Targeted mutation of the murine *goosecoid* gene results in craniofacial defects and neonatal death

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SUMMARY

The *goosecoid* gene encodes a homeodomain-containing protein that has been identified in a number of species and has been implicated in a variety of key developmental processes. Initially suggested to be involved in organizing the embryo during early development, *goosecoid* has since been demonstrated to be expressed during organogenesis – most notably in the head, the limbs and the ventrolateral body wall. To investigate the role of *goosecoid* in embryonic development, we have inactivated the gene by gene targeting to generate mice mutant for the *goosecoid* gene. Mice that are homozygous for the goosecoid mutation do not display a gastrulation phenotype and are born; however, they do not survive more than 24 hours. Analysis of the homozygotes revealed numerous developmental defects affecting those structures in which *goosecoid* is expressed during its second (late) phase of embryonic expression. Predominantly, these defects involve the lower mandible and its associated musculature including the tongue, the nasal cavity and the nasal pits, as well as the components of the inner ear (malleus, tympanic ring) and the external auditory meatus. Although the observed phenotype is in accordance with the late expression domains of *goosecoid* in wild-type embryos, we suggest that the lack of an earlier phenotype is the result of functional compensation by other genes.

Key words: *goosecoid*, homologous recombination, mouse, craniofacial defects

INTRODUCTION

Originally identified in *Xenopus* (Blumberg et al., 1991), the *goosecoid* gene has been shown to be present in many different phyla such as mammals, amphibians, birds and fish (Blum et al., 1992). The *Xenopus goosecoid* gene is expressed in the dorsal lip of the blastopore – the initiation site of gastrulation and has been suggested to encode one of the molecules involved in organizing the ventral cells of the early embryo into axial structures (somites and neural tube). Indeed, injection of *goosecoid* mRNA results in the formation of a double body axis in *Xenopus* (Cho et al., 1991). Furthermore, it has subsequently been demonstrated that *goosecoid* is involved in the control of cell migration in *Xenopus* embryos (Niehrs et al., 1993).

The murine *goosecoid* gene encodes a 256 amino acid protein that contains a homeodomain. The homeodomain is the only DNA-binding motif thus far identified in the protein and it is through this domain that *goosecoid* regulates transcription in vivo. Studies concerning the expression of *goosecoid* during embryonic development have identified two distinct phases of expression: firstly, it is expressed during a short period from day 6.4 to day 6.7, which corresponds to gastrulation (Blum et al., 1992). Secondly, *goosecoid* is expressed during organogenesis (from day 10.5 onwards) in specific structures most notably those that will ultimately form some components of the head, the limbs and the ventrolateral body wall (Gaunt et al., 1993).

Much attention has focused on the early phase of *goosecoid* expression and its potential involvement in gastrulation processes. In the mouse, *goosecoid* is expressed in the developing primitive streak, more specifically in those cells that are undergoing anterior migration – one of the earliest features of gastrulation. The fate of these cells has been demonstrated to lie in the head process (Beddington, 1983). However, this phase of expression is temporally limited – from day 6.4 to 6.7, (Blum et al., 1992). The later phase of *goosecoid* expression concurs well with the earlier phase – *goosecoid* mRNA can be detected in the head with a posterior limit in the region of the first branchial arch. This phase of expression is more persistent than the early phase and appears to continue through morphogenesis (Gaunt et al., 1993). Foremost among these structures are those that arise from the first branchial arch e.g., the lower jaw and parts of the tongue, the first branchial cleft e.g., the auditory meatus and the nasal pits i.e., the nasal chambers. In general, it would appear that *goosecoid* may be involved in

directing the final stages of the formation of craniofacial structures that house much of the animals sensory apparatus i.e., the ear, the nose, the mouth, etc which are of great importance for survival in the early neonatal stages.

By using homologous recombination, we have generated mice that lack the *goosecoid* gene. Mice that are homozygous for the *goosecoid* gene are brought to term but die soon after parturition. These mice display no gross abnormalities but appear to be unable to feed and are lethargic. Histological analysis demonstrated that multiple structures were affected all of which correspond to the later expression domains of the wild-type *goosecoid* gene. The lack of an evident gastrulation phenotype suggests that *goosecoid* is not essential for gastrulation and its absence is compensated for by another functionally related gene.

MATERIALS AND METHODS

Gene targeting and Southern blot analysis

The genomic structure of the murine *goosecoid* gene (129 mouse strain) and the targeting strategy is described in the text. pGS-neo was electroporated into R1 embryonic stem (ES) cells (provided by Dr A. Nagy) and G418-resistant clones were screened for homologous recombinants by Southern hybridization (Bradley, 1987) using an external upstream genomic probe (*Hin*dIII-*Eco*RI) not present in the targeting construct. Morula aggregation was performed as previously described (Nagy et al., 1990). The structure of the targeted allele was confirmed by Southern blot analysis of tail DNA (10 μg) digested with the indicated enzymes using a 582 bp *Sal*I-*Apa*I fragment from exon1. Hybridizations were performed at 67°C in 1 M NaCl, 10% dextran sulphate, 1% SDS, 1 mg/ml yeast tRNA. Blots were washed in 1× SSC and 1% SDS for 60 minutes.

Histological analyses

After genotyping, mice were fixed in Bouins fixative (Sigma), dehydrated through graded ethanol, embedded in paraffin and sectioned. Sections were stained with haemotoxylin and eosin as previously described (Kaufman, 1992; Lohnes et al., 1994). Staining of skeletal preparations was performed with alizarin red and alcian blue as previously described (Kessel and Gruss, 1991; Kessel, 1992).

RESULTS

Targeting the *goosecoid* gene

The murine *goosecoid* gene is composed of three exons with the homeobox being encoded by the 3′ end

of exon 2 and the 5′ end of exon 3 (Blum et al., 1992). The targeting vector, pGS-neo contains the PGK promoter linked to the neomycin resistance gene (Fig. 1B) inserted into the homeobox-coding region of exon 2. As shown in Fig. 1D, *Hin*dIII-digested wild-type genomic DNA generated a 6.6 kb fragment whereas the targeted allele (containing the 1.2 kb targeting vector insertion) hybridized with a 7.8 kb sequence. The neo resistance gene probe only hybridized to the mutated allele (data not shown). A total of 240 G418 resistant clones were screened of which 4 displayed homologous recombina-
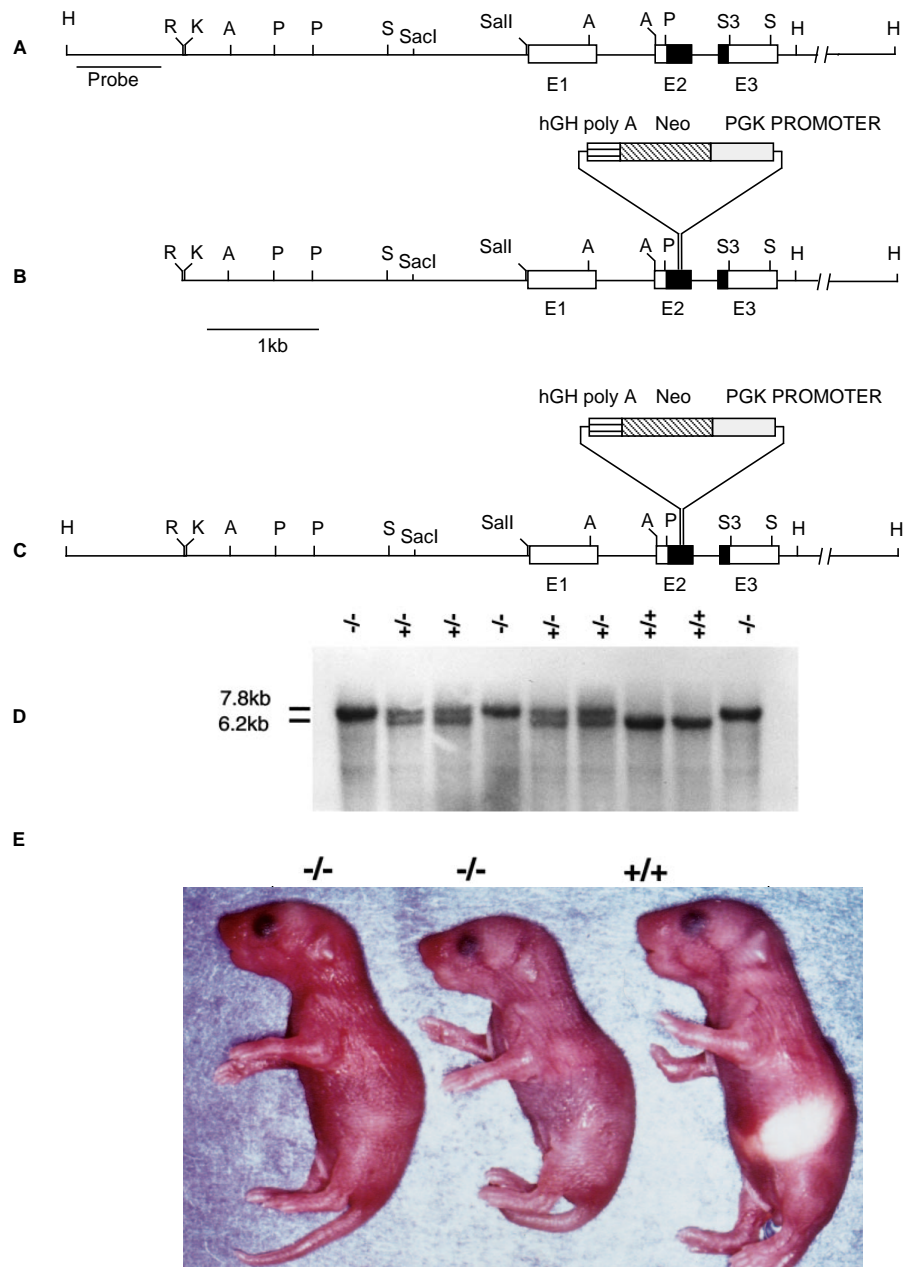

Fig. 1. (A) Schematic illustration of the mouse *goosecoid* gene. Exons are denoted by E1 to E3. Homeobox encoding exons are shown in black. (B) The targeting construct. (C) Organization of the mutated *goosecoid* gene. (D) Genotyping of DNA by Southern blot analysis. (E) Newborn wild-type (+/+) and homozygous (−/−) mice 13 hours after birth, note the absence of milk in the stomach of the −/− mice. Abbreviations : A, *Apa*I; H, *Hin*dIII; K, *Kpn*I; R, *Eco*RI; P, *Pst*I; S, *Sma*I; S3, *Sau*3A; hGH, human growth hormone poly(A) signal.

tion. One of these four clones gave germline transmission. The results of the Southern screen suggest that we have generated a likely null allele of *goosecoid*.

Goosecoid homozygous mice die after birth

Wild-type (+/+), heterozygous (+/−) and homozygous mice (−/−) were born at the expected Mendelian frequency, examples of which are shown in Fig. 1E. However, the −/− mice died within 24 hours of parturition. In each case, these mice did not have milk in their stomachs (Fig. 1E). They were smaller than their littermates and probably died because of starvation and dehydration. All other gross external features were normal. We performed histological analysis on these mice in an effort to identify developmental defects that could affect structures necessary for proper feeding.

Abnormal craniofacial morphogenesis in *goosecoid* mutants

Fig. 2A shows the nasal cavity of a wild-type littermate in which the normal nasal passage topography can be seen. This normal topography is essential for the efficient sensing of airborne substances such as the maternally originating odours. Mice breathe only through their noses and thus the altered morphology of the nasal structures may impair proper breathing. In Fig. 2B, a typical (−/−) *goosecoid* mouse can be seen to have aplastic nasal cavities, nasal capsules and lack the ethmoid-derived turbinals. The innermost nasal epithelium appears unaffected whereas the basal cell layers are aplastic (Fig. 2B). It is also noteworthy that the nasal septum does not fuse with the secondary palate. This may be due either to the underdevelopment of the septum or aplastic development of the
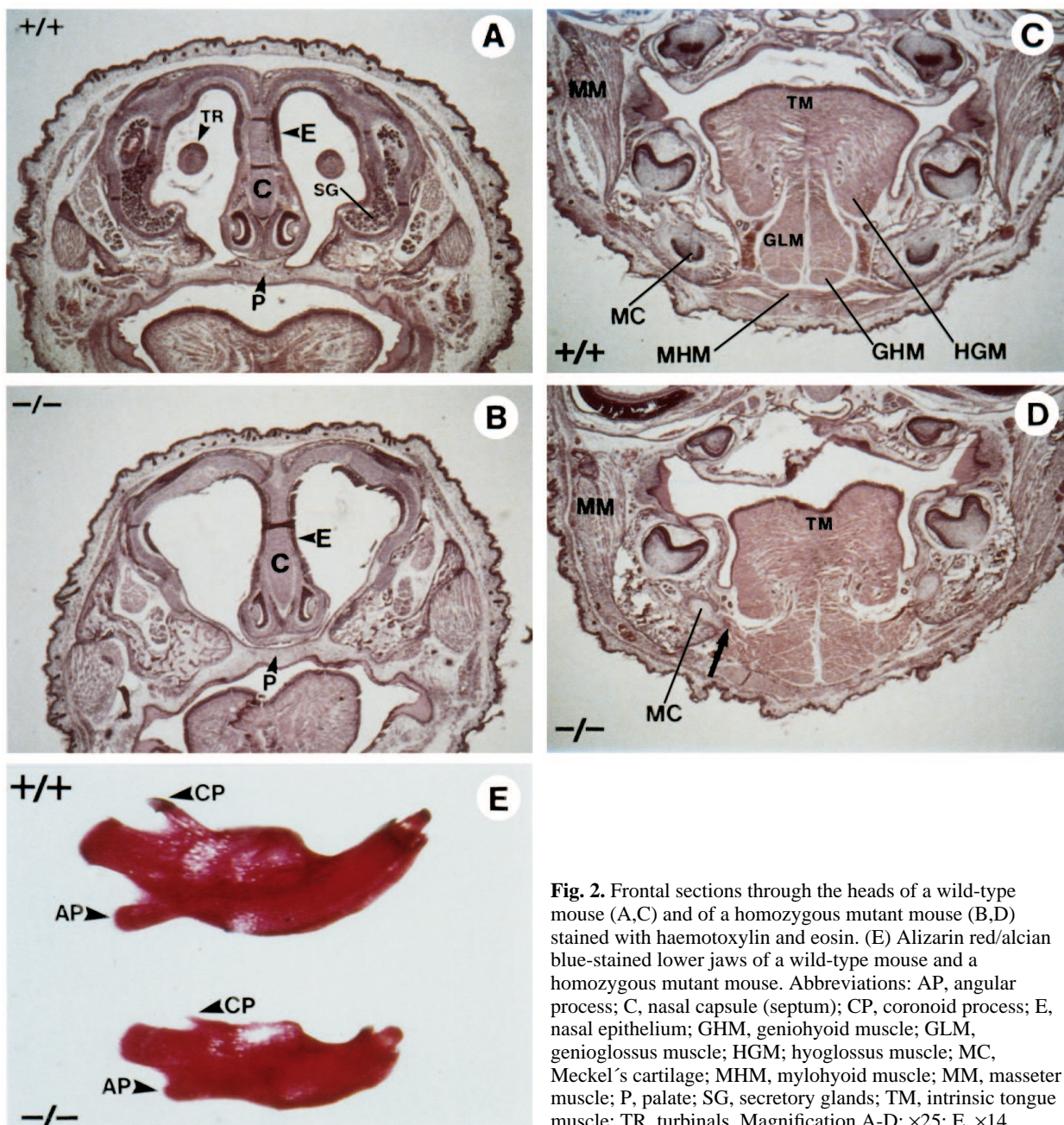

Fig. 2. Frontal sections through the heads of a wild-type mouse (A,C) and of a homozygous mutant mouse (B,D) stained with haemotoxylin and eosin. (E) Alizarin red/alcian blue-stained lower jaws of a wild-type mouse and a homozygous mutant mouse. Abbreviations: AP, angular process; C, nasal capsule (septum); CP, coronoid process; E, nasal epithelium; GHM, geniohyoid muscle; GLM, genioglossus muscle; HGM; hyoglossus muscle; MC, Meckel´s cartilage; MHM, mylohyoid muscle; MM, masseter muscle; P, palate; SG, secretory glands; TM, intrinsic tongue muscle; TR, turbinals. Magnification A-D: ×25; E, ×14.

Fig. 3. (A,B) Haemotoxylin- and eosin-stained sections of homozygous (A) and wild-type (B) ears. (C,D) Whole-mount alizarin red/alcian blue-stained middle ear specimens of homozygous mutant (C) and wild-type (D) mice. (D) The stapes was separated during dissection. Note the absence of the processus brevis and the aplasia of the manubrium of the malleus in the mutant middle ear. The incus and stapes are normal in the mutant mice (C). Abbreviations: TM, tympanic membrane; MC, middle ear cavity; EA, external acoustic meatus; P, pinna; ET, Eustachian tube; TR, tympanic ring; I, incus; VI, long ventromedial process of the incus; M, malleus; MM, manubrium of the malleus; PB, processus brevis of the malleus; S, stapes; G, gonial bone; MC, Meckel's cartilage. Magnification (A,B) ×25; (C,D) ×35.
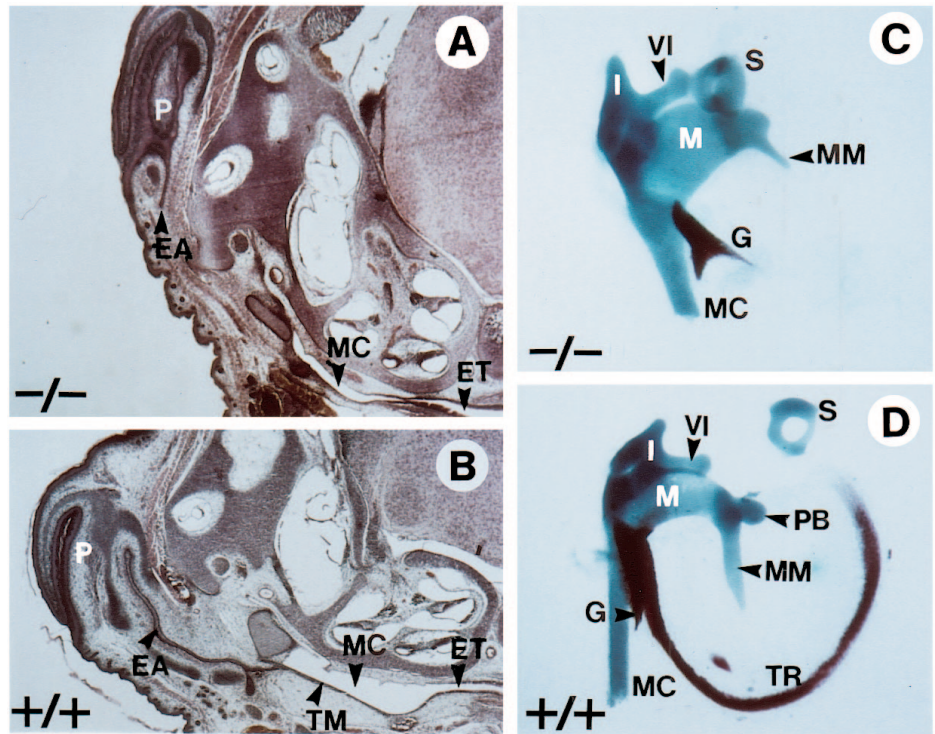
secondary palate. However, the latter appears to be unlikely as the secondary palate appears normal in −/− mice.

In addition to the nasal phenotypes, −/− mice display hypoplasia of the lower jaw (which is formed from the 1st branchial arch) and display reductions in the coronoid and angular processes although the condilar process appears normal (Fig. 2E). Also, a novel groove in the lower mandible could be identified along Meckel's cartilage (data not shown). This may be the result of impaired interactions between the mandibular cells and those of Meckel's cartilage. Meckel's cartilage is also malformed such that aberrant muscle connections are formed in −/− mice (Fig. 2D, black arrow). These abnormalities may represent the primary defects that underlie the disturbances evident in the tongue and somite-derived muscles.

Many of the muscles associated with the structures described above are also affected in *goosecoid* −/− mice. In general, an underdevelopment of muscle alignment is evident (Fig. 2D) e.g., in the genioglossus muscles (which develop from the 2nd to 4th somites), geniohyoid, hyoglossus, mylohyoid and masseter muscles (1st branchial arch). In −/− mice, the muscle cells are recognizable per se, however, the cellular arrangement is disturbed. In addition, alignment of the anterior intrinsic tongue muscles, which are derived from the myotomes of the occipital somites, is confused. The anterior (body) of the tongue expresses *goosecoid* in contrast to the root of the tongue (Gaunt et al., 1993). As proper alignment of these muscles is required for smooth, co-ordinated movement of the tongue and/or the lower jaw, these phenotypes would also enhance the suggestion of neonatal death due to an impairment of suckling. It was also evident that some −/− mice died immediately after parturition and this observation could be explained by suffo-
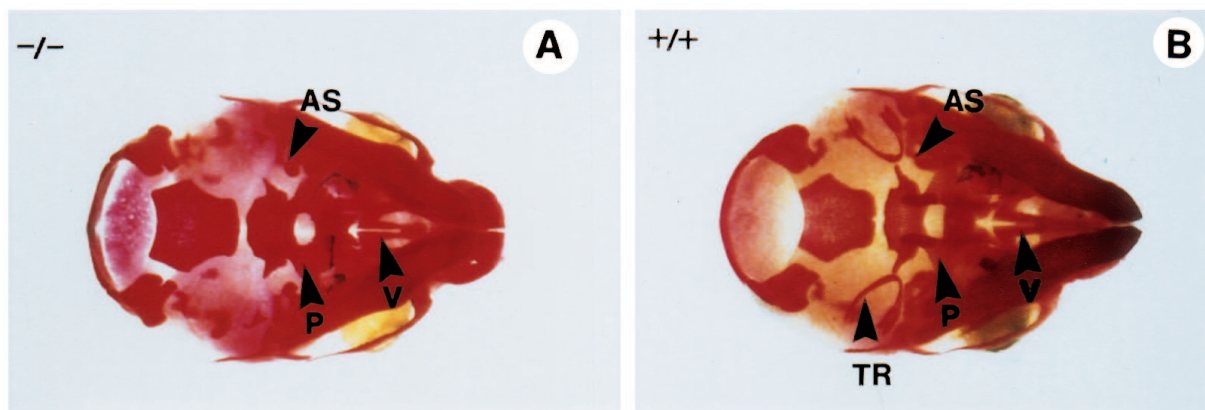

Fig. 4. Whole-mount alizarin red / alcian blue-stained skulls of homozygous (A) and wild-type (B) mice. Abbreviations: P, palatine bone; AS, alisphenoid bone; V, vomer bone; TR, tympanic ring. Magnification: ×12.

cation due to an occlusion of the airways by the inability to coordinate tongue movements properly.

Abnormal ear and bone morphogenesis in *goosecoid* mutants

It has previously been demonstrated that *goosecoid* expression in the branchial arch region persists when these areas undergo morphogenesis e.g., the expression around the first branchial cleft and first branchial pouch remains when they form the external auditory meatus and the middle ear, respectively (Gaunt et al., 1993). Figs 3C and 4A show skeletal preparations of −/− mice that display deletions of the tympanic ring, a neural crest-derived structure. Other defects in the ear include aplasia of the tympanic cavity and the external auditory meatus – both of which normally show *goosecoid* expression. In the case of the middle ear bones, the malleus is dramatically affected with its mandibulum being greatly reduced and the processus brevis being absent. The malleus is derived from an earlier *goosecoid*-expressing structure – the mandibular process. In accordance with wild-type mouse expression of *goosecoid,* other bones of the middle ear, such as the incus and stapes, are unaffected. Thus, the defects apparent in the ear correlate well with the expression domains of *goosecoid.*

The tympanic membrane does not form in *goosecoid* −/− mice, Fig. 3A. The tympanic membrane is formed by an inductive interaction between the acoustic meatus and the tympanic cavity thus raising the possibility that the observed tympanic membrane aplasia may be a secondary and not a direct effect. In addition, the size of the external pinna is slightly reduced as is the gonial bone (Fig. 3A,C). As previously shown, *goosecoid* is initially expressed in the mesenchyme surrounding the hyomandibular cleft and this expression persists in its derivatives suggesting that *goosecoid* may play a role in the morphogenesis of the external and middle ear. Other skeletal components affected include reduced and malformed palatine, alisphenoid, pterygoid and vomer bones, all of which are neural crest in origin (Fig. 4A). The anteriormost part of the sternum (manubrium sterni) is malformed such that the costal cartilage divides it into two parts (data not shown).

DISCUSSION

We have demonstrated that targeted mutation of the *goosecoid* gene leads to a late stage embryonic phenotype, which is incompatible with survival postparturition. We suggest that the primary cause of death in *goosecoid* homozygous mutant mice resides in the malformed nasal structures. Defects involving the nasal structures are in accordance with the expression pattern of *goosecoid* during the later stages of embryogenesis. In addition to the abnormal structures that may affect breathing, it is also likely that impaired olfactory sensing is enhanced by the probable reduction in olfactory secretion by the olfactory glands. Therefore, as it is known that neonates rely heavily on smell to detect the location of their mother, impaired olfactory sensing may be a contributory factor to death in these mice.

The murine *goosecoid* gene has two distinct phases of expression in the developing embryo. In the early phase of gastrulation, it is expressed in the anterior end of the developing primitive streak. The anteriormost mesoderm of the gastrula, the head process, is the only structure maintaining *goosecoid* expression during later primitive streak stages. The second phase of its expression is confined to those areas that form parts of the head and specific regions of the upper body. This later phase is characterized by *goosecoid* transcription in both the undifferentiated cells as well as their differentiated derivatives. It has previously been suggested that *goosecoid* may play a role in spatial programming within distinct embryonic fields (Gaunt et al., 1993). Our results support this view and demonstrate that targeted mutation of *goosecoid* affects only the domains in which the gene is normally expressed late in embryogenesis.

The mesenchymal component of the branchial arches and facial processes (and their subsequent structures, the visceral cranium) are derived almost entirely from cephalic neural crest cells (Noden, 1983, 1988; Keynes and Lumsden, 1990; Hunt and Krumlauf, 1991; Kuratani and Wall, 1992; Le Douarin et al., 1993).

Numerous genes including those that control development have been suggested to be involved in the programming of neural-crest-derived structures (Kessel and Gruss, 1990). Previously, gene targeting studies have demonstrated important roles for developmental control genes in craniofacial structure formation (Gendron-Maguire et al., 1993; Rijli et al., 1993). Mutation of another homeobox gene, *Msx1*, has been reported and the mutant mice display phenotypes which are at least overlapping with those of the *goosecoid* −/− mice (Satokata and Maas, 1994). Further studies on possible genetic interactions between *goosecoid* and *Msx1* may therefore reveal common genetic pathways.

Recently, it has been reported in a number of cases that only compound (multiple) mutations reveal the expected phenotype anticipated by the gene expression pattern. Examples of such cases are the null mutants of *Myo-D* and *Myf-5* (Braun et al., 1992; Rudnicki et al., 1992). Although both genes exhibit overlapping expression patterns in muscle precursor cells during development, only the double mutants exhibit strong muscle phenotypes, suggesting the redundant function of these two genes (Rudnicki et al., 1993). Similarly, only in compound mutants of the paralogous genes *Hoxa-3* and *Hoxd-3*, were indications of a role for *Hoxa-3* in atlas formation evident (Condie and Capecchi, 1994). It is possible that the early development redundancy of *goosecoid* seen in this study may be due to the presence of a second *goosecoid* gene or another functionally related gene. It seems that the higher complexity of mammalian genomes will continue to produce examples of genes that are functionally supported by paralogous family members or other genes (Lohnes et al., 1994; Mendelsohn et al., 1994; Wurst et al., 1994).

A number of human genetic syndromes showing malformations in branchial arch derived structures are known. First arch (pharyngeal arch) syndrome consists of a number of malformations in tissues derived from the pharyngeal arch e.g., external, middle and inner ear, mandible and several bones of the skull (Smith, 1982; Stricker, 1990). Several cloned developmental regulatory genes have already been suggested for or assigned to some human craniofacial syndromes e.g., cleft palate, oligodontia (Jabs et al., 1993; Satokata and Maas, 1994). The molecular cloning and chromosomal assignment of the human *goosecoid* gene revealed that no corresponding human syndrome has yet been mapped to this locus (Blum et

al., 1994). Our results suggest that *goosecoid* mutant mice may provide an animal model for neural crest syndromes.

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