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Nasal and Pharyngeal Abnormalities Caused by the Mouse Goosecoid Gene Mutation

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The *Goosecoid* (gsc) gene is a homeobox-containing gene expressed first in the gastrula, and later during organogenesis in development. The gsc gene transcript is found in the first and second branchial arches, frontonasal mass in its late phase of expression. We have previously shown that targeted mutation of the mouse gsc gene leads to neonatal death and craniofacial defects. In this study, we performed histological studies on craniofacial phenotypes in order to elucidate the processes underlying the neonatal death of gsc mutant mice. We found that gsc mutant mice have aplastic nasal cavities and lack the Sinus Paranasalis. We also showed that secretory olfactory glands in the basal layers are aplastic. This is suggested to be essential defects for olfaction. gsc mutant mice also show several pharyngeal phenotypes, including defects in the pharyngeal muscles and the pharyngeal mucosa. It is therefore suggested that mutant mice develop lethal gastro-intestinal phenotypes caused by defects in breathing and sucking of milk as a consequence of these craniofacial disorders. These results should help elucidating the molecular genetic programs essential to the neonatal development of mammals.

Goosecoid (gsc) is a homeobox-containing gene originally isolated in Xenopus by screening the organizer specific cDNA library and subsequently cloned in various species (1-6). It is expressed during early embryogenesis, i. e., in the dorsal lip of the blastopore in Xenopus and in the developing primitive streak of the mouse (2). The unique feature of the gsc gene function is indicated by its late phase of expression, i.e., branchial-arch- and nasal-pit-derived structures, the nasal cavity and capsule, the tongue muscle, the mandible, the malleus, the tympanic-cavity and the external acoustic meatus. We previously generated gsc mutant mice by gene targeting and showed that they die neonatally, suggesting its role in craniofacial development (7). However, it remains elusive how such histological and physiological abnormalities consequently lead to neonatal death of the gsc mutant mice.

One of the evolutionarily acquired characteristics of mammalian development is a transition from fetal to neonatal growth. During this process, proper sucking of milk and regular aerobic breathing are essential. In this aspect, it is therefore intriguing to elucidate the neonatal lethality by the gsc gene mutation. In this study, we provide evidence of neonatal misfunctions, i.e., breathing and sucking of milk in gsc mice.

Recently, many genes have been implicated in craniofacial development, such as palatogenesis or nasal cavity development (8-10). The correlation between neonatal development and craniofacial morphogenesis is discussed in line with previous findings.

MATERIALS AND METHODS

Newborns were analyzed by Southern blot for genotyping as previously described (7). They were fixed in the Bouin's fixative, dehydrated in graded ethanol, embedded in paraffin, and sectioned in a frontal plane. Sections for Fig.1, Fig.2 C,D were labeled with the biotinylated lectins (WGA or UEA: Vector Laboratories, CA, USA) followed by the avidin-biotin complex method coupled with the haemotoxylin stain as described previously (11-13). In Fig.2, PAS

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Abbreviations used in this paper: gsc, Goosecoid; WGA, wheat germ agglutinin; UEA, Ulex europeus agglutinin; PAS, Periodic acid Schiff; NCC, neural crest cell.

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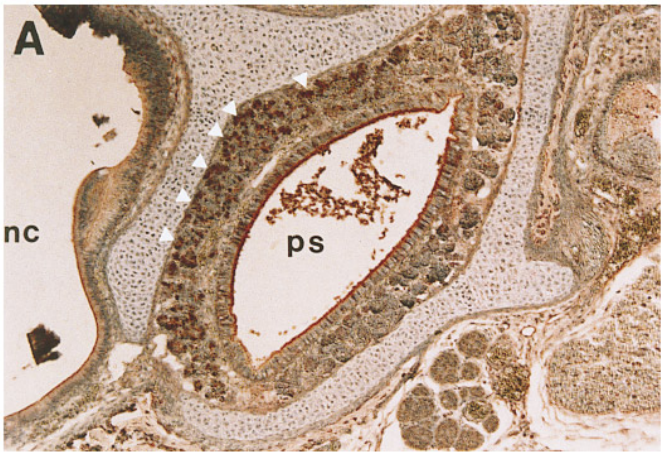
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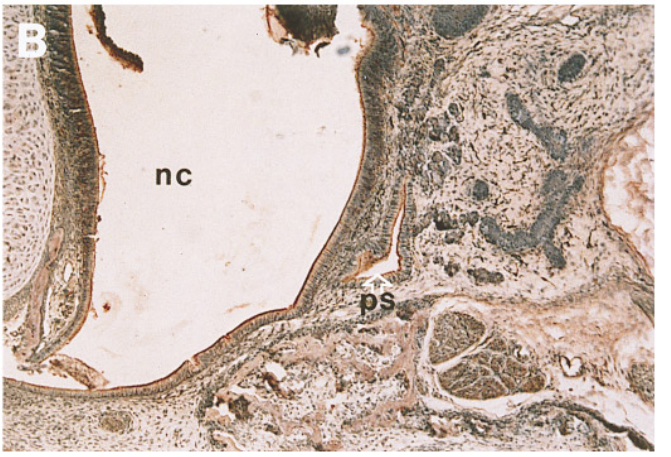
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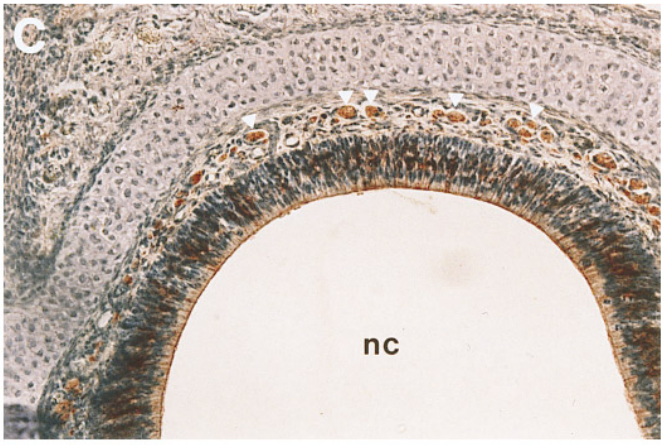
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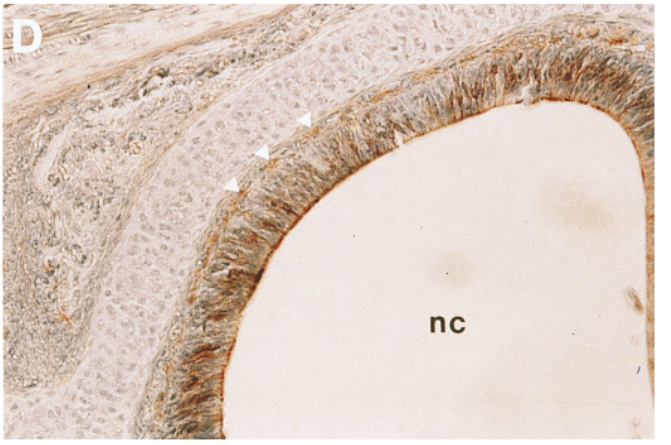
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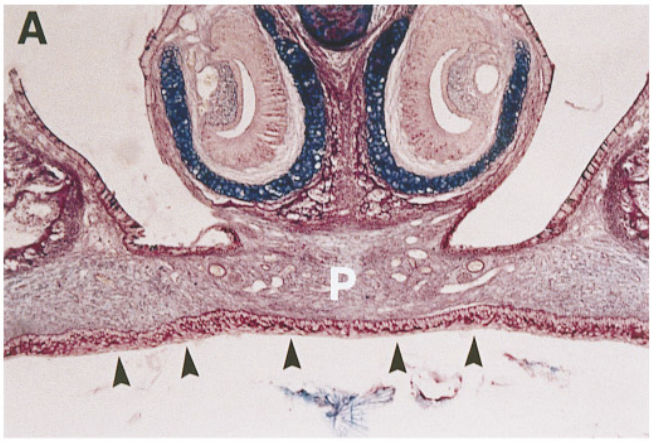
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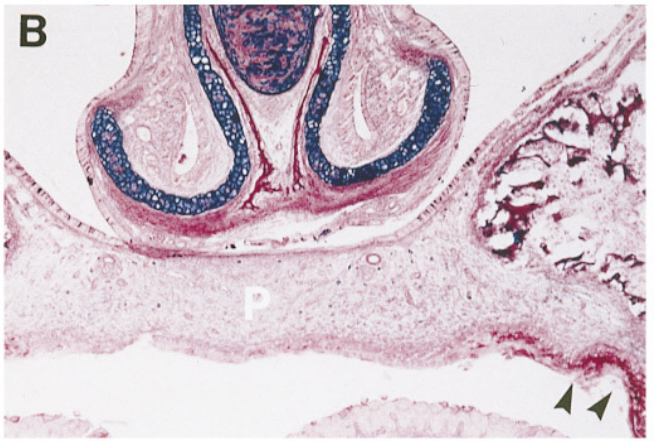
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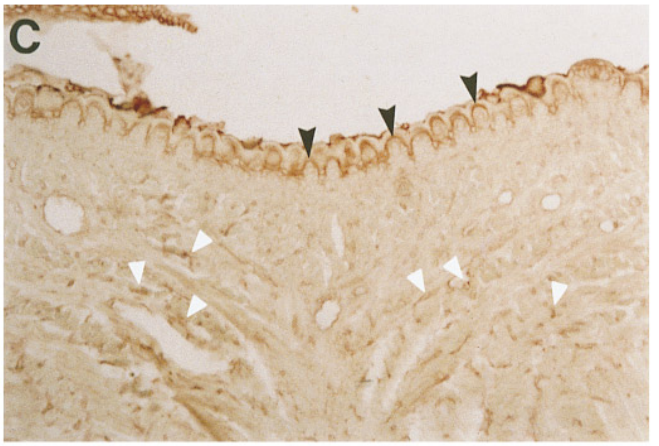
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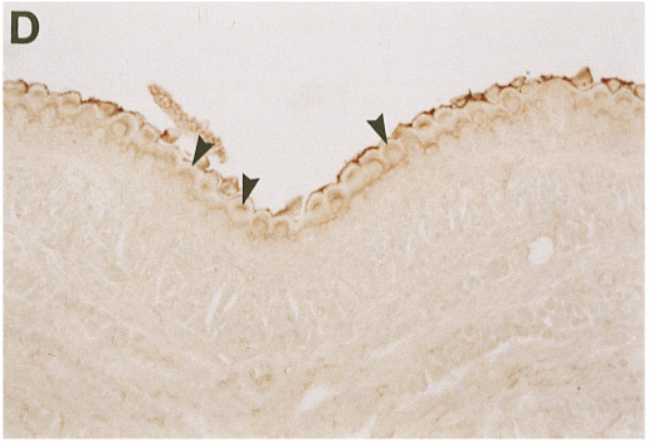
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(Periodic acid Schiff) reaction with alcian blue stain was performed in order to mark neutral mucosubstances as described (11-13). For whole mount immuno-staining, anti-neurofilament associated protein antibody (2H3; ATCC) was utilized to mark tongue nerve innervation for day 13.5 embryos as described (14). Fig.4 shows the haemotoxylin and eosin (H. E.) stain of the pharyngeal region, and photos of dissected newborn mouse abdomens.

RESULTS

Phenotypes in Nasal Cavity

The gsc homotygotes have defects in the nasal cavity and the nasal cartilage. In Fig.1 (A, B), the wild type mice have well developed paranasal sinuses (paranasal cavities) showing the WGA lectin positive secretory glands (marked brown signals), whereas mutants clearly lack these signals. Thus, wild type mice show the highly branched nasal cavity including the paranasal sinus, in order to efficiently caputure odoriferous substances, while the paranasal sinuses of the mutants are characterized by agenesis. This suggests the impairment of olfactory secretions that are considered essential for olfaction.

In Fig.1(C, D), the UEA lectin (specific for fucose) staining shows clear signals in secretory glands in the nasal basal layers of wild type mice (C; marked by white arrows). In contrast, olfactory sensory cell staining could be seen in the nasal epithelium for both groups. The olfactory epithelium thus appears to be unaffected, while the basal cell layers of mutants are clearly aplastic.

Phenotypes in Oral Cavity

To further elucidate the possible histological changes in the mucosa of oral cavity and palate, PAS staining (Periodic acid Schiff reaction for neutral carbohydrates staining) was performed as shown in Fig.2 A, B. Oral cavity mucosa adjacent to the nasal septum show altered carbohydrate staining (black arrows indicate lack of signals in the mutant palate (B)). The nasal septum did not fuse with the palate (Fig.2 B).

We have previously shown that gsc mutant mice have an underdeveloped alignment of tongue and adjacent, i.e., masseter, muscles (7). The gsc mutant mouse tongue shows a hypoplasia of papillae as shown by WGA stain and a lack of tongue secertory glands was also observed (see arrows in the tongue; Fig. 2 C, D). Anti-neurofilament associated protein antibody staining (2H3 antibody) further revealed an alternation of tongue nerve innervation in the root of the tongue (Fig.3 A, B).

Pharyngeal and Gastro-intestinal Phenotypes

Mutant mice show pharyngeal phenotypes including underdevelopment of both (1) pharyngeal muscles, and (2) pharyngeal mucosa (Fig.4 A, B). Proper alignment of these structures is known to be essential for breathing and sucking of milk in mammals. Mutant mice develop gastro-intestinal phenotypes as shown in Fig.4 C, D (Note the presence of air in the stomach and intestine of mutants).

DISCUSSION

One of the characteristic processes in the early development of all mammals is an evolutionarily devised process that establishes the transition from fetal to neonatal development. Craniofacial morphogenesis is a vital element, since facial architectures, including several sensory systems, are required to be properly organized from fetuses to neonates.

Recently, many developmental control genes, including homeobox genes, were isolated and found to be dynamically expressed during craniofacial organogenesis. Therefore, it is becoming increasingly important to analyze functions of these genes during craniofacial development.

The murine gsc gene expression persisted progressively in craniofacial regions. Its expression around the first branchial arch persisted during the oral cavity development and the expression in the nasal pits persisted as they elongate to form the nasal chambers (15). Based on these facts, it has been suggested that the gsc gene might play roles in spatial programming of craniofacial regions (15). In this study, we showed several craniofacial abnormalities which can hamper essential neonatal functions, i.e., olfaction, sucking of milk and breathing.

It has been postulated that the nasal cavity is coated with olfactory mucus to capture odoriferous chemicals. Therefore, olfactory secretion is considered to be one of the essential elements for efficient olfactory sensing. As shown in Fig.1, the lack of both Sinus Paranasalis and olfactory secretory glands could contribute to neonatal lethality because olfactory but not visual sensing

FIG. 1. Lectin staining for the wild type mouse (A, C) and the gsc mutant mouse newborns (B, D). Paranasal sinus (A, B) and nasal cavity were stained (C, D). Wild type mice display well developed paranasal sinuses (WGA lectin positive secretory glands are indicated by white arrows in A). In (C), UEA lectin positive olfactory secretory glands (marked by white arrows) developed well in contrast to (D). Original magnifications: ×30 Abbreviations: nc, nasal cavity; ps, paranasal sinus.

FIG. 2. Frontal sections through the heads of a wild type (A, C) and of a gsc homozygous mutant newborn (B, D) stained with PAS reaction (A, B) and WGA lectin stain (C, D). The wild type palate shows PAS positive signals, as shown by black arrows, in contrast to mutants (B). The wild type tongue mucosa (ectoderm derived) contains well developed WGA lectin positive papillae and the tongue secretory glands (shown by white arrows in C). Original magnifications: A, B: ×30; C, D: ×100. Abbreviations : P, palate.

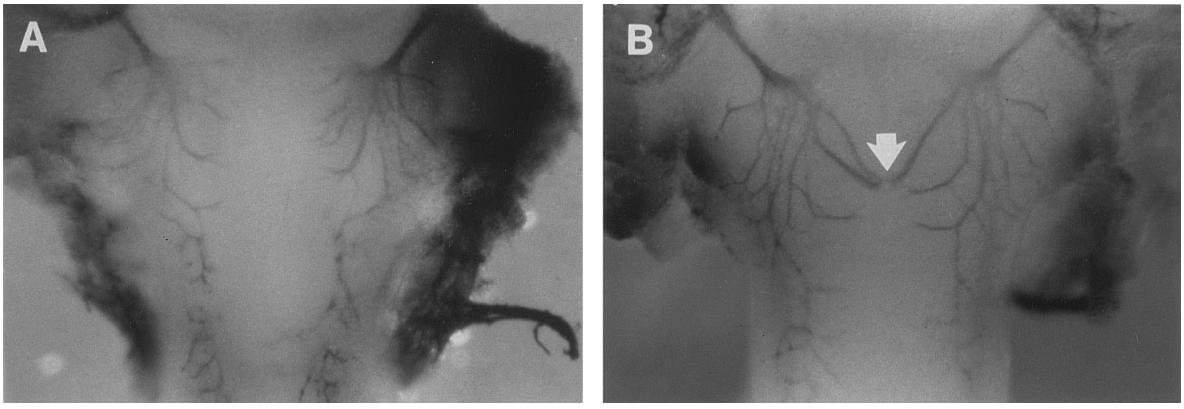

FIG. 3. Whole mount immunostaining by the anti-neurofilament associated protein antibody (2H3 antibody) for the root of the tongue of day 13.5 embryos (A, mutant; B, wild type). The upper region of the photo corresponds to the root of tongue. The immuno-staining was done as described (14). Original magnifications: ×50.

is vital for mammalian neonates to locate mammalian glands. Results of a preliminary functional study on gsc neonates suggest that homozygotes have impaired olfactory sensing (Yamada et.al. in preparation). This phenotype is concordant with the gsc gene expression pattern that is recognizable in nasal pits starting at day 10.5 with strong signals persisting in nasal cavities and chambers (15). The nasal epithelium seems unaffected judged by the lectin staining which can mark several subsets of primary sensory olfactory neurons (13, 16-19,).

As for the palatogenesis, factors regulating the out-

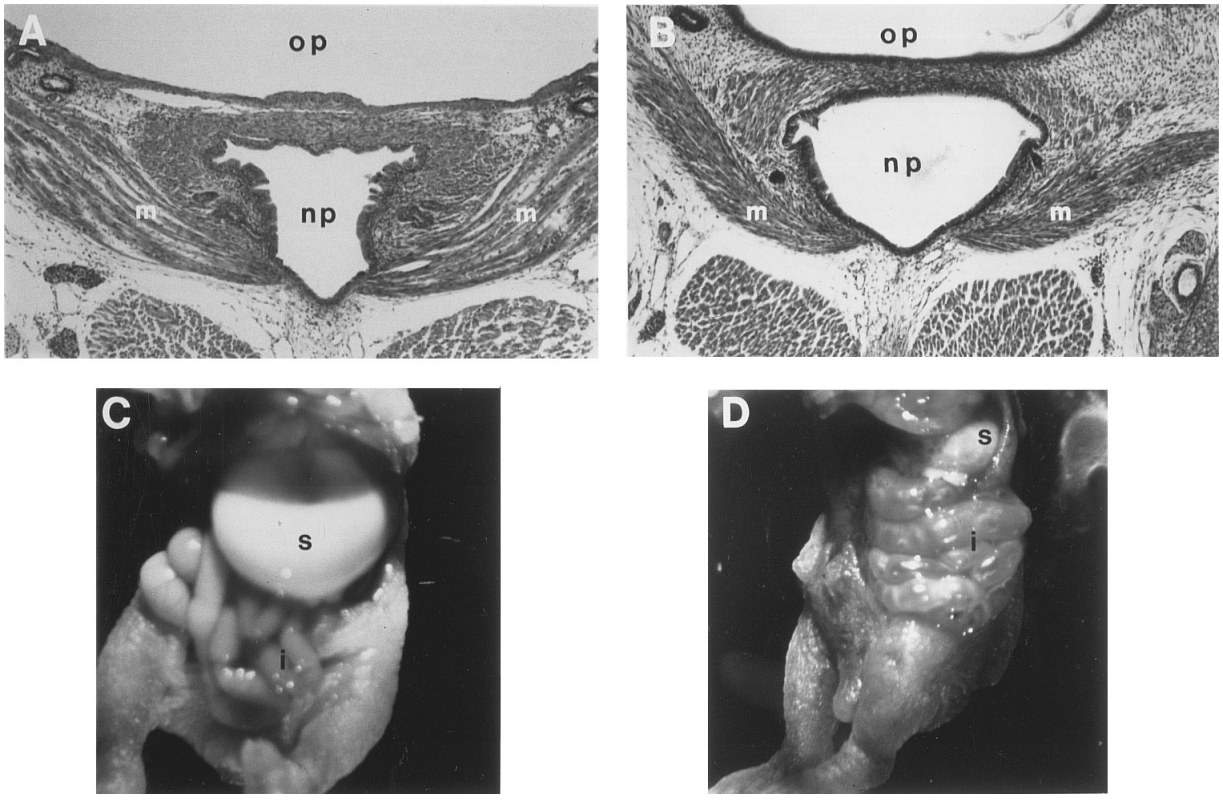

FIG. 4. In the panel A, the wild type naso-pharynx contains well developed mucosa and is connected with the normal pharyngial muscle alignment, in contrast to the gsc mutants (B), as shown by the H. E. stained sections. Photos for dissected abdominal regions of wild type (C) and the gsc mutant (D). Mutant newborn mice digestive organs are filled with air bubbles (see mutant stomach and intestine in contrast to the wild type). Original magnifications: A, B:×25. Abbreviations: op, ortho-pharynx; np, naso-pharynx; m, upper part of naso-pharyngial muscle; s, stomach; i, intestine.

growth, elevation and the subsequent fusion of the vertebrate palatal shelve formation are not well known. It is shown that the oral cavity mucosa adjacent to the nasal septum of homozygous mice display alterations in carbohydrate structures as compared with wild types (Fig.2, A, B).

It has been suggested that an alteration of complex carbohydrate structures may play a role in modulating cell-cell interactions. Alterated expression of glycoconjugate structures have been reported during cartilage development in the chondrocranium, nasal skeleton, and Meckel's cartilage (20). Before palatal fusion, quantitative differences in the distribution of complex carbohydrate structures were reported between oral, nasal, and medial-edge rat epithelial surfaces. As shown in this paper, during palatogenesis, glycosaminoglycans patterns were significantly modified in gsc mutants. Further molecular analysis is necessary to elucidate the cellular processes involved.

In vertebrates, part of the NCC (neural crest cell)-derived mesenchyme of the anterior two-thirds (body) of the tongue is derived from the first pharyngeal arch (21). Interestingly, this anterior tongue (body of the tongue) specifically expresses gsc gene, in contrast to the root of the tongue, which is marked by the boundary foramen cecum and sulcus terminalis (15). WGA-stained gsc mutant mouse tongue shows the aberrant structures of papillae in the anterior tongue (Fig.2 C, D). A lack of tongue secretory glands was also suggested (Fig.2 D). Mutant mice also display aberrant tongue nerve innervations in the proximal root of the tongue (Fig. 3). This could also contribute to the defects in sucking of milk.

It is known that the first and second pharyngeal arches are innervated by the trigeminal (cranial nerve V) and the facial (cranial nerve VII) nerves, respectively. This innervation phenotype could be due to the developmental interaction between nerves and other messenchymal components during craniofacial development.

Mutant mice show several pharyngeal disorders in pharyngeal muscles and pharyngeal mucosa. Because the proper alignment of these structures is critical, as is the coordination of the tongue and lower jaw, this would provide strong reasons for neonatal death. In fact, our present analysis suggests the impairment of these processes, as judged by the inhalation of air by mutant's digestive organs (the gastro-intestinal phenotypes in Fig.4 D). These phenotypes are reminiscent of congenital malformations seen in human children (pharyngral arch syndromes) which are known to include malformations in tissues derived from first pharyngeal arch, e.g., tongue, mandible (21-23). Several cloned regulatory genes have already been suggested as candidates for some human craniofacial syndromes, e.g., cleft palate, oligodontia (8, 9, 24). Our results suggests that gsc mice could constitute with an animal model to study some of the developmental defects of these structures.

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