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goosecoid is not an essential component of the mouse gastrula organizer but is required for craniofacial and rib development

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SUMMARY

goosecoid (gsc) is an evolutionarily conserved homeobox gene expressed in the gastrula organizer region of a variety of vertebrate embryos, including zebrafish, Xenopus, chicken and mouse. To understand the role of gsc during mouse embryogenesis, we generated gsc-null mice by gene targeting in embryonic stem cells. Surprisingly, gsc-null embryos gastrulated and formed the primary body axes; gsc-null mice were born alive but died soon after birth with numerous craniofacial defects. In addition, rib fusions and sternum abnormalities were detected that varied depending upon the genetic background. Transplantation experiments suggest that the ovary does not provide gsc function to rescue gastrulation defects. These results demonstrate that gsc is not essential for organizer activity in the mouse but is required later during embryogenesis for craniofacial and rib cage development.

Key words: gastrulation, skeleton, craniofacial development, goosecoid, mouse

INTRODUCTION

The classic embryological experiments of Spemann and Mangold (1924) led to the development of the concept of the organizer. They showed that, when the dorsal blastopore lip from a gastrula stage amphibian embryo was transplanted to the presumptive ventral side of another comparably staged embryo, a secondary axis developed. Their ability to follow the fates of the grafted tissue demonstrated that the donor tissue was able to change the cell fates of the surrounding host cells to participate in the development of the secondary axis. Candidate genes that may participate in this complex embryological phenomenon have been identified. A subset of these genes encode transcription factors, perhaps the most notable among these is gsc.

gsc homologs have also been isolated in other vertebrate species, including zebrafish, chick and mouse (Stachel et al., 1993; Shulte-Merker et al., 1994; Izpisúa-Belmonte et al., 1993; Blum et al., 1992). In zebrafish, gsc is expressed in the anterior region of the embryonic shield and axial hypoblast, and later during embryogenesis in larval cranial neural crest derivatives (Stachel et al., 1993; Shulte-Merker et al., 1994). Lithium treatment of zebrafish embryos results in radialized embryos that are hyperdorsalized (Stachel et al., 1993). gsc expression in these lithium-treated embryos is elevated and radialized. These results suggest that lithium treatment induces ectopic gsc expression that leads to the generation of multiple organizer fields. In the chick, gsc-expressing cells are first detectable in Koller's sickle, a group of cells near the posterior margin zone of the unincubated egg that, when transplanted into another embryo, can induce a secondary axis (Izpisúa-Belmonte et al., 1993). Thus, studies in zebrafish and chick also implicate gsc as a factor with organizer activity.

In the mouse, gsc-expressing cells are found transiently at the anterior end of the primitive streak of the gastrula between E6.4 and E6.8 (Blum et al., 1992). Transplantation studies into Xenopus or mouse embryos have demonstrated that this region of the mouse gastrula possesses some of the organizer functions of the dorsal blastopore lip (Blum et al., 1992; Beddington, 1994). gsc expression reappears at E10.5 (Gaunt et al., 1993). At these later stages of embryogenesis, gsc transcripts are found in undifferentiated tissues and persist as those tissues undergo morphogenesis (Gaunt et al., 1993). Between E10.5
and E14.5, gsc transcripts are detected in the lower jaw and the tongue, the eustachian tube and base of the auditory meatus, the mesenchyme surrounding the nasal pits that form the nasal chambers, and the proximal limb buds and the ventrolateral body wall that form the proximal limb structures and ventral ribs. These findings suggest that, in addition to a potential role in gastrulation, gsc may also be required later during mouse embryogenesis for craniofacial, limb and thoracic development.

To determine the requirement of gsc during mouse development, we generated gsc-null mice by gene targeting in mouse embryonic stem (ES) cells. Gastrulation progressed normally in gsc-null embryos and all of the primary body axes formed correctly; gsc-null mice were born alive but died soon after birth with craniofacial defects. In addition, rib fusions and sternum abnormalities were detected. These results demonstrate that gsc is not an essential component of the gastrula organizer in the mouse but is required later during embryogenesis for craniofacial and ribcage development.

MATERIALS AND METHODS

Deletion of the gsc gene in mouse ES cells
A 129/SvEv mouse genomic library (Stratagene) was screened with a probe containing nucleotides 1837 to 2166 of the mouse gsc genomic sequences (GenBank accession number M85271; Blum et al., 1992). The probe is a subfragment of the gsc locus generated by PCR amplification of mouse genomic DNA. One phage clone that hybridized with the probe was subcloned into pBluescript, and its gsc identity was verified by DNA sequencing.

A 2.9 kb Asp718-SalI upstream fragment and a 2.0 kb downstream region from the NorI-XhoI sites, were used to construct a replacement vector (Fig. 1A). A PGKNeoAP herpes simplex virus thymidine kinase expression cassette (Soriano et al., 1991) was inserted in either forward or reverse orientation relative to the direction of gsc transcription between the two gsc regions. A MC1tkAP herpes simplex virus thymidine kinase expression cassette was added onto the 3' arm of homology to enrich for homologous recombinants using negative selection with 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-s-iodouracil (FIAU) (Mansour et al., 1988). The targeting vectors can be linearized at unique Asp718 sites outside of the homology. 10 μg of linearized targeting vector was electroporated into 107 AB-1 ES cells that were subsequently cultured in the presence of G418 and FIAU (Soriano et al., 1991; McMahon and Bradley, 1990), 154 G418/FIAU-resistant ES clones were initially screened by HindIII digestion and hybridization with a unique 5' probe external to the region of vector homology. Correctly targeted clones were then expanded for further Southern blot analysis by EcoRI/EcoRV digestion and hybridization with a unique 3' probe external to the vector homology. 39 correctly targeted ES clones were identified. The overall targeting frequency for both vectors from two independent electroporations was approximately 1/4 G418/FIAU-resistant colonies screened.

Generation of chimeric mice and germline transmission of the gsc deletion alleles
Four of the gsc mutant ES clones were microinjected into B6-albino blastocysts and the resulting chimeric embryos were transferred to the uterine horns of day 2.5 pseudopregnant foster mothers (Bradley, 1987). Chimeras were identified among the resulting progeny by their pigmentated fur (ES-derived) and were subsequently bred with B6-albino mates. Three of the mutant ES clones (one with neo in the forward orientation and two with neo in the reverse orientation) were found to be capable of contributing to the germline of chimeric mice.

Fig. 1. Targeted deletion of the gsc gene in ES cells and mice. (A) Strategy for targeted deletion of the gsc gene. gsc is encoded by three exons contained within a 1.9-kb SalI-NorI DNA fragment; the homeobox (shaded) is split between the second and third exons. The targeting vector possesses a total of 4.9 kb of homologous sequence including a 2.9 kb upstream region as a 5' arm of homology and a 2.0 kb downstream region as a 3' arm isolated from a mouse strain 129/Sv genomic library (thick closed bars). A PGK-neo cassette (Soriano et al., 1991) (neo) was inserted between the two arms of homology in either forward (gsc1) or reverse (gsc2) orientation relative to the direction of gsc transcription. The neo cassette replaces the gsc-containing SalI-NorI DNA fragment and introduces novel restriction sites for Southern analysis. A MCI-tk cassette (TK) was added for negative selection (Mansour et al., 1988). Unique Southern probes were identified that lie 5' and 3' outside of the vector homology. (B,C) Southern blot analysis of DNA from gsc1 mutant ES clone 6454, gsc2 mutant ES clones 5812 and 5878, and wild-type (WT) ES cells: HindIII digest hybridized with the 5' probe (B); EcoRI/EcoRV digest hybridized with the 3' probe (C). (D) Southern blot analysis of tail DNA from the offspring of heterozygote matings from the 5878 line. EcoRI/EcoRV digest hybridized with the 3' probe. (E) Southern blot analysis of DNA from wild-type and homozygous mutant mice from the 5812 line. EcoRI/EcoRV digest hybridized with a gsc probe contained within the deletion. A, Apol; As, Asp718; H, HindIII; N, NorI; Nh, NhI; Rl, EcoRI; Rv, EcoRV; S, SalI; X, XhoI. +/-, wild-type; +/-, heterozygous; --/--, mutant.
Tail DNA from the pigmented pups that resulted from those matings was analyzed by Southern blotting with either of the probes used to identify gsc heterozygotes. Chimeras were also bred with 129/SvEv females to establish the gsc deletion alleles on the 129/SvEv inbred genetic background. Mice carrying the gsc mutation were also backcrossed to B6 mice to initiate the generation of a congenic mouse line. gsc heterozygotes at generation 4 or 5 possess an inbreeding coefficient of 0.938 and 0.969, respectively, and were used in this study.

Ovary transplants

gsc heterozygotes (B6×129 hybrid genetic background) were interbred to establish timed matings. On E18.5, the pups were delivered by Cesarian section. gsc-null pups were identified by their abnormal breathing behavior and, upon dissection, by air in the gut. Subsequent Southern blot analysis confirmed that they were gsc-null. Both ovaries from a gsc-null female were transplanted into the bursal sac of a B6×129 F1 hybrid female approximately 3 weeks of age whose ovary had been surgically removed. The oviduct from the other uterine horn was surgically ablated leaving the endogenous ovary intact. After three weeks, the transplanted females were bred with gsc heterozygous males. In this scheme, the only way that gsc homozygous mutant progeny can be obtained is if the gsc-null ovary transplant was successful.

Skeleton preparations

Neonates were killed, skinned, eviscerated and fixed in 95% ethanol. Their skeletons were subsequently prepared by alkaline digestion and stained with alizarin red S for ossified bone and alcin blue 8GX for cartilage (Kochhar, 1973). For fetal cartilaginous skeletons, embryos were fixed in Bouin’s fixative, washed, stained with alcin blue 8GX, dehydrated and cleared in 2:1 benzyl benzoate:benzyl alcohol (Jegalian and De Robertis, 1992).

Histological analysis

Embryos at E18.5 and E15.5 (not shown) were fixed in Bouin’s solution, dehydrated in graded alcohols and embedded in paraffin. 10 μm sections were cut and stained with haematoxylin and eosin.

Table 1. Genotype of newborn offspring derived from heterozygous gsc parents

<table>
<thead>
<tr>
<th>Allele/Clone</th>
<th>Litters</th>
<th>Wild-type</th>
<th>Heterozygous</th>
<th>Mutant</th>
<th>Total</th>
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</thead>
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<tr>
<td>gsc&lt;sup&gt;r&lt;/sup&gt; (5878)</td>
<td>15</td>
<td>23 (19.5%)</td>
<td>69 (58.5%)</td>
<td>26 (22%)</td>
<td>118</td>
</tr>
<tr>
<td>gsc&lt;sup&gt;r&lt;/sup&gt; (5812)</td>
<td>7</td>
<td>11 (30%)</td>
<td>16 (43%)</td>
<td>10 (27%)</td>
<td>37</td>
</tr>
<tr>
<td>gsc&lt;sup&gt;f&lt;/sup&gt; (6454)</td>
<td>11</td>
<td>12 (17%)</td>
<td>47 (67%)</td>
<td>11 (16%)</td>
<td>70</td>
</tr>
</tbody>
</table>

Fig. 2. Craniofacial abnormalities of gsc-null mice. Skeleton preparations of wild-type neonates (A,C) and gsc-null littermates (B,D) (A,B) Lateral view of skull. gsc-null mice have a reduction of the orbital processess (asterisks) of the maxillary (m) and frontal (f) bones. (C,D) Ventral view of skull with jaw removed (the nasal region is at the top). Portions of several bones are reduced or malformed in the mutants. The palatine bone (p) has reduced wings and palatine shelves. The alisphenoid bone (al) has a malformed area of foramens (short solid arrow) and the cartilage that unites it with the basisphenoid bone is split in two (asterisk). The pterygoid bone (pt) is reduced in size (white slanted arrow) and the tympanic ring bone (ty) is absent in the mutants (open arrow). (E) Ventral view of dissected nasal cartilage. Wild-type (left) and gsc-null (right). The anlagen of the turbinal bones (tu) are absent in the gsc mutant. The vomer bone (v) is reduced in the mutant. (F) Medial view of the right mandible of wild-type (top) and gsc-null (bottom) mice. The mandible of the gsc-null mouse is shorter. In gsc mutants, the coronoid (cr) and angular (a) processes are reduced in size, whereas the condilar (cn) process is normal. A groove extending along the mandible is observed in the gsc-null mice, so that the Meckel’s cartilage (white arrow) is now visible on the inner medial surface of the jaw, which is not seen in the control (black arrow).
RESULTS

Generation of two gsc-null alleles in the mouse germline

The mouse gsc gene is encoded by three exons (Blum et al., 1992). To mutate the gsc gene in mouse embryonic stem (ES) cells, we generated two different targeting vectors, both of which delete the entire GSC protein-coding region (Fig. 1A). The targeting vectors differed only with respect to the orientation of the neomycin (neo) selectable marker relative to the direction of gsc transcription. The allele generated by the vector with the neo marker in the forward orientation was designated gsc<sup>d</sup> and the allele generated by the vector with the neo marker in the reverse orientation was designated gsc<sup>c</sup>. When these vectors are homologously recombined with the mouse genome, novel restriction enzyme sites are introduced (Fig. 1A). Correctly targeted clones for both gsc<sup>d</sup> and gsc<sup>c</sup> can therefore be detected by the presence of an additional 4.1 kb mutant band when digested with HindIII and hybridized with a 5′ probe external to the region of vector homology or for gsc<sup>d</sup> by the presence of a 4.0 kb mutant band and for gsc<sup>c</sup> by the presence of a 2.3 kb mutant band when digested with EcoRI and EcoRV and hybridized with a 3′ probe external to the region of vector homology (Fig. 1B). Correct targeting with either vector results in the deletion of the entire GSC protein coding region, thereby creating null alleles. Correctly targeted ES clones were obtained for both vectors at a frequency of approximately 1/4 G418/FIAU resistant colonies screened. Three correctly targeted ES clones (one for gsc<sup>d</sup> and two for gsc<sup>c</sup>) successfully contributed to the germline of chimeric mice generated by blastocyst injection (Fig. 1B,C). The phenotype of gsc-null mice from these three independently derived ES clones were identical. In addition, the phenotype of gsc<sup>d</sup>/gsc<sup>c</sup> mice was also identical to each of the gsc<sup>c</sup> and gsc<sup>d</sup> homozygous mutants.

gsc-null mice are born alive without axial patterning defects

Mice heterozygous for the gsc deletion alleles appeared normal and were fertile. Mice homozygous for either of the two gsc mutant alleles were recovered alive at birth (Fig. 1D) and were overtly indistinguishable from their wild-type or heterozygous littermates. The genotypes of the offspring from heterozygote crosses followed predicted Mendelian frequencies, suggesting that homozygous mutant mice were not being lost during embryonic development (Table 1). Southern blot analysis using gsc coding sequences as a probe confirmed that gsc homozygous mutant mice did not contain gsc coding sequences, demonstrating that a null allele had been generated (Fig. 1E).

The recovery of gsc-null mice at birth without axial defects suggested that the patterning events that take place during gastrulation had occurred correctly without gsc function. Although gastrulation had clearly occurred, it was still possible that the absence of gsc could result in a delay of early embryogenesis and that later in development gsc-null embryos could catch up with their wild-type and heterozygous littermates. However, at E7.5, gsc-null embryos had normal morphology and expressed HNF-3β protein (Sasaki and Hogan, 1993; Ang et al., 1993) correctly in the anterior midline and the node (not shown), a structure that possesses a subset of the functions of the Spemann organizer (Beddington, 1994). These results suggest that gsc-null embryos are able to gastrulate and organize early embryonic pattern with correct developmental timing. Therefore, in the mouse gsc is not required for either mesoderm or axis formation.

gsc-null mice are born from females with gsc-null transplanted ovaries

In zebrafish and Xenopus, gsc transcripts are detected in oocytes (De Robertis et al., 1992; Stachel et al., 1993; Schulte-Merker et al., 1994), suggesting that maternal stores of gsc RNA or protein may play a role in early embryonic patterning. Thus, the recovery of gsc-null mice at birth from heterozygous mothers could be due to the rescue of the mutants during gastrulation by a maternal source of gsc function. It is currently unknown whether gsc RNA or protein is present in the mouse oocyte, and the lack of an antibody to GSC protein precludes a judgement about a maternal source of GSC protein. To address the question of a maternal source of gsc function directly, we transferred the ovaries from gsc-null pups recovered by Cesarian section at E18.5 into histocompatible gsc-wild-type recipient females that had been rendered incapable of producing wild-type oocytes. In this way, the host females would produce oocytes from the gsc-null ovaries that would lack gsc transcripts and protein. The females carrying the transplanted gsc-null ovaries were subsequently bred with gsc heterozygous males. Six pups were born alive from two of these females and four of these pups (three from one female and one from the other) were indistinguishable in phenotype from gsc-null mice born from heterozygous matings. Genotyping by Southern analysis confirmed that these four pups were gsc homozygous mutants; the other two pups were heterozygotes. These results suggest that ovarian tissues do not provide gsc function to rescue gastrulation defects in gsc-null mice.

Neonatal lethality with craniofacial and rib cage abnormalities in gsc-null mice

gsc-null mice never fed and all died within 24 hours after birth. The mutants could not suckle when physically placed upon the mother’s nipples but could accumulate milk in their stomachs when forcefed, suggesting that the pathway from the mouth to the stomach was intact. In addition, the mutants had difficulty breathing which was associated with air in the stomach and intestines, and a pale body color.

Skeletal analysis of gsc-null neonates revealed numerous craniofacial and rib cage abnormalities. In the skull, the orbital processes of the maxillary and frontal bones that support the eye were reduced (Fig. 2A,B). In addition, the tympanic ring bone, which normally supports the tympanic membrane (eardrum), was absent (Fig. 2C,D). Within the middle ear, the manubrium and processus brevis of the malleus were smaller, whereas the incus and staples were normal (not shown). In addition, several bones at the base of the skull were malformed, including the palatine, maxillary, alisphenoid and pterygoid bones (Fig. 2A-D). There were also significant alterations in the nasal region, including the lack of the anlagen for the turbinal bones that form the chambers of the nasal cavity (Fig. 2E). Furthermore, the mandible was shortened and, although the condilar process was normal, the coronoid and angular processes were diminished (Fig. 2F). A groove, extending along Meckel’s cartilage of the mandible, was observed in the mutants but was not found in controls. The variations in mandible development were already apparent at E13.5. The craniofacial abnormalities were detected in all 62 of the gsc-null mice analyzed. Although gsc is abundantly expressed in the developing limbs,
no skeletal abnormalities were detected in the limbs of any of the gsc-null mice.

Additional craniofacial defects were noted upon histological analysis of gsc-null embryos and wild-type littermates at E15.5 and E18.5. As observed in the skeletal preparations, gsc-null mutant embryos lacked the anlagen of the turbinal bones and the ventral lateral walls of the nasal cavity (Fig. 3A,B). In addition, the glandular mucous epithelium that normally covers the nasal sinuses was mostly absent in the mutants. However, midline nasal structures, such as the nasal septum and the vomeronasal organs and cartilages, were present in the mutants, although the nasal septum did not fuse with the palate. In addition, middle ear development in the gsc-null embryos was abnormal. Although the tympanosynovial recess had formed and was present adjacent to the otic capsule, the external acoustic meatus had not migrated very far internally. Therefore, the tympanic membrane, which is formed by the apposition of the tympanosynovial recess and the external acoustic meatus, did not form in gsc-null mice (Fig. 3C,D). In the tongue, the genioglossus muscle showed aberrant insertions on Meckel’s cartilage, rather than inserting on the symphysis of the mandible as in controls (Fig. 3E-H). In addition, the density of muscle fibers of the extrinsic muscles of the tongue was reduced in the mutants (Fig. 3G,H). The thyroid and thymus glands, which express gsc during embryogenesis, were present in the mutants.

Rib fusions were detected in about 35% of the 62 gsc-null skeletons analyzed (Fig. 4A-D). The fusions occurred between the costal cartilages of the first and second ribs, although in one case the fourth and fifth ribs had fused. The rib fusions were unilateral, either on the left or right side, or bilateral. An additional 20% of the gsc-null skeletons had a different defect in rib cage development. In these skeletons, rather than rib fusions, a reduced number of ribs were attached to the sternum in comparison to controls. As in the case of the rib fusions, this variation in rib attachment was unilateral, on either side, or bilateral. Typically, the skeletons with rib fusions or abnormal numbers of attached ribs had sternum abnormalities characterized by modifications in sternebrae ossification, probably the result of incorrect rib attachment. The rib fusions were evident in mutant embryos at E14.0 (Fig. 4E).

The initial skeletal analyses were performed on a C57BL/6 (B6) × 129 F2 hybrid genetic background. We also examined the gsc mutation on a 129/SvEv inbred genetic background and on a genetic background that was theoretically >90% B6 (Table 2). All gsc-null 129 inbred mice or B6 mice died soon after birth with essentially the same craniofacial syndrome exhibited by the gsc-null mice on the F2 hybrid genetic background. Interestingly, whereas the penetrance of the craniofacial abnormalities of the gsc-null mice were the same on both the 129 and B6 genetic backgrounds, the penetrance of the rib cage abnormalities were different on these two genetic backgrounds. Rib fusions or changes in the normal number of ribs contacting the sternum were recorded as deviations of the normal pattern of rib cage development. Whereas approximately 55% of the gsc-null mice on the F2 hybrid background had rib cage abnormalities, only about 15% of the gsc-null mice had such defects on the 129 inbred background. In contrast, 70% of the gsc-null mice had rib cage defects on the B6 background. These results suggest that there is genetic variation between strains 129 and B6 that can suppress or enhance the frequency of rib cage defects caused by the gsc mutation, respectively.

**DISCUSSION**

**gsc and the vertebrate gastrula organizer**

Previous studies had suggested that gsc was an essential component of the vertebrate gastrula organizer (Cho et al., 1991; Blum et al., 1992; Izpisúa-Belmonte et al., 1993). These expectations were based upon the observations that gsc was expressed in the organizer regions of four vertebrate species and that gain-of-function assays in *Xenopus* resulted in the development of secondary axes (Cho et al., 1991, Blum et al., 1992; Izpisúa-Belmonte et al., 1993). However, even in the gain-of-function assays, gsc only had weak organizer activity; trunk duplications were most frequently induced and rarely were complete axes with head structures formed (Cho et al., 1991). In this study, we have determined the requirement of gsc during mouse embryogenesis by generating a loss-of-function mutation in mice by gene targeting in ES cells. Our studies unequivocally demonstrate that embryonic expression of gsc is not required for mesoderm induction or axis formation in mice.

In zebrafish and *Xenopus*, gsc is a maternally expressed transcript (De Robertis et al., 1992; Stachel et al., 1993; Schulte-Merker et al., 1994). Thus, maternally derived gsc RNA or protein could be used by the early embryo for axial development. However, if there were a maternal gsc component in the mouse, it is unlikely that it would persist long enough (to E6.5 when gastrulation is initiated) in the developing embryo to be biologically relevant. Moreover, the recovery of gsc-null pups from a mating between a female mouse carrying gsc-null ovaries suggests that the ovary does not provide gsc activity to the embryo for mesoderm formation or axial patterning. However, it is still formally possible that maternal contributions of GSC function exclusive of the ovary could effect a rescue.

One likely explanation for our results is that gsc serves a redundant role with respect to the mouse gastrula organizer. Thus, other genes may exist in mice that provide organizer activity. Candidates for such genes include *HNF3β* and *Lim1* because mutations in these genes lead to axial defects in mice.
It will be interesting to generate compound mutants with gsc and these genes to reveal a required function for gsc in organizer activity. It is clear that ectopic high level expression of gsc can dramatically alter the axial organization of the Xenopus embryo (Cho et al., 1991). In addition, elevated levels of radialized gsc expression after lithium treatment correlates with the development of dorsalized zebrafish embryos (Stachel et al., 1993). These observations suggest that gsc expression must be restricted both spatially and quantitatively for correct axis formation. It seems reasonable to suggest that it is critical for vertebrate embryos to maintain precise levels of organizer activity. A redundant or compensatory role for gsc could be envisioned in which gsc expression would be modulated, probably by growth factors (Cho et al., 1991; Green et al., 1992; Steinbeisser et al., 1993), to compensate for variations in organizer activity levels to maintain them within a narrow window of action. This would provide a certain amount of flexibility in response to inductive signals or other environmental cues to maintain axial patterning in the embryo. Whatever the case may be, the results presented here provide important information required for the interpretation of abnormalities in germ layer and axis formation.

Fig. 3. Histological analysis of gsc-null mice. Wild-type (+/+) embryos (A,C,E,G) and gsc-null (−/−) littermates (B,D,F,H) were isolated at E18.5 and were sectioned in frontal (A-F) and sagittal (G,H) planes. (A,B) Mutant embryos exhibited multiple defects in the nasal region, including loss of the anlagen of the turbinal bones (tb) and the ventrolateral walls of the nasal capsule (nc), loss of the glandular mucous epithelium (arrowhead) and lack of fusion of the nasal septum to the palate (arrow). (C,D) In mutant embryos, the tympanic ring (arrow) was absent. The external acoustic meatus (arrowhead) did not extend into the region surrounding the otic capsule; thus, the tympanic membrane, which is formed by the apposition of the tubotympanic recess (tr) and the external acoustic meatus, did not form in the mutants. The manubrium (mm) of the malleus (m) of the mutants was smaller than controls. (E,F) In the mandible of wild-type embryos, Meckel’s cartilage (arrow) is completely enveloped by the ossifying dentary bone. In mutants, Meckel’s cartilage is not completely enveloped by the dentary bone and the genioglossus muscle (gg) of the tongue (t) aberrantly inserts on Meckel’s cartilage. (G,H) A sagittal section of the mutant embryo displays both the aberrant insertions of the genioglossus muscle (gg) and the decreased density of extrinsic muscle fibers (arrow) of the tongue. Abbreviations: gg, genioglossus muscle; m, malleus; mm, manubrium of the malleus; nc, nasal capsule; t, tongue; tb, turbinal bone; tr, tubotympanic recess. Scale bar: A,B,E,F, 160μm; C,D, 200 μm; G,H, 100 μm.

Fig. 4. Thoracic skeletal abnormalities of gsc-null mice. Flat mounted rib cages from wild-type (A) and gsc-null mice (B,C,D). In wild-type mice, seven pairs of ribs contact the sternum. (B) gsc-null rib cage with only six pairs of ribs attached to the sternum. (C,D) Rib fusions occurred between the first and second ribs and contacted the sternum close to or at the normal site of attachment for the second rib. (E) Cartilaginous skeletons of E14.0 wild-type (left) and mutant (right) embryos with the forelimbs removed. Rib fusions (solid arrow) and mandible abnormalities (open arrows) were already evident at this stage.
formation in vertebrate embryos with alterations in gsc-expression patterns.

**gsc and craniofacial and rib cage development**

The non-viability of gsc-null mice clearly demonstrates that there are required functions for gsc during development. This essential role for gsc is in craniofacial and rib cage morphogenesis. The craniofacial defects observed in the gsc-null mice were predominantly restricted to derivatives of the first branchial arch, which correlate with the later pattern of gsc expression. In addition, the rib cage abnormalities also correlate with the expression in the developing ventrolateral body wall. One significant region of embryonic gsc expression where no defects were detected were the limbs. Perhaps, like gastrulation, gsc also has a redundant role in limb development.

Craniofacial development is a complex and dynamic process involving numerous tissue interactions (Noden, 1988). The expression of gsc in cranial mesenchyme and the craniofacial defects observed in our gsc-null mice suggest that this homeo-protein is involved in inductive tissue interactions that form the head. Many of the craniofacial structures that were abnormal in the gsc mutants are derived from the neural crest that migrate into the cranial region. Previous studies have shown that gsc expression can modulate cell migration in Xenopus embryos (Niehrs et al., 1993). It is unlikely that gsc is regulating neural crest cell migration because gsc expression in neural crest-derived cells occurs after migration (Hunt et al., 1991). Thus, gsc may be important for regulating postmigratory neural crest-derived cell behaviour in response to inductive signals that is essential for proper tissue morphogenesis. Two other homeobox genes, mhx-1 and Mhx, are expressed in cranial mesenchyme and, when mutated, also result in numerous craniofacial defects (Satokata and Maas, 1994; Martin et al., 1995). Some of the abnormalities found in the these mutant mice overlap with those of our gsc mutants but most are different. Thus, multiple homeoproteins expressed in cranial mesenchyme function uniquely in the formation of the various components of the vertebrate head. It is also interesting to note that gsc-null mice have abnormalities in sensory organs that may alter olfaction and hearing. The involvement of cranial neural crest in the development of craniofacial structures that were abnormal in the gsc-null mice overlap with those of our gsc mutants but most are different. Thus, multiple homeoproteins expressed in cranial mesenchyme function uniquely in the formation of the various components of the vertebrate head. It is also interesting to note that gsc-null mice have abnormalities in sensory organs that may alter olfaction and hearing. The involvement of cranial neural crest in the development of craniofacial and rib cage development is in vertebrate embryos with alterations in gsc-expression patterns.

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