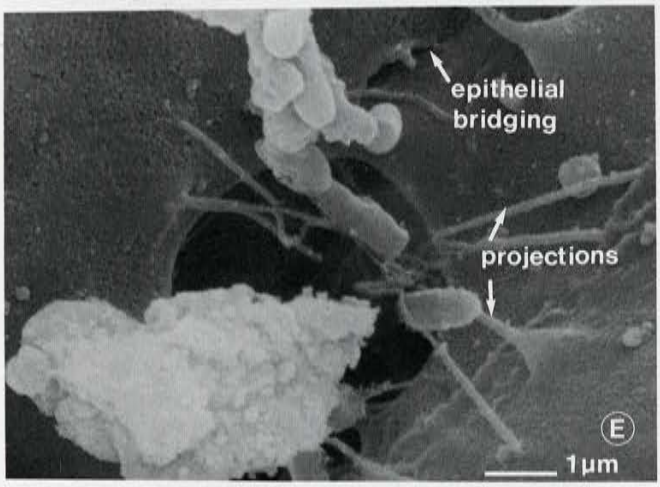
In both strains of mice, the sites of epithelial surface activity (blebs, cellular debris, and cellular projections) were localized to the specific sites (nasal placode, sites of merging, and sites of fusion) The difference between the two strains was in

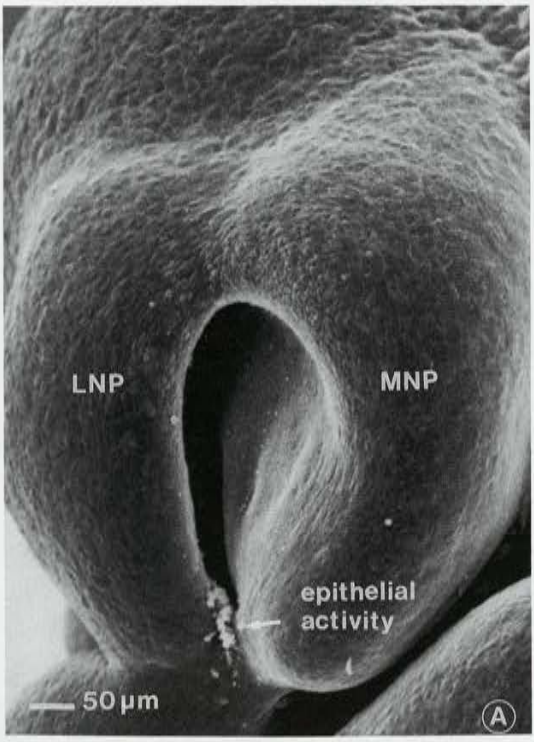
Fig 2. Photomicrographs depicting the different types of epithelial activity· The most conspicuous manifestation of epithelial activity was the presence of globules, blebs, and other cellular debris on the epithelial surface at the putative site of fusion. Other types of activities were cell elevations dissociated cells), cellular projections, and epithelial bridging. **A:** Cell debris and dissociated cells at the site of fusion. The elevated cells with projections were suggestive of motility (A/WySn; nine tail somites) **B:** Composite photomicrograph showing spherical cells (both smooth and ruffled surfaced) and flat cells located at the fusion site (A/J ten tail somites) **C:** Globules, blebs and cellular projections suggestive of cell motility (A/WySn, ten tail somites). **D:** Projections spanning several cells in length across the fusion site (A/WySn; ten tail somites). **E:** Higher magnification of D showing cell bridging at the fusion site (A/WySn; ten tail somites). LNP lateral nasal prominence; MNP medial nasal prominence.

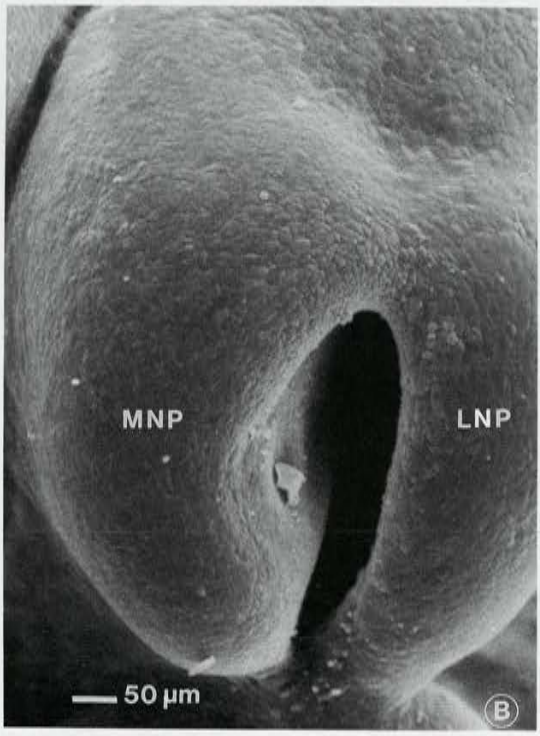

10 µm
nasal pit
dissociated cells
A
nasal pit
smooth surfaced cell
fusion site
ruffled surfaced cell
flat cell
projection
1 µm
B


dissociated cell
fusion site
LNP
MNP
cellular projections
5 µm
C


fusion site
2 µm
D


epithelial bridging
projections
E
1 µm


LNP
MNP
epithelial
activity
50 µm
A


MNP
LNP
50 µm
B

the amount of activity These findings were in close agreement with those of Millicovsky and Johnston [1981], who also found a reduction of epithelial activity in strains of mice that showed spontaneous clefting Millicovsky et al. [1982] found an absence of epithelial activity in about 40% of Cl/Fr embryos, a species that has a spontaneous clefting frequency of 36% The same investigators observed individual epithelial cell prominences bridging the gap between the medial and lateral nasal prominences during late stages of primary palate fusion. Their observations led to the conclusion that this late surface epithelial event (termed secondary fusion) may facilitate successful fusion of the primary palate in some Cl/Fr embryos Secondary fusion may be required for complete union of both medial nasal and lateral nasal prominences Secondary fusion becomes more important if initial epithelial events did not occur during approximation of the medial and lateral nasal prominences Schupbach and Schroeder [1986] speculated that the basal epithelial cells underwent epithelial rearrangement along the medial edge at the site of fusion of the secondary palate

Even with normal epithelial surface activity, primary palate fusion may not take place if the gap between the medial and lateral nasal prominences of the A/WySn or A/J embryo is too large to render the activity effective. The fusion process requires proper temporal coordination of epithelial surface changes and approximation of the medial nasal and lateral nasal prominences A wide gap in the presumptive area of fusion in the A/WySn embryo may thus prevent or delay contact between the approximating prominences

The near absence of cell activity in the potential CL(P) embryos was an interesting finding Trasler and Ohannessian [1983] observed cellular projections only when cells were approaching or in contact; the epithelial surfaces that were not in contact, in controls and where clefts were developing, were smooth It may be speculated that the epithelial activity is therefore dependent on prominence contact and would not occur when approximation of the prominences is not achieved We do not believe that this is the case since similar epithelial activity occurs in the nasal placode during invagination, which is independent of fusion Also, the secondary palate, where fusion does occur, produces epithelial activity along the medial edge, in vitro, independent of shelf contact [Schupbach and Schroeder, 1986] Most likely, prominence contact is not required to initiate the observed cell changes in the epithelium. Instead, prominence contact facilitates and promotes further epithelial activity

This study supports the view that CL(P) is a threshold character or quasicontinuous variant [Gruneberg, 1952], where the continuous variable would be the amount of contact among the prominences and the threshold is the level of contact that permits successful fusion. Either a decrease in the relative size of the prominences, reducing the amount of overlap among the prominences, or a reduction of the epithelial metabolic activity could conceivably alter the potential for successful fusion One possibly related factor, differences in the degree of medial prominence divergence

Fig. 3 Photomicrographs of the A/J (**A**) and A/WySn (**B**) embryos showing the isthmus region at the time of fusion of the medial and lateral nasal prominences, 11.5 days of gestation, nine tail somites. Note the amount of globules, blebs, and other cell debris between the medial and lateral nasal prominences is much greater in the A/J strain compared to the A/WySn. LNP lateral nasal prominence; MNP medial nasal prominence.

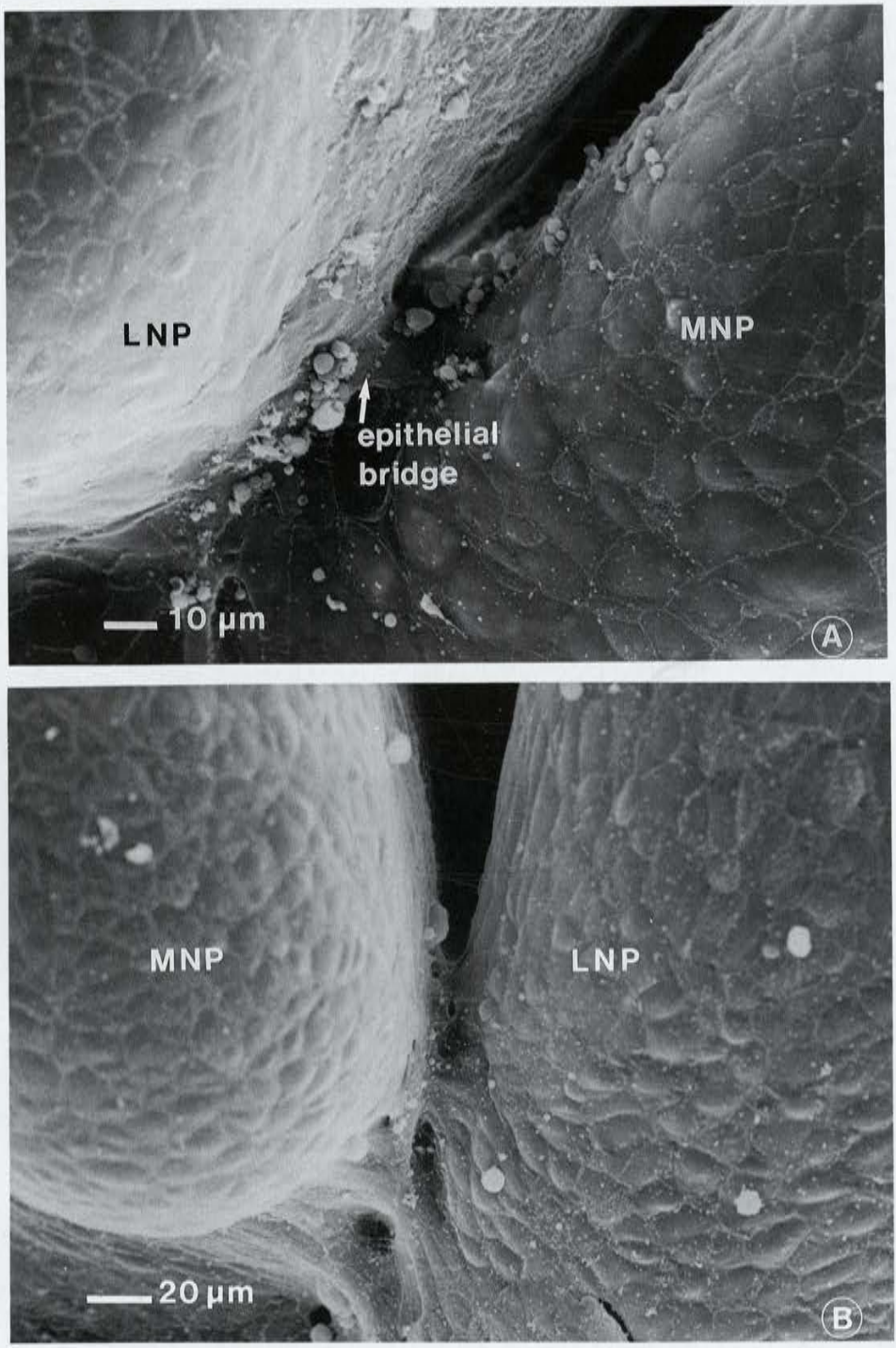


Fig. 4. Photomicrograph of a A/J **(A)** and A/WySn **(B)** embryos, day 11.5 of gestation, nine tail somites. Note the greater accumulation of surface globules and cell debris in the A/J strain, LNP lateral nasal prominence; MNP medial nasal prominence.

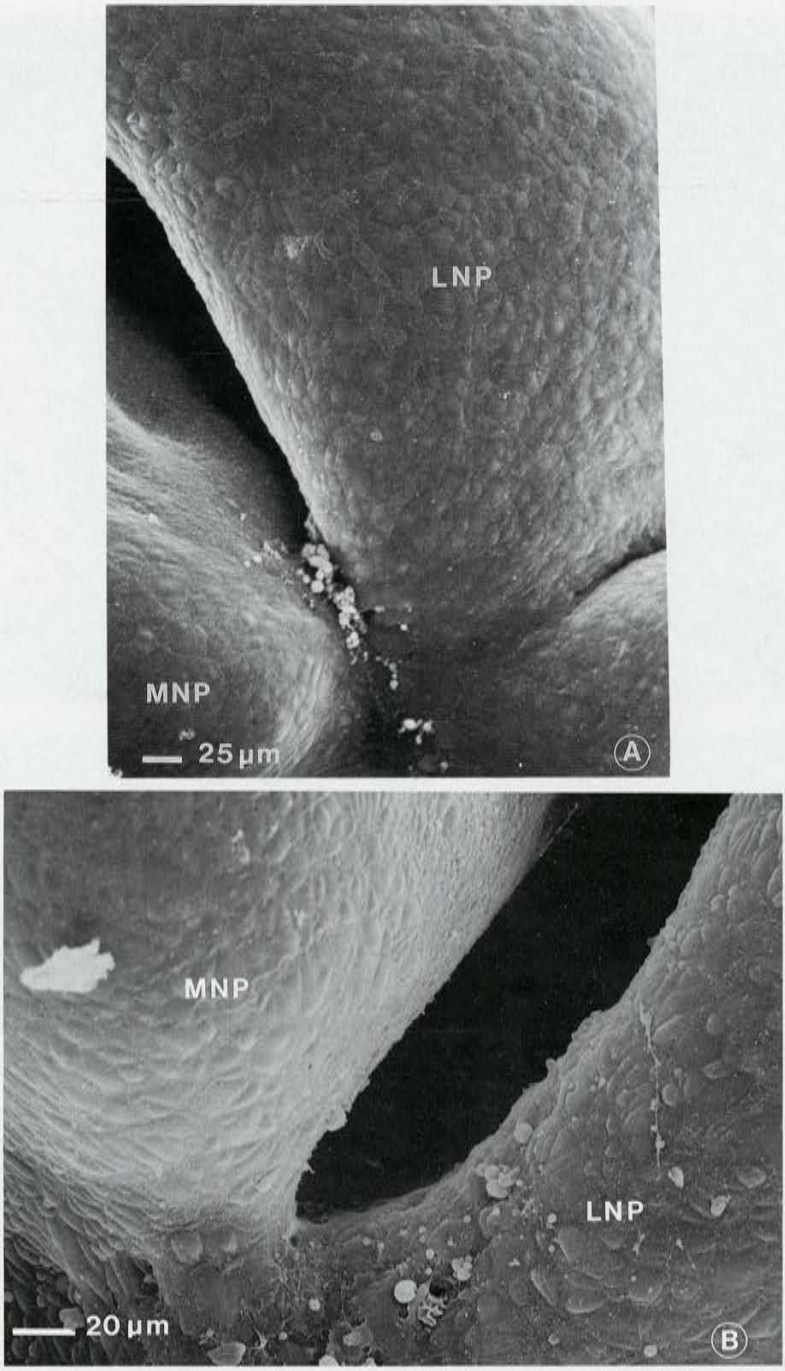


Fig. 5 Photomicrograph of the A/J **(A)** and A/WySn **(B),** day 11.5 of gestation, ten tail somites. Note that the amount of epithelial activity is still greater in the A/J strain. LNP lateral nasal prominence; MNP medial nasal prominence.

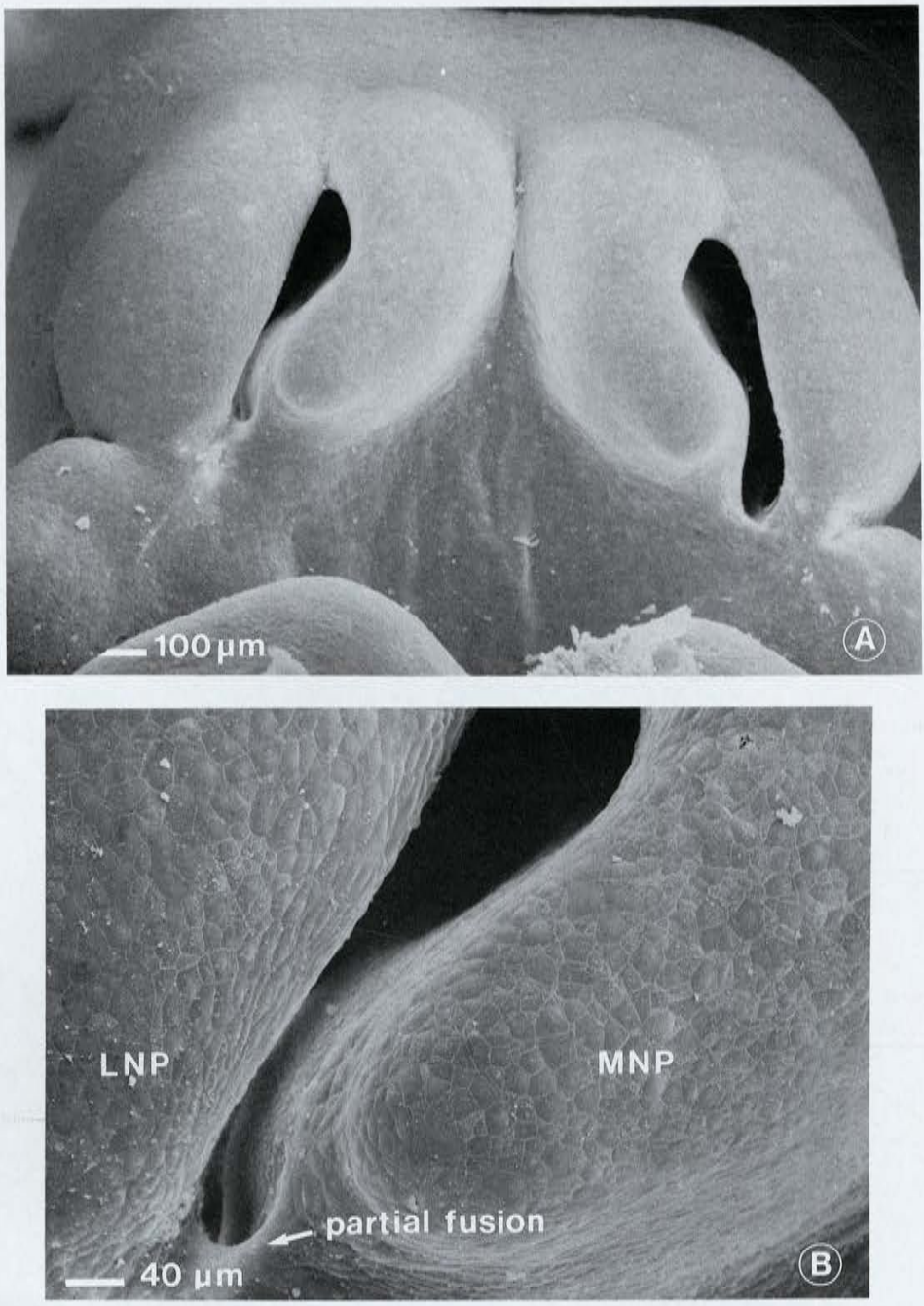


Fig. 6. Photomicrographs of a A/WySn embryo at the time of fusion of the lateral and medial nasal prominences, day 11.5 of gestation, ten somites. **A:** This embryo appears to be developing abnormally and suggests that bilateral clefts of the primary palate will result. On one side a complete cleft will occur and on the other side a partial cleft may result. **B:** Higher magnification showing very little epithelial activity LNP lateral nasal prominence; MNP medial nasal prominence.

was not responsible for the differences in the observed clefting frequencies of the two strains of mice studied. More likely factors such as the degree of surface activity and relative length of the prominences were responsible for differences in facial clefting

ACKNOWLEDGMENTS

The authors wish to acknowledge Dr Malcolm C Johnston, Professor of Orthodontics, University of North Carolina School of Dentistry, for generously providing us with the embryonic specimens used in this study

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